# Cross-talk between iron starvation and $H_2O_2$ signaling pathways in *Schizosaccharomyces pombe*

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WIND IN MY HAIR, I FEEL PART OF EVERYWHERE UNDERNEATH MY BEING IS A ROAD THAT DISAPPEARED

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## Abstract

Hydrogen peroxide ($H_2O_2$), a reactive oxygen species (ROS), is involved in both oxidative stress and signaling cascades in a dosage dependent manner. Its toxicity is partially explained through reactivity with iron via the Fenton reaction. Iron, indispensible for many cellular processes, is thus tightly regulated to balance between need and toxicity. Using fission yeast as a model system, we explored the relationship between $H_2O_2$ and the iron starvation response system, specifically whether cross-talk allowed mutual regulation that could prevent synergistic toxicity of ROS via diminishing iron quantity.

We screened $\sim$2700 haploid *Schizosaccharomyces pombe* deletion mutants in different oxidative stress agents, identifying new genes amongst which *fep1*, *pcl1* and *sib2* are involved in iron homeostasis. $H_2O_2$, unexpectedly, triggers transcriptional iron starvation response, including enhanced iron import and decreased iron consumption in an indirect Sty1- and/or Atf1- dependent manner. Over-expression of several antioxidant proteins, in particular heme-containing catalase, causes strong iron consumption within the cell, triggering the iron starvation pathway accidentally.

Furthermore, glutaredoxin Grx4 contains an iron-sulfur cluster (ISC) involved in iron sensing, underpinning regulation of the iron starvation response. Finally, we identify and characterize the frataxin homolog gene in *S. pombe*, *pfh1*. Deficiencies in frataxin provoke a neurodegenerative disease called Friedreich ataxia; the function of this protein remains controversial. We create $\Delta$*pfh1* strain as a new model system to elucidate the molecular events leading to the disease.

## Resumen

El peróxido de hidrógeno ($H_2O_2$), una especie reactiva de oxígeno (ROS), está involucrado tanto en estrés oxidativo como en cascadas de señalización de una manera dosis dependiente. Parte de su toxicidad se explica, a travós su reactividad con hierro a través de la reacción de Fenton. El hierro, indispensable para muchos procesos celulares, está severamente regulado entre la necesidad y la toxicidad del mismo. Usamos la levadura de fisión como sistema modelo, exploramos la relación entre $H_2O_2$ y la respuesta de inanición del hierro, específicamente si la regulación mutua pudier aprevenir la toxicidad sinérgica de ROS atraves de la disminuyendo del hierro.

Hemos examinado $\sim$2700 mutantes por delecion haploides de *Schizosaccharomyces pombe* en diferentes agentes oxidantes e identificamos nuevos genes, entre ellos textitfep1, *pcl1* y *sib2* que están implicados en la homeostasis del hierro. $H_2O_2$, de forma inesperada, desencadena la respuesta transcripcional por bajada de hierro, aumentando la importación y disminuyendo el consumo del mismo de una manera indirecta pero de una forma Sty1-y / o Atf1-dependiente. La sobre expresión de proteínas antioxidantes, en particular la catalasa (contiene un grupo hemo), genera un alto consumo de hierro dentro de la célula, desencadenando accidentalmente la respuesta de bajada de hierro.

Por otra parte, glutaredoxina Grx4 contiene un cluster hierro azufre (ISC) que participan en la detección de hierro, que sustenta la regulación de la respuesta por bajada de hierro. Por último, identificamos y caracterizamos el gen homólogo de la frataxina en *S. pombe*, *pfh1*. Las deficiencias en la frataxina provocan una enfermedad neurodegenerativa llamada ataxia de Friedreich, la función de esta proteína sigue siendo controvertida. Generamos la cepa $\Delta$*pfh1* como un nuevo sistema modelo para elucidar los eventos moleculares que conducen a la enfermedad.

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# Chapter 1

# INTRODUCTION

## 1.1. Homeostasis of reactive oxygen species (ROS)

### 1.1.1. Reactive oxygen species

The early reducing atmosphere was converted into an oxidizing one due to the emergence of photosyntetic organisms. Evolution is driven by heritable adaptations which improve environmental 'fit'. Dramatical change in the atmosphere composition stimulate the biodiversity of life forms on Earth, leading to the near-extinction of oxygen-intolerant species and the proliferation of organisms that use oxygen ($O_2$). Oxygen is the most powerful oxidizing agent in aerobic organisms, in which it exists with an oxidation number (the charge that the oxygen atom would have in its ionic form) ranging from 0 in $O_2$ to -2 in water ($H_2O$):

$$O_2 + 4e^- + 4H^+ \rightarrow 2H_2O$$

The complete reduction of oxygen to water utilizes oxygen as an electron acceptor. During this process partially reduced forms can be generated, including radical forms (that is, those containing free or impaired electrons) and non-radical (parcially reduced) forms. Owing to their high oxidizing power, these intermediate reduction states of $O_2$ are called reactive oxygen species (ROS). One of the properties of these molecules is their reduction potential, a quantity that measures, in volts(V), the relative affinity of a substance for electrons as compared with hydrogen (which is set at 0). Molecules that have lower reduction potentials are strongly electronegative and are ready to accept electrons. There

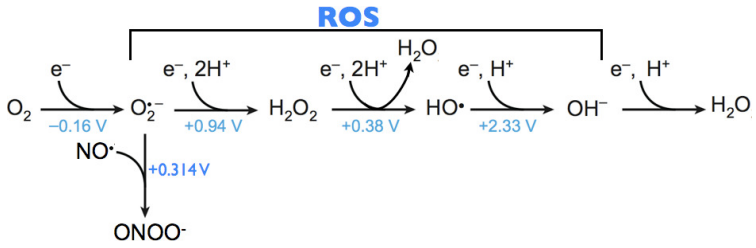


Figure 1.1: Reactive oxygen species (ROS). The redox states of oxygen with standard reduction potentials. The standard concentration of oxygen is 1 M. Abbreviations: $O_2$ oxygen, $e^-$ electron, $H^+$ hydrogen, $O_2^{\bullet-}$ $O_2^{\bullet-}$, $NO^\bullet$ nitric oxide, $ONOO^-$ peroxynitrite, $OH^\bullet$ hydroxyl radical, $OH^-$ hydroxide ion, $H_2O_2$ $H_2O_2$, $H_2O$ water. Adapted from [2, 3].

are many types of ROS in living systems, the three principal intermediate products generated from oxygen reduction are: $O_2^{\bullet-}$ anion ($O_2^{\bullet-}$), $H_2O_2$ ($H_2O_2$) and hydroxyl radical ($HO^\bullet$). The formation of ROS during the process of reducing $O_2$ to water are dividing in the following steps:

(1) $O_2 + 1e^- + H^+ \leftrightarrow HO_2^\bullet \leftrightarrow H^+ + O_2^{\bullet-}$
(2) $HO_2^\bullet + 1e^- + H^+ \leftrightarrow H_2O_2$
(3) $H_2O_2 + 1e^- + H^+ \leftrightarrow [H_3O_2] \leftrightarrow H_2O + HO_2^\bullet$
(4) $HO^\bullet + 1e^- + H^+ \leftrightarrow H_2O$

**$O_2^{\bullet-}$ anion ($O_2^{\bullet-}$)**
$O_2^{\bullet-}$ anion is formed through a single electron reduction of $O_2$, it is a relatively stable intermediate but because $O_2^{\bullet-}$ is charged, it cannot cross membranes. The most important routes of $O_2^{\bullet-}$ creation are the mitochondrial respiratory chain and the heme oxidation. The $O_2^{\bullet-}$ anion is the precursor of most ROS, it can interact with other molecules to generate secondary ROS either spontaneously, or via enzyme- or metal-catalysed processes.

**Hydrogen peroxide ($H_2O_2$)**
$H_2O_2$ has the lower reactivity, the highest stability and the highest intracellular concentration (see Table 1.1). $H_2O_2$ can be formed, for example, by the dismutation reaction of $O_2^{\bullet-}$ catalysed by $O_2^{\bullet-}$ dismutases (SODs) via hydroperoxyl

radical.

**Hydroxyl radical ($HO^{\bullet}$)**
Hydroxyl radical has a high oxidant power that makes it the most dangerous and reactive radical. It has a very short in-vivo half-life of about $10^{-9}$s [4], this means that as soon it is produced it reacts close to its site of formation with the first molecule it meets. Hydroxyl radical is mainly generated when there is an excess production of $O_2^{\bullet -}$ anion and $H_2O_2$ in the Haber-Weiss reaction (see Equation 1.1) [5] or by the metal ion (iron or cooper) catalysed decomposition of $H_2O_2$ (known as the Fenton reaction (see Figure 1.10) [6, 7]. Organisms that suffer an overload of iron (for example in hemochromatosis, $\beta$-thalassemia, hemodialysis) contain higher amounts of free available iron which can react with $HO^{\bullet}$ having deleterius effects. Finally another source of $HO^{\bullet}$ is caused by ionising radiation that decomposes water, resulting in the formation of $HO^{\bullet}$ and hydrogen atoms, as well as photolytic decomposition of alkylhydroperoxides.

Haber-Weiss reaction:

$$O_2^{\bullet -} + H_2O \rightarrow OH^- + HO^{\bullet} + O_2 \tag{1.1}$$

Fenton reaction:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\bullet} + OH^- \tag{1.2}$$

**Singlet oxygen ($^1O_2$)**
Singlet oxygen is an excited form of dioxygen in which the $\pi$-antibonding electrons are spin-paired. It can be formed by energy transfer to oxygen by excited chromophores and is generated in photosynthetic systems. The toxic actions of singlet oxygen have been reviewed elsewhere and are not considered here (review in [8]).

**Reactive nitrogen species (RNS)**
Another group of powerful oxidizing molecules are the reactive nitrogen species (RNS). RNS are derived from nitric oxide (NO) and are able to promote modification in thiol groups to yield *S*-nitrosothiols [9]. One of the most potent RNS

| | $O_2^{\bullet -}$ | $H_2O_2$ | $OH^{\bullet}$ |
|---|---|---|---|
| Redox potential (V) | 0. 94 | 0. 32 | 2. 31 |
| Half-life (sec) | $10^{-6}$ | $10^{-5}$ | $10^{-9}$ |
| Concentration in vivo (M) | $10^{-10}$ | $10^{-7}$ | $10^{-15}$ |

Table 1.1: ROS properties

derivatives is the peroxynitrite ($ONOO^-$); this molecule is the product of the coupling reaction of two free radicals, nitric oxide (nitrogen monoxide, NO•) and $O_2{}^{\bullet -}$. The nitric oxide is a small nonpolar, and rather unreactive free radical formed enzymatically by NOS (nitric oxide synthetase) enzymes. Peroxynitrite is in itself very reactive against specific targets; it can lead to nitration of tyrosine residues that range from efficient detoxification systems, such as peroxiredoxins, to reactions eventually leading to enhanced radical formation (e. g. , nitrogen dioxide and carbonate radicals).

### 1.1.2. ROS origins

ROS are generated in multiple compartments and by multiple enzymes within the cell. Important contributions towards ROS generation include proteins within the plasma membrane, such as: the family of NADPH oxidases [10], various cytosolic enzymes such as cyclooxygenases, lipid metabolism within the peroxisomes, monoxygenase system (cytochrome P450), ionizing radiation [11], as well as the inflammatory cell activation (neutrophils, eosinophils,macrophages) [12]. However, the main source of ROS have traditionally been regarded as products of aerobic metabolism. Mitochondrial respiration is considered to be the major intracellular source of accidental ROS production [13, 14].

Mitochondria take up oxygen and reduce more than 95% of it to water by adding four electrons step by step, a process achived by cytochrome oxidase (complex IV). The cytochrome c oxidase is a large and complex multiprotein assembly, it removes one electron from each of four reduced $Fe^{2+}$-heme groups of cytochrome c molecules, oxidizing them to ferric cytochrome c, and adds the four electrons on to $O_2$. During mitochondrial respiration, oxidative phosphorylation takes place. Controlled oxidation of NADH (reduced form of nicotinamide adenine dinucleotide) or FADH (reduced form of flavin adenine din-

ucleotide) creates a potential energy by the flux of electrons across the mitochondrial inner membrane. This potential energy is used to phosphorylate ADP (Adenosine-5'-diphosphate) via the F1-F0 ATPase and synthetase ATP (Adenosine-5'-triphosphate). Electrons are extracted from reduced subtrates and are transferred to $O_2$ through a chain of enzymatic complexes (I to IV). In the final step of the electron-transfer chain (ETC), cytochrome c oxidase ensures the complete reduction of $O_2$ to $H_2O$ without the formation of oxygen radicals.

However, if the $O_2$ interacts with the ETC upstream of complex IV a partial reduction of $O_2$ can occur resulting in the generation of ROS. Some electrons derived from NADH or FADH can escape from the mitochondrial ETC, especially from complex I and II and react with $O_2$ to form $O_2^{\bullet -}$ [15]. The rate at which these enzymes produce $O_2^{\bullet -}$ depends directly upon the frequency of collisions with $O_2$; thus it is first order in $O_2$ concentration [13, 14]. An important implication is that bacteria can minimize such sources of oxidative stress by dwelling in environments that are low in $O_2$ [13, 14]. The increase in ROS formation is often seen in the condition of electron leakage out due to reverse electron flow, including the use of ETC inhibitors (as the antimicyn A) or a deletion of one of the components of the ETC [16, 17, 18, 19] (see Figure 1.2).

The ability of the mitochondrial matrix to withstand large transient loads of free radicals with minimal damage using these protection mechanisms has been demonstrated to be a highly efficient scavenging system. This suggests that any measured release of ROS from mitochondria may represent only a small fraction of the total ROS generated. The basal value of ROS generation is around 0.2% of the total oxygen consumption [17, 18].

Finally other cause of increase ROS formation in mitochondria is the release of cytochrome c during apoptosis, which results in a block in electron flow. Also, when complex III is inhibited the turnover of the coenzyme Q cycle is blocked, generating an active ubisemiquinone capable of reducing $O_2$ to $O_2^{\bullet -}$ [20].

Another physiological regulator of ROS production is the family of uncoupling proteins (UCPs). Uncoupling protein 1 (UCP1) diverts energy from ATP synthesis to thermogenesis in the mitochondria of brown adipose tissue by catalysing a regulated leak of protons across the inner membrane [21]. In response to oxidants, UPCs cause a mild uncoupling, leading to lowered proton motive force, which reduce the membrane potential ($\Delta\Psi$) and ROS levels as a consecuence. Thus the uncoupling has been proposed as a feedback loop, which constitutes a self-limiting cycle to protect against excessive $O_2^{\bullet -}$ production and

in the modulation of ROS-dependent signalling [22, 21, 23].

superoxide releases iron from aconitase, leading to a cascade of lipid peroxidation and the release of molecules such as hydroxynonenal that covalently modify and activate the proton conductance of UCPs and other proteins. A function of the UCPs may be to cause mild uncoupling in response to matrix superoxide and other oxidants, leading to lowered proton motive force and decreased superoxide production. This simple feedback loop would constitute a self-limiting cycle to protect against excessive superoxide production, leading to protection against aging, but at the cost of a small elevation of respiration and basal metabolic rate.

Besides mitochondria, there are other cellular sources of ROS; for example, dihydrorotate dehydrogenase, aldehyde oxidase and xanthine oxidase. In particular, xanthine oxidase which is a member of a group of enzymes known as molybdenum-iron-sulphur flavin hydroxylases, catalyses the hydroxylation of purines causing oxygen reduction by the transformation of hypoxanthine and xanthine to uric acid. This leads to production of $H_2O_2$ and $O_2^{\bullet -}$.

Another important route through which it is possible to form $O_2^{\bullet -}$ is represented by heme oxidation. The iron of the heme is reduced to $Fe^{2+}$ in deoxyhemoglobin, but when it attaches oxygen, an intermediate structure in which an electron is delocalized between $Fe^{2+}$ and $O_2$ is created:

$$\text{Heme } Fe^{2+}\text{-}O^2 \leftrightarrow \text{Heme } Fe^{3+}\text{-}O_2^{\bullet -}$$

The result is an intermediate between $Fe^{2+}$ bound to oxygen and $Fe^{3+}$ bound to $O_2^{\bullet -}$. Sometimes the oxyhemoglobin molecule goes into decomposition and releases $O_2^{\bullet -}$ anion:

$$\text{Heme } Fe^{3+}\text{-}O_2^{\bullet -} \leftrightarrow O_2^{\bullet -} + \text{Heme } Fe^{3+}$$

The methemoglobin is the product with $Fe^{2+}$ in heme and is unable to bind oxygen. About $3\%$ of hemoglobin in erythrocytes undergo oxidation every day. The burden of ROS is amplified by the presence of free metals, such as iron, copper and manganese, that are released from metalloprotein complexes [24, 25, 26].

Free radicals can also be produced by a host of exogenous processes such as environmental agents and xenobiotics (metal ions, radiation, barbiturates) [27]. Xenobiotics accept an electron from a respiratory carrrier and transfer it to molecular oxygen (a process called redox cycling), stimulating $O_2^{\bullet -}$ formation without inhibiting the respiratory chain to suppress the growth of competitors [28]. The herbicide paraquat (1,1'-dimethyl-4,4'-bipyridium) or the polycyclic

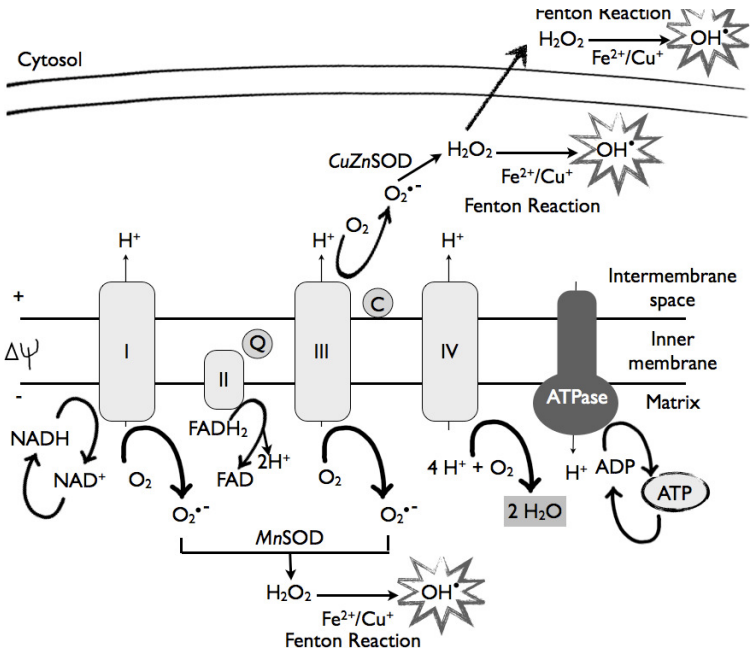


Figure 1.2: Electron transport chain (ETC) the main source of ROS in the cell. Abbreviations: $H_2O_2$ $H_2O_2$, $O_2^{\bullet-}$ $O_2^{\bullet-}$, $Fe^{2+}$ ferrous ion, $H^+$ proton, $OH^\bullet$ hydroxyl radical, $Cu^+$ cuprous ion, *CuZn*SOD cooper zinc $O_2^{\bullet-}$ dismutase, *Mn*SOD cooper zinc $O_2^{\bullet-}$ dismutase, Q coenzime q, C cytochrome c, NADH reduced form of nicotinamide adenine dinucleotide, NAD $^+$ oxide form of Nicotinamide adenine dinucleotide, FADH reduce form of flavin adenine dinucleotide, $FAD^+$ oxide form of flavin adenine dinucleotide, ADP Adenosine-5'- diphosphate, ATP Adenosine-5'-triphosphate, complex I (NADH dehydrogenase), complex II (succinate dehydrogenase), complex III (cytochrome bc1), complex IV (cytochrome c), ATPase ATP synthetase, $\Delta\Psi$ membrane potential.

aromatic ketone, menadione (2-methyl- naphthalene-1,4-dione) are the two redox cycling agents commonly used in yeast studies [29, 30].

Summarizing, aerobic organisms have to deal with the production of ROS in order not to reach toxic concentrations that cause oxidative stress and cell damage. Oxygen derivatives are more reactive than oxygen itself, the majority of them are generated during respiration. ROS includes $O_2^{\bullet -}$ anions, hydroxyl radicals, and $H_2O_2$ (see Figure 1.1). Different ROS have distinct properties, including reduction potential, half life, intracellular concentration and diffusibility through membranes (see Table 1.1).

### 1.1.3. Redox state and oxidative stress

Organisms are constantly exposed to a multiplicity of systems that generate ROS. Each cell is characterized by its 'redox state'. The redox state describes the ratio of the interconvertible oxidized and reduced form of a specific redox couple, such us $NAD^+$/NADH couple, oxidized/reduce GSH or oxidized/reduced thioredoxin [31]. When the redox state is unbalanced oxidizing damage in macromolecules occurs; this event is known as oxidative stress. Oxidative stress represents an imbalance between the production and detoxification (enzymatic or non enzymatic) of ROS. The imbalance that increases ROS could be due to: metabolic changes, damage in the detoxifying enzymes, radiation or drug exposure. Damage to macromolecules upon oxidative stress has been associated with the ageing process and with chronic diseases including cancer, coronary heart disease and neurological disosrders in humans [32, 33, 34, 35].

### 1.1.4. ROS toxicity: target molecules

Moreover, oxidative stress is commonly viewed as a condition under which the generation of ROS within a cellular system exeeds the buffering capacity of endogenous antioxidant defenses leading to oxidative damage involving lipids, deoxyribonucleic acid (DNA), proteins and carbohydrates. But the basis of this universal phenomenon is not intuitive; after all, the structural molecules from which cells are made (amino acids, lipids, and nucleic acids) are reasonably stable in aerobic buffers.

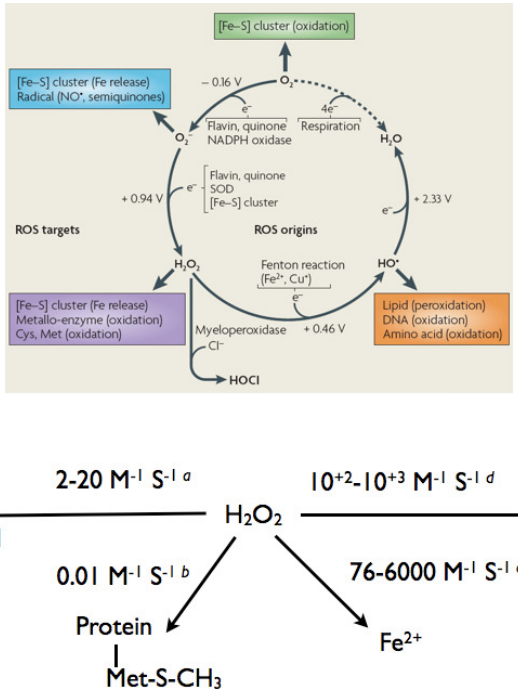


Figure 1.3: ROS sources, biochemical properties and targets damaged. Modified from [36].

### DNA oxidation

Common forms of oxidative DNA damage involve single- or double-stranded DNA breaks, depurination/depyrimidation or deoxyribose modifications, DNA-DNA or DNA-protein cross-links [37] and chemical modification of sugar the moieties [38]. These modifications lead to mutations, especially in hot spots, due to exacerbation of polymerase-specific conformations that are error prone [39], replication errors and genomic instability. This adduct formation interferes with normal cell growth altering normal gene transcription and affecting several signalling pathways. The formation of hydroxylated bases of DNA are considered an important event in chemical carcinogenesis [40] and ageing [41, 42]. The most extensively studied DNA lesion due to oxidative damage is the formation of 8-hydroxy-2'-deoxyguanosine (8-OHdG) produced by hydroxylation in the C-8

position of deoxyguanosine residues by the hydroxyl radical [43] and has been used as a measurement of oxidative DNA damage.

### Lipid peroxidation

ROS also attacks polyunsaturated fatty acids residues of phopholipids to produce lipid peroxyl radicals and lipid hydroperoxides [44]. Once formed, peroxyl radical ($ROO^{\bullet}$) can be subsequently propagated into malondialdehyde (MDA) through a cycle reaction (see the lipid peroxidation steps described Figure 1.4) [45]. MDA is consider carcinogenic in rats and mutagenic in bacterial and mammalian cells, but the major toxic product of lipid peroxidation is the 4-hydroxy-2-noneal (HNE) [45]. MDA is detected in Parkinson's disease [46].

Lipid radicals ($R^{\bullet}$) can diffuse through membranes, thus modifying the structure and function of the membrane and resulting in a loss of cell homeostasis. In addition, lipid peroxides may result in the interaction with cellular DNA and cause the formation of DNA-MDA adducts [47].

**Steps of lipid peroxidation:**

**Initiation**

$$\text{Lipid} + R^{\bullet}/HO^{\bullet} \rightarrow \text{Lipid}^{\bullet}$$

**Propagation**

$$\text{Lipid}^{\bullet} + O_2 \rightarrow \text{Lipid-O-O}^{\bullet}$$

$$\text{Lipid-O-O}^{\bullet} + \text{Lipid} \rightarrow \text{Lipid-O-O}^{\bullet} + \text{Lipid}^{\bullet}$$

**Termination**

$$\text{Lipid}^{\bullet} + \text{Lipid}^{\bullet} \rightarrow \text{Lipid-Lipid}$$

$$\text{Lipid-O-O}^{\bullet} + \text{Lipid}^{\bullet} \rightarrow \text{Lipid-OO-Lipid}$$

Figure 1.4: Lipid peroxidation cycle. Reprinted from [24].

**Protein oxidation**

Proteins are also easily attacked by ROS directly or indirectly (through lipid peroxidation) producing covalent modifications. There are numerous different types of protein oxidative modifications due to the existence of many mechanisms that induce protein oxidation and because all amino acids side chains can become oxidatively modified. Also, products of free amino acid oxidation can also form covalent attachments to proteins [48]. The oxidative modifications of proteins are summarized in Table 1.1.4 and some of them will be discussed briefly below.

**Modification of radicals in amino acids**

The most prone amino acid residues to oxidative attack are Cys (Cys) and methionine (Met) residues [49, 50, 51], both of which contain susceptible sulphur (S) atoms. Under physiological conditions the major product of oxidation of Met residues is Met sulphoxide [49], that could be reversed by Met sulphoxide reductase (see section 1.1.5.2). In the case of Cys residues, they are susceptible to reversible and irreversible oxidation. Mainly solvent-exposed Cys residues with a low $pK_a$ are susceptible to oxidation by $H_2O_2$. The $pK_a$ of a particular Cys residue is dependent on the charge of the adjacent aas in the tertiary and quaternary protein structure [52, 53, 54]. The oxidation of thiol (SH) group of Cys residues leads to the formation of disulfide bonds (S-S), mixed disulfides (e. g. , with GSH, S-S-GSH) and thiyl radicals [55, 56]. The capability of Cys residues to engage into electron transfer reactions has made them critical residues in cell signaling, enzimatic reactions and metal ion coordination events. Thus, the catalitic centers of proteases, tyrosine phosphatases, ribonucleotide reductases, antioxidant proteins, haemoglobin, chaperones or transcription factors do contain essential Cys residues. Therefore, oxidation of this essential Cys residues may be one cause of $H_2O_2$ toxicity.

Proteins irreversibly inactivated by formation of methionine sulfones, Cys sulfinic ($SO_2H$) or sulfonic ($SO_3H$) acids and carbonyl derivatives cannot be repaired and have to be recognized and degraded by cellular proteolytic processes. It is worth mentioning that $SO_2H$ in peroxiredoxins can be reversed by two families of enzymes (for a review, see [57]).

Other amino acids may require more stringent conditions for oxidative modification such as site-specific metal-catalyzed oxidation (MCO) or gamma irradiation. A potent mode of direct oxidative attack on a protein derives from site-

specific MCO, in the presence of transition metals (e. g. $Fe^{2+}$, $Cu^{+}$) an oxidative scission can be observed [58, 59, 60, 61, 62]. His is the most susceptible amino acid to MCO and generates oxo-His or aspartate (Asp) [63, 64]. A discussion of mechanisms of induction of protein carbonyls by MCO can be found in [65] and [66]. Gamma-irradiation causes direct formation of hydroxyl radical in addition to inducing MCO and may cause more generalized protein damage, which is not restricted to the metal-binding site in the protein [62]. For this reason, gamma irradiation may cause a higher degree of protein agregation and chemical degradation than the MCO. Hydrophobic amino acyl residues such as valine, leucine, and tyrosine (Tyr) are oxidized to hydroxy- and hydroperoxy- derivatives upon exposure to gamma irradiation in aerobic conditions [55, 67].

Another target of radical attack are Tyr residues, that leads to formation of dityrosyl cross-links [68].

**FeS cluster attack**

In addition, it was early studies in *E. coli* that revealed that hyperoxia specifically blocks the ability to synthesize branched-chain amino acids[69]. However, oxygen was not the direct toxin; instead toxicity was due to increasing intracellular ROS: $O_2^{\bullet -}$ and $H_2O_2$. ROS inactivate the [4Fe-4S] family of dehydratases, including key enzymes of the branched-chain and TCA pathways: dihydroxyacid dehydratase, aconitase and fumarase. These enzymes utilizes a solvent-exposed [4Fe-4S] cluster as a Lewis acid during substrate dehydration [70, 71, 72, 73]. Three of the four iron atoms in the enzyme cluster are each coordinated by four sulphur ligands, but the fourth iron atom has a coordination site occupied in the resting enzyme only by a $H_2O$ molecule. The damage occurs when $O_2^{\bullet -}$ directly oxidizes the FeS cluster, converting the $[4Fe\text{-}4S]^{2+}$ form to an unstable $[4Fe\text{-}4S]^{3+}$ state, which releases iron (see Figure 1.5). The resultant $[3Fe\text{-}4S]^{1+}$ cluster lacks the catalytic iron atom, so the enzyme becomes inactive and the pathway fails. $H_2O_2$ can oxidized these clusters in similar fashion [74]. The rate constants with which dehydratase clusters react with $O_2^{\bullet -}$ and $H_2O_2$ are extremely high: $3 \times 10^{6}$ $M^{-1}$ $s^{-1}$ and $4 \times 10^{3}$ $M^{-1}$ $s^{-1}$ respectively [73]. Therefore, *E. coli* must synthesize enough $O_2^{\bullet -}$ dismutase, catalase and peroxidase to restrict $O_2^{\bullet -}$ and $H_2O_2$ to non-toxic levels: $10^{-10}$ M [75] and $10^{-7}$ M [76], respectively. Below these low levels, ROS participates in cell signalling processes as cell growth, metabolism and defense.

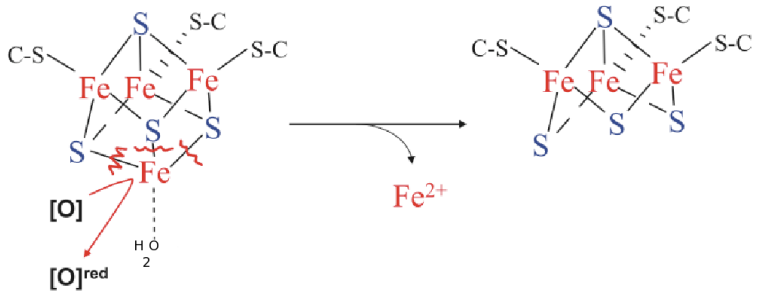


Figure 1.5: FeS cluster destruction by oxidation. Univalent oxidants, including $O_2^{\bullet -}$, $H_2O_2$, molecular oxygen and peroxynitrite (collectively denoted [O]) convert the exposed $[4Fe\text{-}4S]^{2+}$ cluster of aconitase-family enzymes to the unstable +3 oxidation state. The cluster then spontaneously decomposes to the inactive $[3Fe\text{-}4S]^{1+}$ form. Further degradation gradually occurs in vitro and in vivo, although the rate and ultimate products are not well established and probably vary from protein to protein. Reprinted from [26].

**Carbonylation**

However, the major form of protein oxidation is carbonylation; carbonyl groups are widely used as an indicator of oxidative stress [36], in part based on the fact that carbonyl modification can be produced by a wide variety of ROS including as products of lipid oxidation. When proteins react with hydroxyl radical an hydrogen atom is removed from protein polypeptide, forming a carbon-centred radical which readily reacts with dioxygen to form peroxyl radicals under aerobic conditions. Specific protein carbonylations are thought to be of additional significance, beyond their use as a biomarker, because they can work as biological signals or confer irreversible loss of protein function in connection with disease [77, 78, 79]. There are three types of aa oxidative modifications that can give rise to the formation of carbonyl groups:
1) direct oxidation of specific amino acids (lysine (Lys), arginine, proline and His) and protein backbone cleavage (at proline, glutamate and Asp residues) [62]
2) modification of His, Cys and Lys residues by lipid peroxidation products such as MDA or HNE [77]. Lipid peroxidation lead the addition of aldehyde moieties to proteins [80, 81, 82, 83, 84].
3) reaction with reducing sugars, forming advanced glycation end product and

adducts [85]. Carboxymethyllysine represents a form of protein modification generated by the oxidation products of sugars [86, 87, 88].

Table 1.2: Oxidative Modifications of Proteins. Reprinted from [1]

| Modification | aas involved | Oxidizing source [a] |
|---|---|---|
| Disulfides, glutathiolation | Cys | All, $ONOO^-$ |
| Methionine sulfoxide | Met | All, $ONOO^-$ |
| Carbonyls (aldehydes,ketones) | All (Lys, Arg, Pro, Thr) | All |
| Oxo-histidine | His | $\gamma$-Ray, MCO, $^1O_2$ |
| Dityrosine | Tyr | $\gamma$-Ray, MCO, $^1O_2$ |
| Chlorotyrosine | Tyr | HOCl |
| Nitrotyrosine | Tyr | $ONOO^-$ |
| Tryptophanyl modifications (N-formyl)kynurenine | Trp | $\gamma$-Ray |
| Hydro(pero)xy derivatives | Val, Leu, Tyr, Trp | $\gamma$-Ray |
| Chloramines, deamination | Lys | HOCl |
| Lipid peroxidation adducts (MDA, HNE, acrolein) | Lys, Cys, His | $\gamma$-Ray, MCO (not HOCl) |
| aa oxidation adducts | Lys, Cys, His | HOCl |
| Glycoxidation adducts | Lys | Glucose |
| Cross-links, aggregates, fragments | Several | All |

[a] MCO (metal catalyzed oxidation), All = $\gamma$-Ray, MCO, HOCl, ozone
$^1O_2$, aa (amino acid), valine (Val), histidine (His), leucine(Leu), tyrosine (Tyr), cysteine (Cys), histidine (His), tryptophane (Trp), arginine (Arg), proline (Pro), methionine (Met), lysine (Lys).

### 1.1.5. Antioxidant response

The partially reduced oxygen species are inevitable by-products of aerobic metabolism. The organisms that developed antioxidant defence mechanisms which prevent and neutralise toxic $O_2$ intermediates became wide- spread and

the predominate biological systems. As a defense against oxidative injury, cells have evolved a multilayered interdependent antioxidant system that includes enzymatic and non enzymatic components [89]. The antioxidant system is mainly driven by catalases, $O_2^{\bullet -}$ reductases, thioredoxins, glutaredoxins and peroxidases that will be described in this section. When yeast cells are treated with low concentration of either $H_2O_2$ or menadione, adaptative response is induced, which protect against a subsequent challenge with the higher concentration of these oxidants [90]. This enhance of tolerance is due to induction of various enzyme activities of oxidative defense system [90, 91].

**Detoxifying enzymes**

**$O_2^{\bullet -}$ dismutases (SODs)**

The discovery made by McCord and Fridovich in 1968-1969 of the existence of SOD [92]; the concomitant proof of that $O_2^{\bullet -}$ can be produced by enzymes [93]; and that free radicals can be substrates for an enzyme, completely revolutionized the thinking on the role of free radicals and related oxidants in biology [94].

SODs have an antioxidant function by catalyzing dismutation of $O_2^{\bullet -}$ to $H_2O_2$ and $O_2$, and their activity requires redox active metal ions. Several common forms of SOD exist: they are metalloproteins cofactored with copper (Cu) and zinc (Zn), or either manganese (Mn), iron (Fe), or nickel (Ni). Thus, there are four major families of SODs, depending on the metal cofactor: CuZnSOD type (which binds both copper and zinc) [92], MnSOD type (which binds manganese) [95, 96], FeSOD type (which binds iron) [96] and the Ni type (which binds nickel) [97]. The SOD-catalysed dismutation of $O_2^{\bullet -}$ could be written with the following half-reactions:

$M^{(n+1)}$-SOD + $O_2^{\bullet -} \rightarrow Mn^{+}$-SOD + $O_2$

$M^{+}$-SOD + $O_2^{\bullet -}$ + $2H^{+} \rightarrow M^{(n+1)+}$-SOD + $H_2O_2$.

where M = Cu (n=1) ; Mn (n=2) ; Fe (n=2) ; Ni (n=2).

In this reaction the oxidation state of the metal cation oscillates between n and n+1.

Iron or manganese are used by prokaryotes, protistas and in mitochondria. Meanwhile, CuZnSOD are the most common cytosolic enzymes in eukaryotes. The eukaryotic cells contain a CuZnSOD cytosolic; also a glycosylated extracellular CuZnSOD and a mitochondrial MnSOD [94]. MnSOD has been the subject of

particular interest because represents the first line of defense againts $O_2^{\bullet -}$ radicals produced as byproducts of oxidative phosphorylation [98].

*S. pombe* possess two SODs: the Cu,Zn- depending cytosolic Sod1 and the Mn-depending Sod2, which localizes at the mitochondrial matrix[99]. *S. pombe* cells deficient in Cu,Zn-SOD or Mn-SOD are hypersensitive to $O_2^{\bullet -}$ generators, but the disruption of Sod1 gene do not show a defective growth in aerobic conditions [99, 100, 101]. This means that in fission yeast under normal cell growth the $O_2^{\bullet -}$ levels generated are sublethal.

**Catalase**

Catalase is an enzyme that reduces $H_2O_2$ to $H_2O$ and $O_2$, using the redox properties of a heme group complexed to the polypeptide.

$$2\ H_2O_2 \overset{catalase}{\rightarrow} 2\ H_2O + O_2$$

*S. pombe* has just one catalase called Ctt1, which is cytosolic. Ctt1 expression is induced under enviromental stress such as: oxidative, osmotic, heat or cadmium stresses [102]. A mutant, which is deficient in catalase activity in fission yeast, showed increased susceptibility to $H_2O_2$ but showed no growth defect in medium without $H_2O_2$ [103]. This suggests that the level of $H_2O_2$ generated in vivo in aerobically growing cells is low and does not affect the growth or the mutation rate of cells lacking catalase. Meanwhile, cells overexpressing catalase showed increased resistance to $H_2O_2$ relative to the wild type [103].

**Thioredoxin system**

Thioredoxin (Trx) are small thiol oxidoreductases which contain two conserved Cys residues at its active site. Due to its low redox potential (-270 mV) Trx proteins participate in protein thiol reduction [104]. Trx reductase (TrxR) completes the Trx system. TrxR reduces oxidised Trx to the active thiol form using NADPH [105]. All enzymes involved in thiol-disulfide metabolism have a conserved motif CGPC, also referred to as the Trx fold motif in the active site. While these enzymes are capable of catalyzing both disulfide bond formation and reduction, they have evolved to perform one or the other reaction more efficiently. The reactions that Trx undergoes are:

$$\text{Trx-(SH)}_2 + \text{X-S}_2 \overset{TrxR}{\longleftrightarrow} \text{Trx-S}_2\text{+ X(SH)}_2$$

$$\text{Trx-S}_2\text{+ NADPH} \overset{TrxR}{\longleftrightarrow} \text{Trx-(SH)}_2 + \text{NADP}^+$$

The genome of *S.pombe* harbors three genes for thioredoxins, *trx1*, *trx3*/*txl1* and *trx2*, which encode two cytosolic and one mitochondrial thioredoxins, respec-

tively. The Δ*trx1* mutant was found sensitive to diverse external stressors such as various oxidants, heat, and salt, whereas Δ*trx2* mutant was not sensitive except to paraquat (a $O_2^{\bullet -}$ generator). The Δ*trx1* mutant exhibited Cys auxotrophy, which can be overcome by adding sulfite. At a more general level, the thioredoxin system has been suggested participates in moduling the redox state of cell protein sulphydryls and consequently in protein folding [106, 107].

**Glutaredoxin system**

Glutaredoxins (Grxs) are a class of small proteins with an active site containing two redox sensitive Cys like all enzymes involved in thiol-disulfide metabolism and a thioredoxin-like fold. Grxs act as thiol oxidoreductases responsible for the reduction of protein disulphides or GSH-protein mixed disulphides. These proteins are thought to act in much the same way as Trxs but they differ in that Grxs use GSH as hydrogen donor [105, 108, 109]. The Grx system also includes NADPH and GSH reductase. Glutathione reductase is the enzyme which regenerate GSH from oxidised GSH disulphide (GSSG) formed during the Grx reaction. Classical dithiol Grxs contain the CPYC motif at the active site, whereas that of the monothiol enzymes is CGFS [110].

Dithiol mechanism:

$$\text{R-S}_2 + \text{Grx-(SH)}_2 \rightarrow \text{R-(SH)}_2 + \text{Grx- S}_2$$
$$\text{Grx- S}_2 + 2\ \text{GSH} \rightarrow \text{Grx-(SH)}_2 + \text{GSSG}$$

Monothiol mechanism:

$$\text{R-S-SG} + \text{Grx(SH)}_2 \rightarrow \text{R-SH} + \text{Grx-S-SG}$$
$$\text{Grx-S-SG} + \text{GSH} \rightarrow \text{Grx-(SH)}_2 + \text{GSSG}$$

where R-S-SG is a mixed disulphide with GSH.

**Peroxidases (Prxs)**

The ROS-detoxifying role of SODs and catalase depends on the redox properties of the metal group associated to the enzyme. This is not the case of peroxidases, which reduce inorganic and organic peroxides into the corresponding alcohol and $H_2O$ using their Cys thiol active site [111, 112, 113, 114, 115]. The absence of peroxidase has been implicated in increased genome instability, tumor incidence, and associated with accelerated ageing [116, 117, 118]. Peroxiredoxin is a family of bifunctional proteins that exhibit peroxidase and chaperone activities upon heat shock [119, 120]. However, the main role of Prxs have classically been related to their peroxidase activity due to their high affinity for $H_2O_2$. The

$H_2O_2$ $K_m$ value for Prxs is $\sim$ 20 $\mu$M, that is higher than the $K_m$ for other peroxide scavengers such as catalase or GSH peroxidase, which suggests that Prxs are highly efficient at removing low concentrations of $H_2O_2$. Peroxidase activity requires electron donors for recycling the thiol groups. Depending on the latter reductant that is used, two classes of peroxidases are distinguised: GSH peroxidases (Gpxs), which employ GSH and Trx peroxidases (also called peroxiredoxins), which employ Trxs. There exist two types of Gpxs (for review see [121]). Classical Gpxs are multimeric, soluble and act on inorganic and organic hydroperoxides. Meanwhile, there are three types of structurally different Prxs: 1-Cys, 2-Cys and atypical 2-Cys [113]. All three types of Prxs are dimers in solution, and all of them share the same catalytic mechanism. The catalytic cycle of breaking down $H_2O_2$ by either of the two types of 2-Cys Prxs involves the oxidation of the peroxide-reactive peroxidatic Cys, located close to the N-terminal domain, followed by the formation of a sulfenic acid (RSOH) by either $H_2O_2$ or alkyl hydroperoxides. In both types of 2-Cys Prxs, the RSOH then reacts with the C-terminal (or resolving) Cys, in the case of the classical 2-Cys Prxs with the C-Terminal Cys of a neighboring Prx molecule to form an intermolecular disulfide, and in the atypical 2-Cys Prxs with the C-Terminal Cys of the same monomer to form an intramolecular disulfide. In both cases, Trx system then reduces this disulfide to restore the active peroxidase. In the 1-Cys Prxs the sulfenic is directly reduced to thiol, because there is no nearby Cys available to form a disulfide bond; the source of the reducing equivalents for regenerating this thiol is not known, although GSH has been proposed to serve as the electron donor in this reaction

Paradoxically, Prxs are susceptible to inactivation by $H_2O_2$-induced hyperoxidation of the peroxidatic Cys to sulfinic derivatives resistant to reduction by Trx [122, 113, 112]. In those circumstances sulfiredoxin (Srx) enzyme reduces these hyperoxidized sulfinic derivatives in the presence of ATP, regenerating the active Prx [123]. Srx specifically binds to 2-Cys Prx enzymes by recognizing several critical surface-exposed residues of the Prxs, and transfers the gamma-phosphate of ATP to their sulfinic moiety, using its conserved Cys as the phosphate carrier. The resulting sulfinic phosphoryl ester is reduced to Cys after oxidation of four thiol equivalents.

*S. pombe* has three isoenzymes of Prx including Tpx1 (thioredoxin peroxidase 1), Bpc (bacterioferritin comigratory protein) and Pmp20 (peroxisomal membrane protein 20, may act as a chaperone rather than peroxidase in the fis-

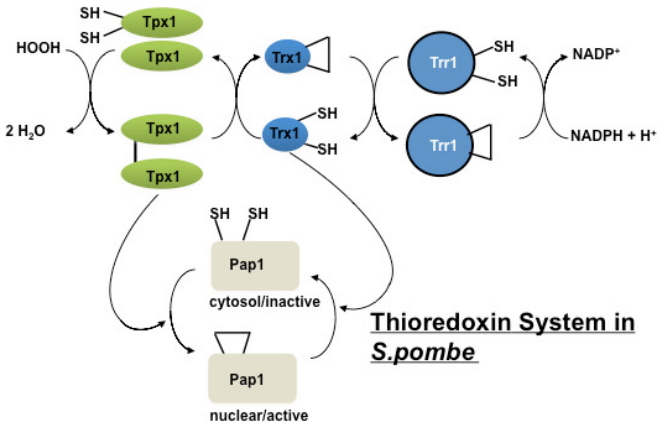


Figure 1.6: Thioredoxin system in *S. pombe*

sion yeast) and one Gpx called Gpx1 [124, 125]. Tpx1 is essential for aerobic growth [126]. For this reason, Tpx1 is consider as the main $H_2O_2$ scavenger in *S. pombe*, and its role is emphasised by the fact that strains lacking either of the other Prxs do not show any growth defects, even in the presence of extracellular $H_2O_2$ [127, 128].

Finally, it was through studies in *S. pombe* which showed the first evidence that Prxs participated in signaling transduction pathways in antistress response [129, 127, 128].

**Pentose phosphate pathway enzymes**

Enzymes such as glucose-6-phosphate dehydrogenase (ZWF1), transketolase (TKL1) and ribulose-5-phosphate epimerase (RPE1) are involved in the pentose phosphate metabolic pathway, and are crucial for the production of cellular reducing power in the form of NADPH. The enzymes GSH reductase and TrxR both require NADPH as a reductant to reduce GSSG, Grx and Trx. Therefore, given that GSH and thioredoxin are important antioxidants, it is perharps not surprising that mutations in *ZWF1*, *TKL1* and *RPE1* genes, negatively affecting the pentose phosphate pathway, render the cells hypersensitive to oxidants [130, 131, 132, 133].

**Methionine sulfoxide reductase (Msr)**

Methionine residues of proteins are especially susceptible to oxidation by ROS. It has been proposed that Met residues exposed on the surface of an enzyme can act as antioxidants to protect the active site of the enzyme [134]. Met residues would be oxidized to methionine sulphoxide, which leads to a mixture of *S*- and *R*-epimers of methionine sulphoxide. The enzyme methionine sulphoxide reductase (Msr) can reverse this process. This system therefore has a repair function and consists of two enzymes in *E. coli*, MrsA and MrsB, that repair the *S*- and *R*-epimers respectively [135]. Trxs act as electron donors for Msr-catalyzed reduction of methionine sulphoxide. In that way, the Trx and Msr systems can be considered as part of a cycle that would detoxify ROS at the expense of NADPH, through methionine sulphoxide intermediates [136]. The participation of Trxs in regulating the redox state of Msr enzymes has not been demostrated in the case of *S. cereviciae*. However, there are two genes (*MRX1* and *YCL033c*) coding respectively for the MsrA and MrsB activities and in their absence, hypersensitivity to ROS [137] and heavy metals [138] as well as accumulation of carbonylated proteins occurs [139]. This emphasizes the importance of Msr for protection against ROS *in vivo*.

**Non-enzymatic systems**

Besides the enzymatic protection against ROS and RNS, there are small molecules acting as antioxidants, such as GSH, manganese (Mn) or vitamin E [140]. Another antioxidant defence is the sequestration of transition metal ions such as: Fe (ferritin for storage, transferrin for transport), Cu (caeruloplasmin).

**Glutathione (GSH)**

Perhaps the best-known example of a non-enzymatic defence system is GSH, a tripeptide gamma-L-glutamyl-L-cysteinylglycin, which exists either in a reduced form with a free thiol group (GSH) or in an oxidized form, with a disulfide between two identical molecules (GSSG). GSH acts as a radical scavenger, the redox-active sulphydryl group reacts with oxidants to produce GSSG. GSH is the most abundant redox scavenging molecule in cells [141, 142, 143]. On average, GSH concentration in the cytosol is 1-10 mM [144] and the redox potential of the couple 2GSH/GSSG is -240 mV at pH 7.0 [31, 145]. Therefore, it has a central role in maintaining cellular redox state and protein redox regulation. Many researchers estimate the redox state of the system by taking the ratio of

[GSH]/[GSSG] [146]. Furthermore, GSH is an electron donor for antioxidant enzymes such as glutaredoxins and GSH-dependent peroxidases. The genes involved in GSH biosynthesis have been identified in *S. pombe*: *gcs1* and *gsh2*, encoding gamma-glutamylCys synthetase and GSH synthetase respectively and pgr1 which encodes GSH reductase that reduces GSSG to GSH [147] at the expense of NADPH [148].

Furthermore Grx and GSH regulate protein activity via glutathionylation [149].Proteins can bind GSH, Cys, homoCys and $\gamma$-glutamyl-Cys to form mixed disulfides, but GSH is the dominant ligand. The oxidation of the thiol form or the reduction of a disulfide form of an enzyme can result in the activation or inactivation of the enzyme [149, 150]. This process is reversible and occurs as an early cellular response to oxidative stress [151].

However, GSH is necessary not only for maintaining the redox balance, but also for xenobiotic detoxification, heavy metal detoxification, and more generally for cell signaling [152, 153].

Another emerging function for Grx and GSH is related to the capacity of some Grxs to bind iron sulphur centers and for some of them to transfer FeS clusters into apo-proteins [154, 155, 156]. GSH, also has a role in FeS cluster assembly.

The redox state of the cells is primarily maintained by the redox pairs GSSG/2GSH and TrxSS/Trx($SH_2$). However, the three redox systems NADP+/NADPH, 2GSH/GSSH and TrxSS/Trx($SH_2$) are not isolated systems. NADPH is an essential cofactor for the GSH- and thioredoxin-dependent enzymes that constitute major cellular defenses against oxidative stress [157]. Both Trx- and GSH-systems use NADPH as a source of reducing equivalents; thus, they are thermodynamically connected to each other. The redox environment of cells and tissues is influenced by the half-cell potentials of these linked redox couples [31].

**Phytochelatins**

In plants and fission yeast, phytochelatins play an anologous role to GSH. Phytochelatins are small cadmium-binding peptides that have the structure (gamma-glutamylCys)n-glycine and they are derivatives of GSH [158].

**Polyamines**

In adition to GSH, aa-derived polyamines have also been implicated in protecting yeast against oxidant stress. Indeed, both spermine and spermidine have been

found to be essential for aerobic growth of *S. cereviciae* and an spe2 null mutant was found to be hypersensitive to oxygen [159, 160].

**Manganese (Mn)**

Manganese (Mn) represents an essential trace element that is accumulated and utilized by virtually all forms of life. Mn is a redox-active metal that plays several different roles in biological systems, acting as a Lewis acid catalyst or switching between different oxidation states [161]. Mn is used as a co-factor for a wide range of metalloenzymes, including oxidases, dehydrogenases, DNA and RNA polymerases, kinases, decarboxylases, sugar transferases and especially for all-important catalases, $O_2^{\bullet -}$ dismutases and peroxidases that defend against ROS [161]. Experiments *in vitro* have shown that $Mn^{2+}$ can act catalytically as a scavenger of either $O_2^{\bullet -}$ or $H_2O_2$ [162, 163, 164, 165, 166]. The capacity of Mn as an antioxidant was first reported by Archinbald and Fridovich in 1981 [162], who discovered that in *Lactobaccilus plantarum* that lacked SOD, enzymes were resistance to $O_2^{\bullet -}$ due to accumulation of vast quantities of Mn. Incredibly, *L. plantarun* accumulates up to 20 mM intracellular $Mn^{2+}$ compared with the low $\mu$M levels typical of other organisms [167, 168, 169, 162]. This high level of Mn was essential for aerobic survival of *L. plantarun* and correlated with the presence of a $O_2^{\bullet -}$-scavenging activity in cell lysates that was non-proteinaceous but Mn-dependent in nature [162]. The antioxidant activity of Mn seemed to be logical for an organism like *L. plantarun* that evolved without SOD but the same characteristic was observed in various SOD-expressing organisms as well [170, 171, 172]. The catalytical action of this metal as an antioxidant is done by associating with anions including phosphate [173], and metabolic intermediates such as lactate or malate [174, 162, 163, 175]. Relative efficiencies of cellular anions that are known to promote the steady-state scavenging of $O_2^{\bullet -}$ by $Mn^{2+}$ are: lactate $>$ orthophosphate $>$ succinate $>$ malate [162, 163, 166, 176, 177]. High non-physiological levels of Mn can restore SOD-null mutants or alleviate SOD deficient activity in yeast models of Friedreich ataxia [178] either by suplementing the growth medium with Mn or by genetic augmentation of Mn uptake [179, 180, 172, 181, 182].

Another mechanism for Mn-based oxidative stress resistance is by substitution as a cofactor for iron in certain enzymes susceptible to oxidative attack. Iron is often considered a pro-oxidant in biology under situations in which Mn is an antioxidant. Mn operates as a safety cofactor without the deleterius side ef-

fects of Fenton chemistry (reviewed below) due to a higher reduction potential. Studies in *E. coli* reveal that during oxidative stress cells shift from an Fe- to a Mn-centered metabolism, whereas mononuclear iron enzymes such as ribulose-5-phosphate-3- epimerase switch to using Mn as a cofactor [183, 184].

Furthermore, Daly et al [185, 186] have shown that Mn protects proteins rather than DNA from deleterious effects of iron-dependent ROS caused by ionizing radiation (IR). These findings challenge the classical model where DNA is the most affected molecule by IR, but this could be explained by the idea that an irradiated cell, through the accumulation of Mn-complexes, would be able to protect sufficient enzymatic activity needed to repair DNA and survive. Hence, Mn-antioxidant activity is best served in a cellular environment that has low iron [185, 186]. It has been proposed that, by scavenging ROS, intracellular levels of Mn in pathogen microorganisms could contribute to virulence caused by them [187, 188].

However, in addition to serving as an essential nutrient and antioxidant molecule, Mn can also be toxic [189, 190] . In *S. cereviciae* more than 5 mM of Mn results in toxicity and impairs viability [191] and in humans, exposure to manganese can cause severe neurological damage, leading to a Parkinsonian-like disorder known as manganism [192, 193, 194, 195, 196]. Mn homeostasis is complex and appears to overlap with peroxide defence and iron homeostasis in many organisms [197, 198, 199, 200]. While the biological importance of Mn has long been recognized, there is scarce understanding regarding the mechanisms of manganese homeostasis.

**Vitamin E**

Vitamin E acts as a lipid-based radical chain breaking molecule with scavenging capacity for free radicals such a lipid peroxyl, alkoxyl and hydroxyl radiacals [201]. Its analogue soluble form, Trolox (model compound for alpha-tocopherol), decreases the levels of $H_2O_2$ and $O_2^{\bullet -}$ as well as increases the synthesis and the activity of enzymatic antioxidants in yeast [202, 203].

### 1.1.6. Beneficial roles of ROS

However, oxidative stress is not always bad; one example of that is shown by the antimicrobial responses of both plant and mammalian higher organisms as a strategy to stave off infection [204, 205]. Local $H_2O_2$ concentrations at plant wound sites and in macrophages rise to micromolar levels, which are suf-

ficient to suppress microbial growth. Because of that, all microbes engage specialized stress responses to fend off exogenous $H_2O_2$. Moreover, in non-toxic levels some reactive oxidative species can even act as messengers through a phenomenon called redox signalling.

## 1.2. Iron homeostasis

### 1.2.1. Iron as an essential biometal and as a toxic molecule

Iron is a vital metal for most biological organisms. This is due to its physicochemical properties including, reversible variation in its oxidation state, change in its redox potential and in its electronic spin configuration in response to the interaction with different coordinating ligands. These abilities make iron more suitable for biological purposes in comparison to other transition metals such as copper or nickel, because iron allows fine tuning in response to the particular needs of the enzyme in which it operates. This unique properties make it enable to participate in an astonishing array of biological reactions. Thus, this transition metal is used as a cofactor (e.g. heme moieties and iron-sulfur clusters) in a variety of active centers of many enzymes and electron transporters. That is why iron exerts a key role in cellular metabolic processes, including DNA synthesis, cell cycle progresion and respiration [206].

Nevertheless, this redox-active transition metal presents a dilemma to cells, because iron can also catalyze the deleterious oxidation of biomolecules via Haber-Weiss/Fenton chemistry (see section iron toxicity) [207]. Accordingly, concentration of iron in biological fluids is tightly regulated by control of its uptake and intracellular storage.

### 1.2.2. Different types of iron containing proteins: FeS cluster, heme group and coordinated Fe

Despite its relative abundance in nature the amount of bioavailable iron is very limited, because atmospheric $O_2$ rapidly oxidizes iron to form sparingly soluble ferric oxyhydroxides. In biological systems iron is commonly found in three oxidation states: Fe(II), Fe(III), and (to a much lesser extent) Fe(IV). At physiological pH, Fe(II) is soluble, while Fe(III) precipitates as oxyhydroxide polymers. On the other hand, Fe(II) is unstable in aqueous media and tends to

react with molecular oxygen to form Fe(III). Thus, to effectively use iron as a cofactor in enzymatic systems, nature has employed coordinating ligands such as O, N, S, or other metaloid atoms to shield iron from molecular oxygen and the surrounding media. When oxygen is bound to iron in an enzyme active site, reactions of the bound oxygen and reduced oxygen intermediates become less energetically favorable. In addition, proteins have complete control of steric factors and can prevent undesirable side reactions between bound reactive intermediates and non-substrate molecules. The relative redox potentials of iron are highly dependent on coordination, and many enzymes exploit this property using the redox couple as a fundamental means of controlled oxidation and reduction of biomolecules.

Most of the iron acquired by eukaryotic cells is consumed by two major pathways: synthesis of iron-sulfur clusters and heme groups. The cross-talk between FeS proteins and heme biosynthesis might be used for regulating the iron distrubution between these two processes in mitochondria.

### FeS clusters

FeS clusters are cofactors in a wide variety of proteins. Proteins with FeS clusters play important roles in cellular processes; FeS cluster function being involved in redox reactions, electron transport and catalytic activities. The two principal types of FeS clusters are rhombic (2Fe-2S) and cubic (4Fe-4S). FeS clusters bind to proteins by coordination of the iron ions with Cys or His residues. Other alternatives also exist like Asp, Arg, Ser CO or $CN^-$. The capability of proteins with FeS clusters to transfer electrons is due to the susceptibility of Fe to switch its oxidation state ($Fe^{2+/3+}$). Thus, an FeS cluster can function as a donor or acceptor of electrons in a variety of biological processes[208]. In some cases the function of the FeS cluster is well known, for example, aconitase, which has a 4Fe-4S cluster and is responsible for the dehydratation of citrate in isocitrate, acting as a Lewis acid (previously described in section 1). Other examples are bacterial and mitochondrial respiratory complexes I-III, photosystem I, ferredoxins, hydrogenases, SAM (S-adenosyl-L-methionime enzyme biotin synthase which binds two FeS clusters) or Isa1 involved in FeS cluster assembly pathway (see below).

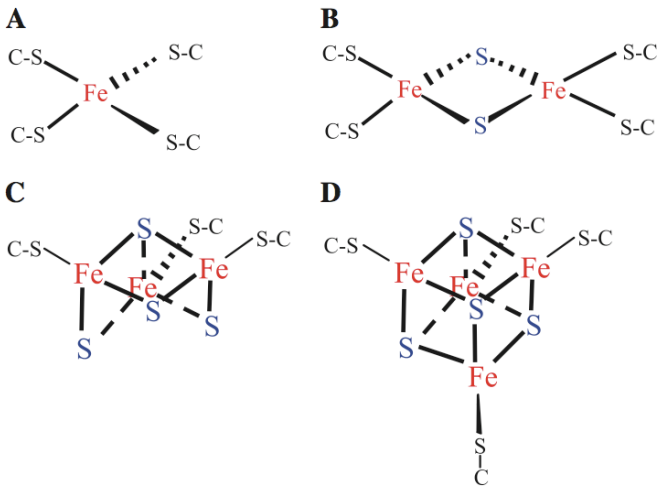


Figure 1.7: FeS cluster types. Structures of common iron-sulphur clusters. A. Rubredoxin-style iron centre. CS represents coordinating Cys residues from the polypeptide backbone. The iron atom may be in the +2 or +3 valence. B. [2Fe-2S] cluster. Typical stable cluster oxidation states are +1 and +2 (with the charges of coordinating cysteinate residues not being considered). C. [3Fe-4S] cluster. Stable oxidation states are 0 and +1. D. [4Fe-4S] cluster. Stable oxidation states are +1 and +2 for ferredoxin-type clusters and +2 and +3 for high-potential [4Fe-4S] ferredoxin clusters. Electrons may be delocalized, so that the valences of individual iron atoms lie between the ferrous and ferric forms. Reprinted from [26].

**FeS cluster biogenesis** Biogenesis of iron-sulphur (FeS) proteins in eukaryotes is an essential process involving the mitochondrial iron-sulphur cluster (ISC) assembly and export machinery and the cytosolic iron-sulphur protein assembly (CIA) apparatus [209]. The mitochondrial ISC biogenic model begins when a sulphur is transferred from IscS or Nfs1 to a conserved Cys residue on IscU/Isu1 [210, 211]; scaffold proteins then bind $Fe^{2+}$, resulting in ISC synthesis. The mitochondrial ISC assembly machinery is not only required for biosynthesis of mitochondrial FeS proteins, but also for the biosynthesis of cytosolic and nuclear FeS proteins [212, 213]. Furthermore, maturation of extra-mitochondrial FeS proteins depends on mitochondrial proteins, designated 'ISC-export machinery', and proteins, the majority of which are cytosolic but are also found in the nucleus machinery and specifically GSH [155]. The components of the ISC-assembly machinery and the CIA machinery are highly conserved in eukaryotes.

**ISC assembly in mitochondria**
This is a complex process that involves more than 20 proteins, but it can be split into two main steps: a) the *de novo* assembly of an FeS cluster on a scaffold protein plus sulphur donor [214]; b) the transfer of the FeS cluster from the scaffold to target apo-proteins and its subsequent assembly into the polypeptide chain. Each of these steps could be divided in partial reactions executed by specific proteins and cofactors, which will be described in detail below:

1) **Sulphur donor**. A pyridoxal phosphate-dependent Cys desulfurase (termed IscS, NifS and SufS in bacteria and NFS1 in the mitochondrial ISC-assembly machinery) releases the sulphur required for the FeS cluster formation from a Cys to produce Ala [212, 213, 215, 216, 217]. The sulfur atom is first transferred to a conserved Cys residue of these enzymes to create a persulfide as an intermediate reaction [218, 219]. Sulphur is then transferred directly to the scaffold protein, namely IscU in bacteria and ISU1 (and ISU2 in yeast) in eukaryotes [2, 220, 221, 222, 214]. Usually, the scaffold and sulphur donor form hetero-tetrameric complexes, which might facilitate the sulphur transfer. IscU and ISU1 are small proteins of 14 kDa, and are among the most conserved proteins in evolution, with three conserved Cys residues involved in ISC synthesis exposed to the surface [223, 224]. Either a [2Fe-2S] or a [4Fe-4S] cluster can be assembled on an IscU dimer, suggesting that Cys residues from both subunits participate in ISC assembly [225].

2) **Iron donor**. Iron is unlikely to be free in solution. To guarantee its accu-

rate delivery to scaffold proteins, specific iron donors are needed. Such a donor function is performed by bacterial (CyaY) and mitochondrial (YFH1) ISC components which bind iron, desulphurase and the scaffold protein ISU1-IscU.

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

4) **Scaffold proteins**. These proteins serve as a plataform for *de novo* biosynthesis of an FeS cluster. They contain conserved Cys residues and bind an FeS cluster in a labile manner, meaning that this cluster can be transferred to target proteins and stably integrated. The most highly conserved scaffolds are bacterial IscU and SufU, and eukaryotic ISA1. Other scaffolds include bacterial NifU, plastid NFU proteins and bacterial IscA and SufA.

5) **Cluster transfer proteins**. Specific factors are involved in transferring the labile FeS clusters from scaffolds to apo-proteins, which are converted from the apo-form to the holo-form. These transfer proteins induce dissociation of the scaffold-bound FeS cluster; to guarantee its accurate and specific transfer to bona fide FeS apo-proteins, and promote correct assembly of the FeS cluster at the proper acceptor sites. In many cases, these cluster transfer proteins can be bypassed in *in vitro* experiments but are essential in living cells. Examples are the mitochondrial SSQ1 and JAC1 and bacterial HscA and HscB chaperones of the ISC assembly systems, and Nar1 and Cia1 of the CIA machinery.

Model for FeS cluster assembly via the CIA system will be describe in Figure 1.9. A nucleotide-dependent cycle for FeS cluster assembly on a Cfd1-Nbp35 scaffold and transfer to apo-targets is depicted. ATP binding, hydrolysis, and release of ADP by Cfd1 and Nbp35 are proposed to occur based on the high homology of these proteins to known deviant P-loop ATPases [226]. FeS cluster assembly on the apo-Cfd1-Nbp35 scaffold complex is proposed to depend on the mitochondrial ISC and export systems, the CIA factor Dre2, and a source of iron and sulfur. Nar1 and Cia1 interact with the Cfd1-Nbp35 complex, facilitating cluster transfer to a first apo-protein of cytosolic or nuclear. It is proposed here that dissociation of Nar1, Cia1, and Cfd1 frees Nbp35 to support FeS cluster assembly in a second round of load an apo-protein. Finnaly, Apo-Cfd1 and apo-Nbp35 reform the heterocomplex to restart the process.

However, ISC synthesis seems to have an important role in iron homeostasis

because some consequences of defects in ISC synthesis are a drastic accumulation of iron in mitochondria [227] and decreased heme biosynthesis and cytochrome content.

### Hemoproteins

Hemoproteins have a broad spectrum of catalytic functions like oxygen binding, electron transport, peroxidation and oxygenation and they are located in different subcellular compartments. Some examples are catalase and cytochrome c; in budding yeast these genes are under positive control of heme and HAP1 protein (part of HAP-complex than act upon iron starvation, orthologue to Php-complex in *S. pombe*). In fission yeast *ctt1* is also under control of Php4 and diminishes under iron starvation [228].

Heme synthesis is rather complex, involving several mitochondrial and cytosolic enzymes that initially convert $\gamma$-aminolevulinate to protoporphyrin IX (for review see [229]). The final step of the pathway is the incorporation of $Fe^{2+}$ into the porphyrin ring and is catalyzed by ferrochelatase (HEM15 in yeast) which is located at the matrix side of the mitochondrial inner membrane [230, 231]. Ferrochelatase, which catalyses the last step of heme biosynthesis, is reversibly inhibited when the biosynthesis of mitochondrial FeS proteins is impaired, seen to be the cause of the heme-synthesis defect in ISC mutants [232]. This is one of the connections between FeS protein biogenesis and heme-synthesis although the cross-talk between these two pathways and iron homeostasis is not completed well understood yet.

### Di-iron containing proteins

Proteins containing dinuclear non-heme iron centers catalyse reversible binding of $O_2$, hydroxylation of alkanes, and oxidation of tyrosine to a tyrosyl radical or phosphoryl transfer reactions. Di-iron centers are bridged by His residues and additional carboxylate ligands. The di-iron sites in the major class of hydroxylase-oxidase enzymes, which contain ribonucleotide reductase (RNR) and methane monooxygenase, show significant flexibility in the geometry of their coordination of three or more carboxylate groups. This flexibility, combined with a relatively low coordination number, and a buried environment suitable for reactive oxygen chemistry, explains their efficient harnessing of the oxidation power of

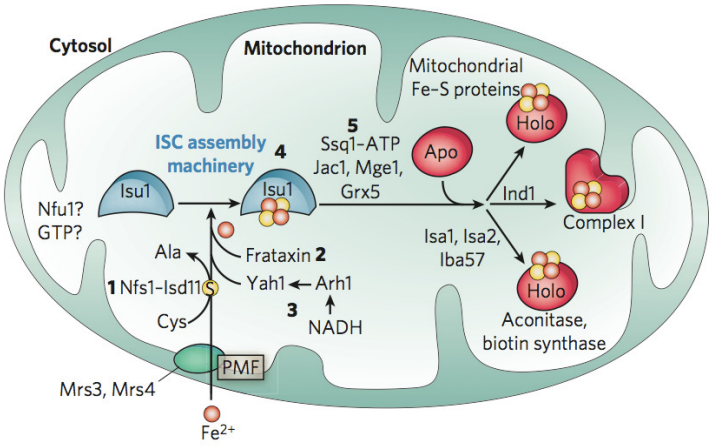


Figure 1.8: A model for FeS protein assembly in mitochondria in *S. cereviciae*. Mitochondria import $Fe^{2+}$ from the cytosol in a membrane potencial dependent manner, using the proton-motive force (PMF) as a source of energy. Importation is aided by the inner membrane carriers Mrs3 and Mrs4 (known as mitoferritin in mammals). The maduration of mitochondrial FeS proteins (to the holo form) starts with sulphur liberation from Cys by the Cys desulfurase complex Nfs1-Isd11. The synthesis of a transient bound FeS cluster on the scaffold protein Isu1 (ans Isu2 in yeast) futher depends on the iron-binding protein frataxin (Yfh1 in yeast) as an iron donor and the electron-transport chain consisting of NADH, ferredoxin reductase (Arh1) and ferredoxin (Yah1), which possibly provides electrons for the reduction of sulphur to sulphide. The release of FeS cluster from Isu1, and its transfer and incorporation into recipient apo-proteins (Apo) are facilitated by ATP-dependent Hsp70 chaperone Ssq1, the DnaJ-like co-chaperone Jac1, the nucleotide- exchange factor Mge1 and the monothiol glutaredoxin Grx5. Proteins of the aconitase family and radical SAM proteins such as biotin systhase specifically need Isa1, Isa2 and Iba57 in addition for maturation of their FeS clusters. Assembly of respiratory complex I also requieres the P-loop NTPase Ind1. The bold numbers refer to the biogenesis steps defined in the text. Reprinted from [208].

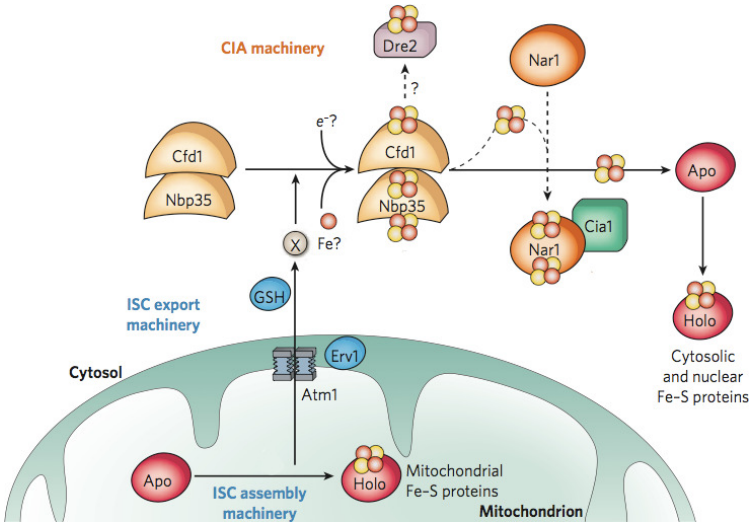


Figure 1.9: The roles of mitochondria and the CIA machinery in FeS-protein biogenesis in the cytosol and nucleus of eukaryotes. Cytosolic and nuclear FeS-protein biogenesis requieres both the mitochondrial ISC assemby and the export machineries. The ABC transporter Atm1 of the mitochondrial inner membrane exports an unknown compound (X) to the cytosol for use in FeS-protein assembly, and is assisted by GSH and the intermembrane -space sulphydryl oxidase Erv1, which introduces disulphide bridges ino substrates. In the cytosol, the components of the CIA machinary catalyse FeS protein maturation in two main steps. First, FeS clusters are assembled on the P-loop NTPase complex Cfd1-bp35 are labile, and aided by the iron- only hydrogenase- like protein Nar1 and the WD40-repeat protein Cia1, can be transferred to cytosolic and nuclear apo-proteins (Apo). The site of involvement of the two FeS clusters on Nar1 depends on mitochondria and Cfs1-Nbp35 function. Reprinted from[208].

molecular oxygen. RNRs are one of the more studied examples, this is the enzyme that catalyze deoxyribonucleotides synthesis, these are essential for DNA synthesis. The R2 subunit of RNR of *E. coli* is a homodimer containing one dinuclear iron centre per monomer. A tyrosyl radical is essential for catalysis, and is formed via a reaction in which the $Fe^{2+}$ of the iron center activates dioxygen.

#### Mononuclear Fe enzymes

Finnally, another example of iron containing proteins are the non-redox mononuclear enzymes such as Rpe (ribulose-5-phosphate 3-epimerase involved in pentose-phosphate pathway), Pdf, Tdh, and Cda in *E. coli*. These enzymes catalyze very distinct reactions and such iron-charged enzymes are vulnerable to $H_2O_2$. However, these enzymes can be converted into a form that is unaffected by $H_2O_2$ if Mn replaces the iron atom (see above Mn as antioxidant defence) [184, 233].

### 1.2.3. Iron Toxicity: Fenton Reaction and Metal Catalized Oxidation (MCO)

#### Fenton Chemistry

The Fenton reaction was developed in the 1890s by Henry John Horstman Fenton as an analytical reagent [6]. Ferrous iron ($Fe^{2+}$) is oxidized by $H_2O_2$ to ferric iron ($Fe^{3+}$), a hydroxyl radical and a hydroxyl anion. Afterwards, $Fe^{3+}$ is reduced back to $Fe^{2+}$ with the consequent reduction of an $O_2^{\bullet -}$. Thus, the metal ion-catalysed univalent reduction of $H_2O_2$ generates a highly reactive oxidant, OH•. This reaction can also be catalyzed by copper (see Figure 1.10).

**Fenton reaction**

$$H_2O_2 + Fe^{2+} \rightarrow OH^- + FeO^{2+} + H^+ \rightarrow Fe^{3+} + OH^- + HO\cdot$$

$$H_2O_2 + Cu^+ \rightarrow Cu^{2+} + OH^- + HO\cdot$$

Figure 1.10: Fenton reaction. Abbreviations: $H_2O_2$ $H_2O_2$, $Fe^{2+}$ ferrous ion, $OH^-$ hydroxide ion, $FeO^{2+}$ ferrous oxide, $H^+$ proton, $Fe^{3+}$ ferric ion, $OH^\bullet$ hydroxyl radical, $Cu^+$ cuprous ion, $Cu^{2+}$ cupric ion.

Table 1.3: Metal-Catalyzed Oxidation of aa Residues in Proteins. Reprinted from [1]

| aa residue oxidized | Product(s) |
|---|---|
| Histidine | Aspartate, asparagine, oxo-histidine |
| Proline | Hydroxyproline, glutamate, $\gamma$-glutamylsemialdehyde |
| Arginine | $\gamma$-Glutamylsemialdehyde |
| Lysine | Amino-adipicsemialdehyde |
| Threonine | Amino-ketobutyrate |
| Tyrosine | Tyr-Tyr (dityrosine) |
| Cys | -S-S- (disulfide cross-links) |

**Metal Catalyzed Oxidation**

MCO is a site-specific process involving the interaction of $H_2O_2$ and $Fe^{2+}$ at a metal binding site on a protein. The most well-studied irreversible protein oxidation caused by MCO is carbonylation. Among other modifications, carbonyl derivatives of some aa residues are formed (see Table 1.2.3). These derivatives can be marks for protein degradation. Furthermore, MCO of proteins is the basis of biological mechanisms for regulating changes in enzymatic levels in response to shifts from anaerobic to aerobic metabolism, or from one nutritional state to another.

The biological significance of MCO reactions is highlighted by much evidence that suggests that there is an acummulation of intracellular pools of catalytically inactive and less active, thermolabile forms of enzymes. These enzymes are acummulated in cells during aging, oxidative stress, and in various pathological states, including premature aging diseases (progeria, Werner's syndrome), muscular dystrophy, rheumatoid arthritis, cataractogenesis, chronic alcohol toxicity, pulmonary emphysema, and during tissue injury provoked by ischemia-reperfusion. MCO is also involved in the killing of bacteria by neutrophils and in the loss of neutrophil function following repeated cycles of respiratory burst activity.

A further complication due to MCO is $H_2O_2$-induced FeS cluster destruction in proteins. This releases ferrous iron atoms which can then bind to DNA. Subsequently, $H_2O_2$ can in turn react with such DNA-bound iron atoms to generate hy-

droxyl radicals that directly cause substantial DNA damage [234, 235, 236, 25] with carcinogenic consequences [237]. In addition, one of the major ligands for iron is ATP, when ADP-$Fe^{2+}$ complex is formed, the maximal rate of lipid peroxidation is promoted [238, 239].

Therefore, it is not surprising that there exists a link between systems of metal-ion detoxification or metal-ion homeostasis and the defences against ROS. In general, cells minimize the formation of OH• by ensuring intracellular sequestration of transition metals such as copper and iron to avoid consequent cellular damage.

### 1.2.4. Mechanisms of intracellular iron concentration control: siderophores, Fe importers, ferritin, vacuoles.

Despite this careful and wise selection of nature, the widespread use of iron in living organisms gave rise to a paradox, the properties that make iron essential can also make it toxic under certain conditions, if not tightly controlled. Iron excess has the ability to generate ROS that can damage cellular components via the Fenton Reaction [240]. Consequently, cells have developed accurate homeostatic mechanisms in order to optimize iron up-take while keeping its reactivity under control.

To satisfy their iron need, fungi have developed various mechanisms of iron aquisition (reviewed in [241, 242, 243]), including a reduced iron assimilation system through high-affinity transporters. This consists of solubilization of iron by enzymatic reduction of ferric iron ($Fe^{3+}$) and subsequent up-take of ferrous iron ($Fe^{2+}$) and a non reduce assimilation pathway through the mobilization of iron by siderophores.

Once iron has been introduced within the cell, in all types of organism an intracellular storage form of non-toxic, soluble and bioavailable iron is required. In a wide variety of organisms the major form of iron storage is in a metaloprotein such as ferritin. In animals and certain fungi, it is known as ferritin, in plants phytoferritin and some bacteria as bacterioferritin [244, 245, 246, 247, 248, 249]. In *S. cerevisiae*, a ferritin-like molecule was purified from iron-loaded cells, but its iron content was very low and was not representative of the cellular iron content [250]. In most fungi siderophores play a dual role, acquisition and intracellular iron storage [251]. In the case of the eukaryotic model *S. cerevisiae* which does not have ferritin and is a siderophore-lacking yeast, it centralises its storage

in vacuoles [252, 253, 200, 250, 254].

### Iron assimilation pathways

Despite the fact that iron is one of the most abundant elements on earth, its bioavailability remains highly limited at physiological pH owing to its oxidation into insoluble ferric hydroxides under atmospheric oxygen conditions [255]. To overcome this issue *S. pombe* uses two iron aquisition systems that involve either a reductive or nonreductive mechanism [256, 257, 258].

**Reductive iron assimilation pathway by high-affinity transporters.** In *S. pombe* the reductive iron assimilation pathway relies on ferrireductase Frp1, whose role is to solubilize extracellular iron by reducing its $Fe^{3+}$ to $Fe^{2+}$ [257]. The following step is the entrance of $Fe^{2+}$ across the plasma membrane mediated by an oxidase-permease complex composed of multicopper oxidase Fio1 and the transmembrane permease Fip1 [256]. Fio1's role consists of re-oxidizing $Fe^{2+}$ to $Fe^{3+}$, which is transported into the cell by the carrier Fip1. In *S. cereviciae*, a high affinity iron uptake complex is formed by FET3 and FRP1 in the membrane and FET5, FTH1, and SMF3 in vacuoles.

**Non reductive iron assimilation pathway. Dual function of siderophores: low affinity system of iron up-take and intracellular storage.** To overcome iron-associated toxicities and iron scarcity, organisms have first evolved a series of molecules known as siderophores. These are low-molecular-weight chelating agents (200-2000 Da) produced by bacteria, fungi and plants to facilitate uptake of iron [259, 260, 261]. Siderophores have an extremely high affinity for $Fe^{3+}$ with stability constants generally around $10^{30}$ $M^{-1}$ [262, 263]. Despite their preference for iron, they can also chelate numerous other metals including heavy metals. Siderophores are produced and excreted in the desferri-form mainly during iron stavation. Siderophores have a variety of chemical structures and form a family of at least 500 different compounds [260, 261], but they can be divided into three groups based on chemical composition: (1) catechols; (2) carboxylates; (3) hydroxamates. With exception of carboxylates produced by zygomycetes, fungal siderophores are hydroxamates [230]. There are four major families of fungal hydroxamate-type siderophores, with representative structures

and characteristics including rhodotorulic acid, fusarinines, coprogens, and ferrichromes [242, 264]. When siderophores are produced by bacteria or fungi to extract iron from organic substances they are considered to be virulence factors [265, 266, 267].

After extracellular iron chelation, siderophores in ferri-form are recovered by cells either by a non-reductive system with specific transporters able to internalize the siderophore-iron complex (reviewed in [268, 269, 270]). Remarkably, numerous fungi possess specific uptake systems not only for native siderophores but also for siderophore-types synthesized exclusively by other fungi. The eukaryotic model microorganism, *S. cerevisiae*, lacks the ability to synthesize siderophores [271], although it can utilize siderophores produced by other species [272, 273, 274, 275, 276, 277].

The ferri-siderophore pathways must have high metal specificity to transport and accumulate only the appropriate metal(s). If the siderophore uptake receptor does not distinguish between a metal-siderophore and ferri-siderophore complexes, siderophores may provide a secondary mechanism for toxic metal uptake, thereby implicating siderophores in heavy metal tolerance [278, 279, 280].

**Non reductive assimilation pathway in** *S. pombe.* Fission yeast have a non-reductive iron assimilation pathway that consists of production, secretion and acumulation of only one type of siderophore, hydroxamate [242]. *S. pombe* produces both extracellular and intracellular hydroxamate- type siderophores [281]. Once secreted, this ferric ion chelating compound, named ferrichrome, can be recaptured and imported into the fission yeast cell as a siderophore-iron complex and accumulates intracellularly. Under iron-limiting conditions, the cellular ferrichrome pool was present in desferri-form, while under iron-rich conditions, in ferri-form. *S. pombe* is also able to take up another type of siderophore, ferrioxamine B, when it is secreted into the enviroment by another microorganism [256]. The products of the genes *str1*, *str2* and *str3* have been identified as siderophore-bound iron up-take [270]. Each siderophore transporter exhibits certain specificity, Str1 for ferrichrome-iron, whereas Str2 is specific for ferrioxamine B-iron but also transports ferrichrome-iron when there is an excess of it. The specific siderophore for Str3 has not been identified, although Str3 may participate in the mobilization of iron bound to siderophores [270].

**Siderophore biosynthesis** Ornithine, is the precursor of ferrichrome. Plattner and Diekmann proposed a general biosynthetic pathway of fungal hydroxamate siderophores in which the first step in the synthesis of ferrichrome is the $N^5$-hydroxylation of ornithine by L-ornithine $N^5$-oxigenase, Sid1 in *Ustilago maydis*, SidA in *Aspergillus nidulans* and Sib2 in *S. pombe* [242]. The subsequent step in the biosynthesis of ferrichrome is the formation of the hydroxamate group. The $N^5$-hydroxy- ornithine are acylated by an $N^5$-transacetilase, which is predicted to be encoded by the *SPBC17G9.06c* locus in fission yeast. In the last step the hydroxamate group is processed by a nonribosomal peptide synthetase (NRP) called Sib1 in *S. pombe* [242]. The *sib1* and *sib2* mutant strain exhibits a severe growth defect on iron-poor media. Both *sib1* and *sib2* genes are repressed by the GATA-type transcriptional repressor Fep1 in response to high levels of iron. The loss of Fep1 results in increased ferrichrome production (see section 1.4).

### The vacuole as an iron storage compartment

Studies have shown the fundamental contribution of the yeast vacuole as a site for storage and detoxification of metals. Furthermore, transmembrane proteins are responsible for iron transport into and out of the vacuole. In budding yeast vacuoles present an oxidase-permease complex called FET5 and a FTR1 [283]. The FET5-FTH1 complex most likely mobilizes stored iron from the vacuole to the cytosol when cells undergo a transition from iron excess to iron-limiting conditions [284]. *S. pombe* does not have any homologs of *S. cerevisiae* proteins FET5, FTH1, and SMF3 [285], but uses its vacuoles as reservoirs of metals. Nevertheless, Abc3 could serve to transport iron from the vacuole to the cytoplasm and Pcl1, viceversa [286].

### Metalloproteins as iron storage reservoirs.

In bacterial cells, metalloproteins acquire the appropriate metal and thus control and limit the number of metals in the cytoplasm. Consequently, as proposed by Waldron and Robinson: 'metals are not in competition for a limited pool of proteins, but rather the proteins compete for a limited pool of metals' [287]. The principal Fe-storage protein is ferritin which is strongly induced by OxyR and PerR system in some $H_2O_2$-stressed bacteria [288, 289]; among these genes are

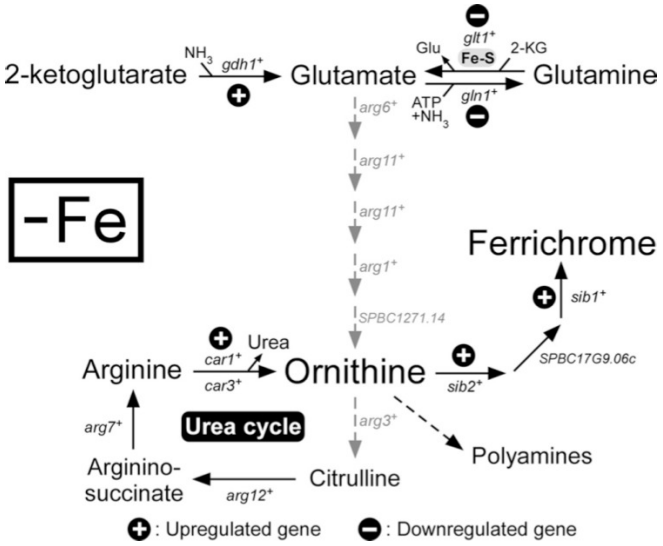


Figure 1.11: Siderophores biosynthesis in *S. pombe* grown under iron limited conditions. The metabolic relationships between glutamate, ornithine, and ferrichrome in fission yeast are depicted. Transcription of genes that are induced (+) or repressed (-) by low concentrations of iron is indicated. Enzymatic reactions that are predicted to occur in mitochondria are depicted with dashed arrows in gray. ÒFe-SÓ designates a gene encoding an iron-sulfur cluster-containing enzyme. Abbreviations: 2-KG, 2-ketoglutarate. Reprinted from [282]

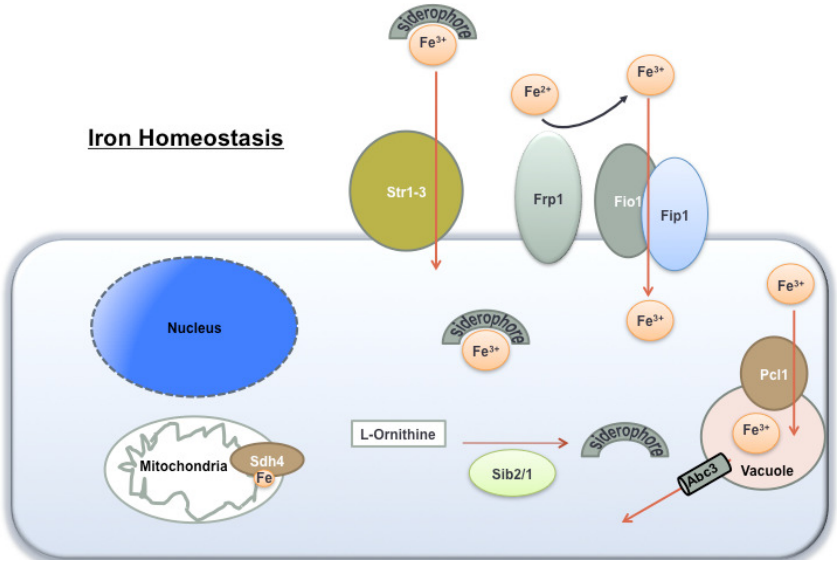


Figure 1.12: Iron homeostasis in *S. pombe*.

ones encoding the Suf cluster-assembly complex, suggesting that Suf is important for FeS clusters assembly or repair during $H_2O_2$ stress [290]. Dps is an alternative iron-storage protein in bacteria [291, 292], is activated in many others transcription factors [293]. Dps appears to use $H_2O_2$ rather than molecular oxygen as the electron acceptor during iron oxidation, perhaps because this arrangement offers a mechanism whereby the storage function may be deactivated when $H_2O_2$ stress has ended. Dps protein sequesters the iron that spills from damaged clusters, thereby minimizing the formation of hydroxyl radicals [294]. *E. coli* dps mutants are hypersensitive to $H_2O_2$ [295]; in fact , *E. coli* catalase-peroxidase mutants are unable to grow in aerobic enviroments if the dps gene is inactivated, as endogenous $H_2O_2$ creates overwhelming amounts of DNA damage [294].

In mammals, metalloproteins, such as ceruloplasmin, metallothionein, and ferritin, are well known for their critical role in metal homeostasis and function as storage reservoirs and/or chaperones for essential trace metals, such as copper, zinc, and iron. Evidence indicates that these proteins are induced during the acute-phase response in inflammation [296, 297] and under oxidative stress [298, 299]. It has been speculated that they ameliorate the deleterious effects of

ROS. The antioxidant properties of these proteins have been attributed primarily to their binding of the redox active metals copper and iron, thus minimizing their capacity to catalyze ROS production via the Fenton reaction. In turn, multicellular organisms have developed Fe-binding proteins known as transferrins, which complex iron, transport it in the circulation (serum transferrin) or in other media (ovotransferrin, lactoferrin) and are taken up by the cell mainly (serum transferrin) via a receptor mediated mechanism. Once transferrin is taken up and iron is released within the cell, iron becomes stored by ferritin. Mammalian ferritin consists of a spherical protein shell composed of 24 structurally equivalent subunits. Each subunit has a molecular mass of 20 kDa. The inner cavity contains up to 4500 iron atoms as a ferric oxyhydroxide polymer [264]. Bacterioferritins have a similar structure except that their subunit molecular mass is smaller (15-17 kDa) and they all contain heme groups associated with the protein [247, 248, 249].

**The labile iron pool (LIP).**

Moreover, a critical point in understanding iron regulation and iron homeostasis appears to be the demonstration of the existence of an intermediate, low molecular weight transit pool, now referred to as labile iron pool (LIP), for the explanation of intracellular traffic, metabolism, and distribution after iron has been transported through the membrane. This entity was postulated in early studies by Greenberg and Wintrobe, and Jacobs [300, 301], but up to few years ago it remained a poorly characterized compartment, in spite of the great deal of experimental approaches aimed to demonstrate it in an unequivocable way. Crichton [264] described this entity as 'somewhat like to the Loch Ness monster, only to disappear from view before its presence, or indeed its nature, can be confirmed'. Cabantchik and coworkers [302, 303, 304] were able to prove the existence of the LIP by detecting it with a method based on the quenching of the fluorescent chelator calcein by metal ions. This pool has finally appeared as a substantial reality. LIP represents only a minor fraction of the total cellular iron (3-5%), and it is composed primarily of $Fe^{2+}$. This cytosolic pool represents iron in transit between transferrin and ferritin. It has been proposed that iron from LIP is complexed by diverse low-molecular weight organic chelators, such as citrate, phosphate and other organic ions, carbohydrates and carboxylates, nucleotides and nucleosides, polypeptides and phospholipids [302, 305]. The balance between iron homeostasis and energy production, cell growth, etc. is critical and requires biological flexibility and complexity, thereby an accurate

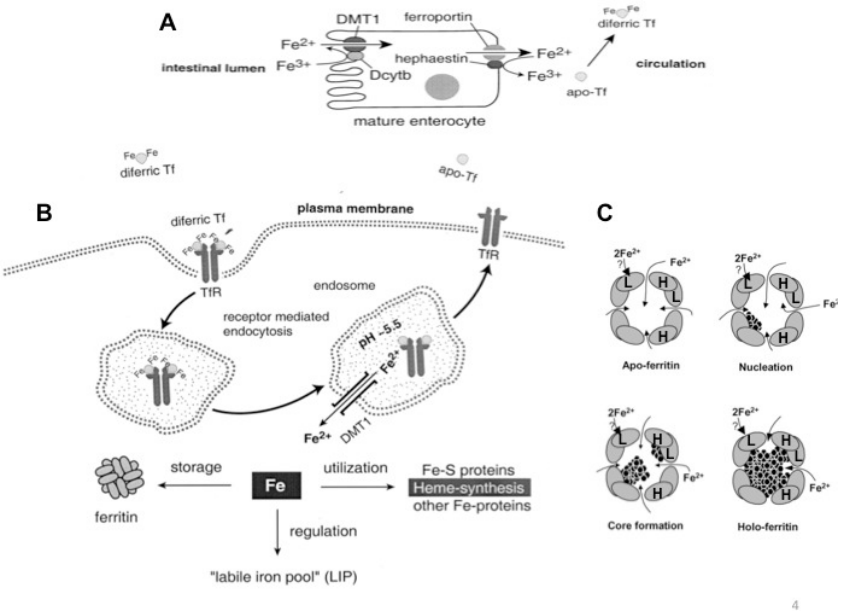


Figure 1.13: Modified from[306, 307]

control of iron up-take, storage and usage offers to cells an efficient means of tuning this important metabolic pathway.

## 1.3. Cellular response to oxidative stress in *S. pombe*

### 1.3.1. *S. pombe* as a model organism in the study of stress responses

In our laboratory we work with an eukaryotic unicellular organism known as *S. pombe*. It was first described by P. Lindner in 1893 after isolating it from an East African beer. The name derives from the Swahili word for beer, pombe. *S. pombe* forms a rod-shaped non-motile cell that grows by elongation and divides by medial fission with a doubling time of 2-4 hours in simple culture conditions [308] (see Figure 1.14).

It proliferates in haploid state. Furthermore, it divides by bipartition, forming a septum at a central position of the cell; for this reason the organism is also

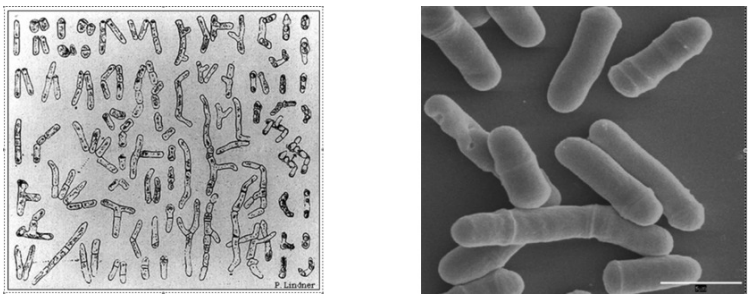

Figure 1.14: The fission yeast *S. pombe* has become today one of the most important model organisms. Left panel, Lidner's drawing of the isolated strain. Right panel, scanning electron microscopy image of some *S. pombe* cells.

known as fission yeast. This feature allows easy identification of the phase of the cell cycle using a microscope, making it a widely used model organism in cell cycle studies. The complete taxonomic classification is: Cellular organism, *Eukaryota*, *Fungi/Metazoa group Fungi*, *Dikarya*, *Ascomycota*, *Taphrinomycotina*, *Schizosaccharomycetes*, *Schizosaccharomycetales*, *Schizosacharomycetaceae*, *Schizosaccharomyces*, *S. pombe*. But the phylogenetic position of *S. pombe* in the tree of life can be in different branches, depending on the set of proteins considered [309]. Futhermore, if the set of proteins chosen for clustering are nuclear proteins and two mitochondrial proteins (a tyrosil-tRNA synthetase and a cytoplasmic iron-sulphur protein), *S. pombe* can be considered phylogenetically more similar to animals than to fungi.

Moreover *S. pombe* genome has been fully sequenced [310]. Its genetic material is organised in three chromosomes, containing 5036 genes scattered around 14 megabases (Mb) of total DNA making it the smallest free-living eukaryote [310]. Its genome contains few duplicated genes, making it susceptible to powerful genetic tools such as gene disruption and phenotypic analyses together with more sophisticated functional screens [308, 311, 19]. This further makes it a model species for basic studies in cell biology. Yeasts such as *S. cereviciae* and *S. pombe* are useful models for studying gene-related human diseases including cancer, metabolic or neurodegenerative diseases [310]. However, several groups of genes that are conserved in *S. pombe* and humans are missing in *S. cereviciae*. *S. pombe* has been seen as evolutionarily closer to higher eukaryotes than *S. cereviciae* [309]. Specially the stress-activated cell signaling pathway in *S.*

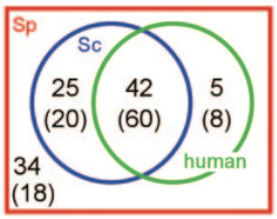


Figure 1.15: The inset shows a schematic representation of protein kinase orthologs in *S. pombe* (*Sp*), *S. cerevisiae* (*Sc*), and human. One hundred six eukaryotic protein kinase catalytic-domain-containing proteins were selected in *S. pombe*, 119 in *S. cerevisiae*, and 491 in human. Analysis of orthologs showed that of 106 *S. pombe* protein kinases, 67 (25 plus 42) have orthologs in *S. cerevisiae* and 47 (42 plus 5) in human. Among these, 42 appeared to have orthologs in both *S. cerevisiae* and human. Numbers in parentheses indicate the numbers of nearest homologs. Reprinted from [313].

*pombe* and mammalian cells are highly conserved, this suggests that *S. pombe* can serve as a good model system to study stress responses [312]. Eukaryotic protein kinases are key molecules mediating signal transduction, and play a pivotal role in the regulation of various biological processes, including cellular response to environmental changes, cell cycle progression, cellular morphogenesis and development. A total of 106 eukaryotic protein kinase catalytic-domain-containing proteins have been found in the entire fission yeast genome, 44% (or 64%) of which possess orthologues (or nearest homologues) in humans, based on sequence similarity within catalytic domains [313] (see Figure 1.15). This indicates that studies on biological functions of fission yeast protein kinases would facilitate our understanding of protein kinase-mediated signaling pathways in humans. All this data make *S. pombe* a very useful and attractive model to study stress responses.

### 1.3.2. Environmental stress

All cells employ specific sensory and signalling systems to obtain and transmit information from the environment in order to adjust cellular meta- bolism, growth and survival. Unicellular organisms have to cope with a wide range of

fluctuations, such as variations in nutrient availability, pH, temperature, osmolarity, ultraviolet (UV) radiation, and also exposure to several toxic compounds. One could consider stress imposed on cells as any disturbance of the normal or optimal growth conditions. Survival and proliferation of cells depends on an appropriate response to these environmental stresses. Understanding the mechanisms involved in sensing stress, the signaling paththway transmitting this information within the cell, and gene expression is essential in order to understand how cells adapt and survive under non-ideal conditions. They have developed elaborate systems to sense fluctuations in the intensity, concentrations or presence of such variables and to adapt by applying the appropriate response. Exposure to low levels of stress often triggers an adaptive response resulting in a transient resistance to higher levels of the same stress. This adaptation to stress can also lead to increased resistance (or cross-protection) to other types of stress [314, 315]. Changes in gene expression play a critical role in the adaptive response because it is short-lived and requires *de novo* protein synthesis and protein down-regulation. The phenomenon of cross-protection suggests either that different stress conditions can activate similar defence mechanisms or that there is a general stress response that can confer a basic level of protection when the cells are challenged by the environment [316, 314, 315, 102].

### 1.3.3. Genomic expression programs to environmental stress

Global transcriptional approaches have been decisive to elucidate the molecular responses of cells to diverse environmental stresses. Whole genome expression profiling studies have revealed general responses to stress in the majority of stress conditions in budding yeast *S. cereviciae* and fission yeast *S. pombe* [314, 315, 102]. This core response is largely conserved between these two distantly related yeast species. Induced genes are involved in various processes, including carbohydrate metabolism, detoxification of ROS, protein folding and degradation, vacuolar and mitochondrial functions, autophagy, and metabolite transport. Otherwise, repressed genes are generally involved in energy consuming, growth-related processes, including RNA processing, transcription and translation, biosynthesis of ribosomes and nucleotides. This stereotypical response has been termed as, environmental stress response (ESR) [314] or common environmental response (CER) [315] in budding yeast and were called 'common environmental stress response' (CESR) in fission yeast [102].

**Identification of CESR and SESR Genes**

Induced genes that were upregulated at least twofold were identified after either 15 or 60 min of treatment. Among those genes, induced CESR genes were selected as those that were up-regulated in at least four of the five stress conditions. Genes that were up-regulated twofold or greater in at least one stress were called induced SESR genes. Repressed CESR genes were selected as those being down-regulated twofold or greater in at least three of the five stress conditions. The five stress conditions were: oxidative stress caused by $H_2O_2$, heavy metal stress caused by cadmium, heat shock caused by temperature increase to 39°C, osmotic stress caused by sorbitol, and DNA damage caused by the alkylating agent methylmethane sulfonate. To regulate their core stress response, cells use different strategies, involving differential regulation of signal-transduction pathways and translating extracellular signals into specific intracellular responses. To implement this specific response, the stress-specific Mitogen Activated Protein Kinase (MAPK) pathways are the most important key regulators. However, the stress-specific responses were less dependent on the Sty1 MAPK pathway and may involve specific regulatory factors. In this thesis we will focus in the oxidative stress response and the cross-talk with iron starvation response.

### 1.3.4. Oxidative stress signaling pathways in *S. pombe*

*S. pombe* triggers two independent but cross-talking signalling pathways depending on the severity of the oxidative stress subjected, Pap1 and Sty1 (also known as Spc1 and Phh1) pathways [317, 318]. The Pap1 transcription factor is activated upon low doses of $H_2O_2$ and induces the adaptation response, whereas the MAPK Sty1 is in charge of the survival response upon doses at least fivefold higher of the oxidant. These two pathways are not required to mantain cell viability during aerobic growth, but they seem to be essential for cell survival under oxidative stress conditions. Both pathways are highly conserved through the evolution in all eukaryotes [319]. These signalling pathways will be discussed below.

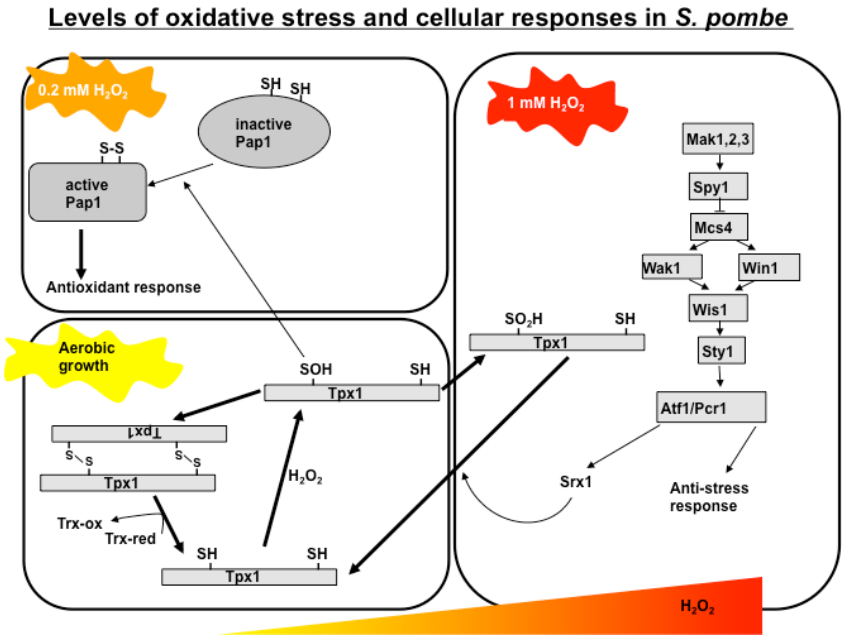


Figure 1.16: Oxidative stress pathways in *S. pombe*. Reprinted from [320]

### 1.3.5. Sty1 pathway

The Sty1 pathway is activated by a wide range of cellular insults such as UV light, DNA damage agents, heavy metal toxicity and different stress including oxygen, heat or osmotic stress [321, 322, 323, 324, 325]. Sty1 is activated by phosphorylation by the MAPK kinase (MAPKK) Wis1, which is activated through phosphorylation by two MAPKK kinase Wak1 (also know as Wis4 and Wik1) [326, 325, 327] and Win1 [328, 329]. Components of this MAPK cascade are homologous to the HOG1 osmosensing MAPK (Mitogen Activated Protein Kinase) pathway in *S. cereviciae* and the mamalian c-Jun NH2-terminal kinase (JNK) and p38 stress activated protein kinase cascade pathways [330]. The *atf1* gene of *S. pombe* encodes a b-ZIP transcription factor which is a homologue of the mammalian factor ATF-2. ATF-2 is regulated through phosphorylation by the stress-activated MAP kinases SAPK/JNK and p38; whereas, Atf1 transcription factor (also known as Gad7 or Mts1) is a target for the Sty1 stress-activated MAP kinase pathway [331]. These findings highlight a remarkable level of conservation in transcriptional control by stress-activated MAP kinase pathways between fission yeast and mammalian cells. Stress induces transient nuclear localization of Sty1. Nuclear translocation of Sty1 is coupled with disassociation from its activator kinase Wis1. However, Sty1 does not concentrate in the nucleus in $\Delta$*wis1* cells; therefore Wis1 does not retain Sty1 in cytoplasm. Wis1 phosphorylates Sty1 to localize it in the nucleus. Atf1 is constitutively nuclear, is the key nuclear substrate of Sty1 and regulates localization of Sty1 stress-activated kinase [332]. Pcr1 forms a heterodimer with Atf1 [333] to stimulates transcriptional response [334]. Furthermore, a number of target genes have been identified that are induced rapidly in a Sty1- and Atf1- dependent manner. These genes include *gpd1* (which is important for the response of cells to osmotic stress), *ctt1* (which is important to combat oxidative stress), and *pyp2* (which encodes a tyrosine-specific MAP kinase phosphatase). Induction of Pyp2 by Atf1 is direct in that it does not require de novo protein synthesis and results in a negative feedback loop that serves to control signaling through the Sty1/Wis1 pathway [331].

### 1.3.6. Pap1 pathway

The b-ZIP containing transcription factor Pap1 (pombe AP-1) (homologue of c-Jun in mammals), responds to non toxic doses of $H_2O_2$ . It regulates transcription of genes required for normal tolerance to peroxides [335, 317, 336].

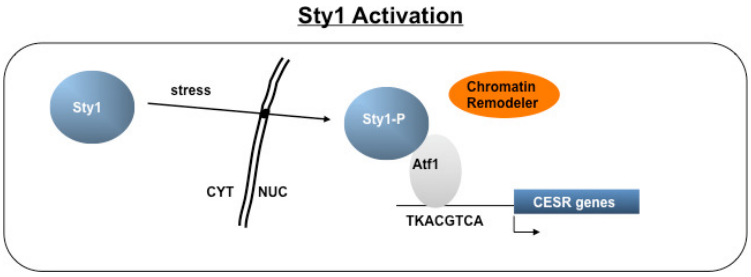


Figure 1.17: Activation of Sty1 pathway in *S. pombe*

The peroxiredoxin Tpx1 acts as a sensor in this signaling pathway. Tpx1 scavenge $H_2O_2$ and transduce the redox signal to Pap1 [127, 128]. The localization of Pap1 is cytosolic prior to stress [337]. The transcription factor has a double nuclear import signal (NLS) which is reconized by Imp1 (importin-$\alpha$) [338] and a nuclear export signal (NES) Crm1- (exportin) dependent [339]. In response to $H_2O_2$ two Cys residues form at least one intramolecular disulfide bond in Pap1; this causes a conformational change, which hinders its NES from Crm1, resulting in a transient nuclear accumulation [339, 340, 318]. Oxidation and nuclear accumulation of Pap1 induces $>$ 2-fold expression of more than 50 Pap1-dependent genes [336]. Low doses of $H_2O_2$ induce two types of responses: adaptation to oxidative stress and tolerance to toxic drugs [341]. A subset of Pap1-dependent genes, such as Caf5 (those coding for an efflux pump), Obr1 (an ubiquitin-like protein) or SPCC663.08c (coding for a dehydrogenase), only require nuclear Pap1 for activation, whereas another subset of genes, those coding for the antioxidants Ctt1, Srx1 or Trr1, need oxidized Pap1 to form a heterodimer with the constitutively nuclear transcription factor Prr1. The ability of Pap1 to bind and activate drug tolerance promoters is independent on Prr1, whereas its affinity for the antioxidant promoters is significantly enhanced upon association with Prr1. The thioredoxin system contribute to both maintaining Pap1 in a reduced state in the absence of stress and returning it to the inactive-reduced-cytoplasmic form once the gene response has been engaged [318, 342]. In addition, *trx1* gene expression was induced by $H_2O_2$ and menadione, being mediated through a stress-responsive transcription factor Pap1. Moreover, at high doses of $H_2O_2$ as described for other eukaryotic peroxiredoxins, Tpx1 is temporarily inactivated and delays Pap1 activation, whereas the Sty1 pathway remains fully functional under these conditions. As part of the Sty1-dependent

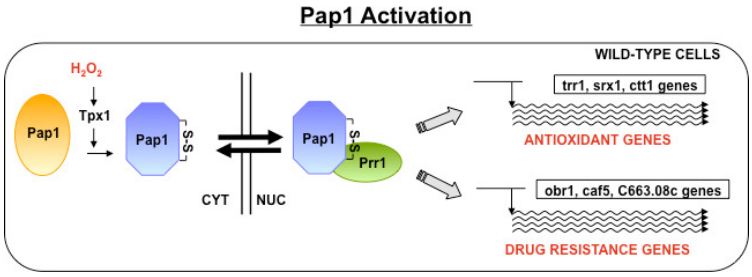


Figure 1.18: Activation of Pap1 pathway in *S. pombe*. Association of oxidized Pap1 and Prr1 is required for the activation of the antioxidant, but not the drug resistance, genes. In wild-type cells, oxidation of Pap1 upon $H_2O_2$ stress induces its nuclear accumulation and its association with Prr1. The heterodimer is then able to activate both sets of promoters, the antioxidant (*trr1*, *srx1*, *ctt1*) and the drug resistance (*obr1*, *caf5*, *c663.08c*) genes. Reprinted from [341]

transcriptional response, the expression of Srx1 is induced and this reductase re-activates the over-oxidised Tpx1. This temporary redox inactivation seems to suggest that Prx enzymes might have a role in signal transduction, with their inactivation shunt causing a temporary or local increase in $H_2O_2$ concentration that could trigger antioxidant cascades [114]. Recently, it was proposed that maybe the hyperoxidation of Tpx1 promotes the survival of cells exposed to acutelly toxic levels of $H_2O_2$ by allowing Trx1 to reduce other vital oxidized proteins such as methionine sulfoxide reductase (Mxr1) [343].

## 1.4. Cellular responses to iron fluctuations

Iron is in short supply in many habitats, may be because of that most organisms have a concert response upon iron starvation, but not under iron repleted. In iron depletion conditions all the organism have a wide range of mechanisms for combat iron starvation (see below). The general response to iron starvation include increase iron importers, diminish iron storage and decrease iron containing proteins. However, under iron repleted conditions the cell could be like a bunker protecting itself just by diminish the amount of iron transporters in the membrane, increasing the storage and the Fe-containing proteins using the same actors than in iron depletion but as a negative feedback loop.

### 1.4.1. *E. coli*

**Fur (ferric uptake regulator): a transcriptional repressor of iron-regulated promoters by virtue of its $Fe^{2+}$-dependent DNA binding activity.**

Because the hydroxyl radical is formed by reaction between $H_2O_2$ and $Fe^{2+}$, its toxicity is limited when their concentrations are minimized. The primary control of iron homeostasis in most bateria is mediated by the Fur protein, a transcription factor activated by the binding of $Fe^{2+}$ [344]. Under iron-rich conditions Fur binds the divalent ion, acquires a configuration able to bind target DNA sequences (generally known as Fur boxes or iron boxes) [345, 346, 347]. On the contrary, when iron is scarce, the equilibrium is displaced to release $Fe^{2+}$, the RNA polymerase accesses cognate promoters, and the genes for the biosynthesis of siderophores and other iron-related functions are expressed [348, 349]. Metallated Fur binds to the promoter regions of operons that encode iron-import proteins, thereby effecting feedback control. Fur mutants exhibit high iron levels and are more sensitive to DNA damage to $H_2O_2$ and high rates of mutagenesis [350]. The toxicity that *E. coli* fur mutants experience from excessive iron import can be compensated for by the engineered over synthesis of the classic iron-storage protein, ferritin [350].

*ryhB: small non coding regulatory RNA that negatively regulate many mRNAs encoding iron-using or iron-detoxifying proteins*

Other way to control iron in *E. coli* is inducing a small non coding regulatory RNA, designated ryhB under iron deficiency [351]. *ryhB* negatively regulates a large number of mRNAs encoding iron-using or iron-detoxifying proteins under conditions in which the essential co-factor is scarce [352]. Under iron-replete conditions, the repressor Fur is active and binds the promoter region of *ryhB*, shutting down its transcription and under these conditions, *ryhB*-targetd mRNAs are expressed [351].

### 1.4.2. *S. cerevisiae*

**AFT1: activator of iron up-take genes**

In budding yeast, two transcription factors, AFT1 and AFT2 are iron-responsive transcriptional activators of genes encoding components of iron transport system in response to iron deprivation [353, 354, 355, 356]. When the iron concentration is low, AFT1 translocates in the nucleus and induces expression of its target genes [357, 358]. Its nuclear import is not regulated by limited iron [359]. Conversely, in response to iron-replete conditions, AFT1 moves from the nucleus to the cytoplasm, where it remains inactive; this event is iron- and MSN5 (exportin)-dependent [359]. In vitro studies have shown that both transcription factors bind to an iron-responsive element (FeRE) that is present in the upstream region of the genes of the iron regulon [354].

**CTH2: posttrancriptional regulator in charge of the metabolic reprogramming in order to facilitate the utilization of limited cellular Fe levels**

CTH2, a RNA-binding protein is also syntethized when the availability of iron is limited [360]. Induction of CTH2 transcript is mediated by AFT1 and AFT2 transcription factors [360]. CTH2 is a posttrancriptional regulator which drives a widespread metabolic reprogramming by downregulation of mRNAs encoding proteins that participate in many Fe-dependent processes [360]. This means that in order to facilitate the utilization of limited cellular Fe levels, CTH2 is bound to specific AU-rich elements (AREs) in the 3' untranslated region (UTR) of the mRNAs targeted and triggers them for degradation [360].

### 1.4.3. Mammals

In mammals, the response to iron scarcity is regulated posttranscriptionally involving two iron regulatory proteins (IRP) which determine the translational efficiency of a few proteins involved in iron uptake, distribution and storage [361, 362]. IRP1 assembles a cubic iron-sulphur cluster which dissociates upon iron scarcity allowing the apo-protein to bind to specific palindromic mRNA stem-loop structures known as iron-responsive elements (IREs) of certain mRNAs of iron-regulated proteins. IRP1 is a bifunctional protein that at high Fe

levels, becomes a holoenzyme and acts as a cytosolic aconitase; however, under low iron conditions it loses the 4Fe-4S cluster and this induces an mRNA-binding activity of the enzyme. The IRE/IRP system controls the expression of ferritin, transferrin receptor and erythroid 5-aminolevulinic acid synthase [363]. IRP1 binding to IREs in the 3'UTR of the transferrin receptor 1 isoform stabilizes the mRNA, thereby increasing protein levels and enhancing Fe uptake via Fe loaded transferrin [364, 361] and at the same time binds to the heavy and light subunits of ferritin inhibiting its translation. IRP2 also binds such IREs under low iron condition. Thus, in iron-replete cells, IRP1 has iron-sulphur cluster associated, which prevents IRE binding, while IRP2 undergoes ubiquitin-proteasomal mediated degradation [365]. Once bound to the 5'-UTR, IRP prevents the recruitment of 43S translation preinitiation complex and consequently inhibits mRNA translation. On the contrary, IRP binding to the 3'-UTR stabilises mRNA and enhances its translation. IRP1 and IRP2 also respond, albeit differentially, to iron-independent signals, such as $H_2O_2$, hypoxia, or nitric oxide. However, IRPs simultaneously 'sense' and 'control' cell iron level, with the latter function being aimed at keeping the intermediate pool of transit or chelatable iron at a critical level to ensure an adequate iron supply for important cell functions, while avoiding the toxic events such as oxidative stress, associated with an expansion of this pool [366].

Despite the fundamental difference of the fungal and mammalian systems for iron regulation, both are severely influenced by the efficiency of mitochondrial FeS protein biogenesis thus linking the efficiency of cellular iron uptake to its intracellular consumption during the generation of cellular FeS proteins.

### 1.4.4. *S. pombe*

In the model organism *S. pombe*, Fep1 and Php4 act as key regulators of iron homeostasis by controling iron aquisition and iron utilization, respectively [258, 367, 368].

**Fep1: repressor of iron up-take genes. Transcriptional regulation of genes required for iron acquisition.**

Under conditions of iron starvation, the mRNA levels of some genes involved in Fe up-take and Fe availability are strongly induced, such as *frp1*, *fio1*, *fip1*, *php4*, *abc3 sib1*, *sib2*, *str1*, *str2* and *str3* [257, 256, 270, 368, 282].These

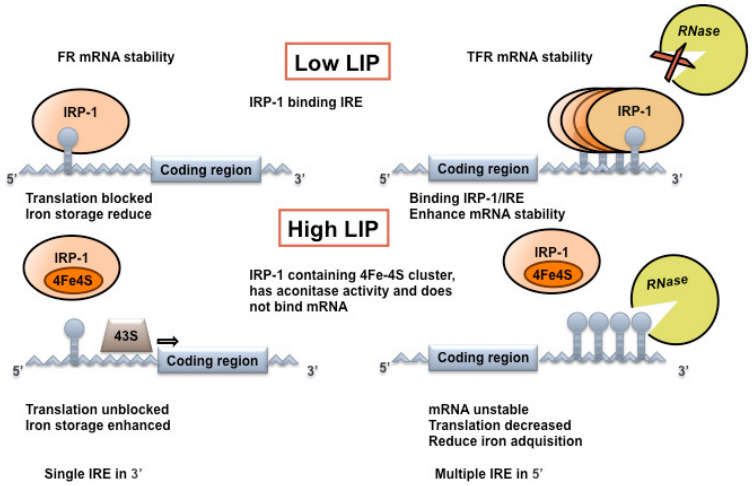


Figure 1.19: Posttranscriptional control of the expression of ferritin (FT) and ferritin tramnsport (TFR) mRNA by the IRE/IRP system. Abbreviations: 43S, pre-initiation complex, LIP, labile iron pool. Modified from[306, 307]

genes possess a GATA-type regulatory sequence 5' -(A/T) GATAA-3' at their promoters [270, 368]. Both mechanisms reductive or nonreductive iron adquisition are directed transcriptionally by the repressor Fep1, a member of the GATA factor protein family [270, 368]. Fission yeast cells in which *fep1* has been disrupted display a elevated level of expression of *fio1*, *sib1*, *sib2*, *str1*, *str2* and *str3* [368, 282, 270]. Moreover the *fep1* disrupted strain is hypersensitive to pheomycin (an antibiotic that cleaves nucleic acids in the presence of excess iron) and increase the activity of the surface reductase Frp1 [368]. Fep1 acts with two proteins Tup11 and Tup12 as a correpresors [368]. Tup11 and Fep1 physically interact with each other for down-regulation of iron uptake genes, this interaccion is through Fep1 C-terminal aas from 405 to 541 [369], whereas the N-terminal of Fep1 241 amino acids include the DNA binding motif [368]. Furthermore the N-terminal region of Fep1 is highly similar to the N-terminal regions of the Urbs1, SRE, SREA and Sfu1 proteins that have been shown to play a role in the regulation of iron-responsive genes of *Ustilago maydis, Neurospora crassa, Aspergillus nidulans and Candida albicans*, respectively [370, 371, 372, 373, 374, 375, 376].

**Regulation of iron utilizing and storage genes through Php4 repressor.**

The CCAAT-binding subunit Php4 is a transcriptional co-repressor that down-regulates genes that encode iron consuming and storage proteins, such as the tricarboxylic acid cycle (TCA), the electron transport chain (Sdhs), the iron-sulphur cluster biogenesis machinery (Isa1) and the iron importer to vacuole (Pcl1) [228]. A genome-wide microarray analysis has revealed that Php4 is capable of coordinating the represion of 86 genes in response to iron starvation [228]. Php4 asociates with its target genes by recognintion of the CCAAT-binding complex, which is composed of Php2, Php3 and Php5 [377, 367]. The Php2/Php3/Php5 heterotrimer binds CCAAT cis-acting elements, whereas Php4 lacks DNA binding activity. Php4 is responsible for the capability of the Php complex to repress transcription in response to iron starvation. Fep1 posess a CCAAT box and it is regulated by Php4 [378], when the availability of iron is limited Fep1 fails to act as a repressor resulting in php4 transcription. At the same time Php4 has a GATA-sequence in its promoter. Thus, create a negative regulatory feedback loop between both iron-responsive sensors [228]. Part of the regulation in this system also includes the localization of Php4. Under low iron conditions Php4 acummulates in the nucleus [379]. Php4 posesses a rich leucine nuclear export signal (NES) within the region of aas 93-100 [379]. Php4 binds Crm1 (an exportin that recognizes leucine-rich NES) and is sensitive to leptomycin B (LMB, abolishes its nuclear export behavior by covalent binding to a Cys residue in Crm1). This means that in the presence of LMB, Php4 is constitutively nuclear but it can still be inactivated by iron, resulting in the derepression of Php4 downstream genes [379]. Grx4 is a binding partner of Php4 as well. In summary Php4 translocated from the nucleus to the cytoplasm in a Crm1-, iron-, Grx4-dependent manner [379]. One of the requirements for iron excess sensing by Php4 is GSH. Mutant cells defective in GSH biogenesis such as $\Delta gcs1$ ($1^{st}$ enzyme in the GSH biosynthesis pathway) show a decreased transcription of genes encoding iron-using proteins as a result of constitutively active Php4 [228]. GSH has also been associated with cellular iron sensing in *S. cereviciae* [380]. Likewise, in *S. cerevisiae* as for Php4, AFT1 is constitutively active during GSH deficiency [380].

### 1.4.5. Grx4: the common regulator of Fep1 and Php4.

*S. pombe* possess two CGFS-type monothiol glutaredoxins Grx4 and Grx5, which are localized primarily in the nucleus and mitochondria respectively [381]. Grx5 plays a role in the FeS assembly process through interaction with FeS scaffold proteins Isa1 and Isa2 in mitochondria [381]. Grx4 is critically required for aerobic growth in *S. pombe*. The *grx4* gene encodes a protein (244 aa) highly homologous in sequence with GRX3 and GRX4 of *S. cereviciae*, with 42% and 41% aa identities, respectively[381]. The localization of Grx4-GFP protein was corroborated later by S. Labbe's laboratory. It was found throughout the cell but with a predominance of the signal being observed in the nuclei [382]. Grx4 contains an N-terminal (1-106 aa) thioredoxin (TRX)-like domain, which contributes to its nuclear localization, and Grx4 also posesses a C-terminal glutaredoxin (GRX)-like domain with the CGFS motif. Biochemical characterizations revealed that both prokaryotic and eukaryotic monothiol glutaredoxins with CGFS motif incorporate a GSH-ligated [2Fe-2S] center [383]. In this thesis we will determine if the same occurs in *S. pombe*.

The deletion of *grx4* leads to permanent Php4 nuclear accummulation and a constitutive repression of iron utilizing genes [379]. Likewise, in *S. cereviciae* Grx4 homologs, GRX3 and GRX4 regulate iron-responsive gene expression by modulating the activity of the activator AFT1 [384]. When cells encounter sufficient iron, it has been proposed that GRX3 and GRX4, with the aid of FRA1 and FRA2, transmit an inhibitory signal from mitochondria to inactivate AFT1 [385].

Grx4 and Fep1 interact physically with each other, and the deletion of *grx4* gene led to constitutive promoter occupancy by Fep1 and caused repression of the iron transporters genes [382]. Grx4 monothiol glutaredoxin is required as an inhibitory partner of Fep1 in iron limitation conditions [382]. The TRX domain of Grx4 interacts strongly and constitutively with the C-terminal region of Fep1; it is Cys35 which is required for this interaction [382]. Furthermore, the GRX domain of Grx4 associates weakly and in an iron-dependent manner with the N-terminal region of Fep1 through Cys172 [382].

## 1.5. Human diseases related to iron pertubations: Friedreich's ataxia (FRDA).

The mechanisms of iron transport are remarkably conserved between yeast and humans. Importance of iron homeostasis were highlighted above. Studies of the yeast homologs of human disease genes involved in metal homeostasis have shed light on the pathophysiology of these disorders. Due to *S. pombe* has a homologue gene of FRDA-encoding frataxin, mutations in this gene are responsible for FRDA and frataxin is a mitochondrial protein well conserved throughout evolution, from bacteria to yeast and humans, we decide to develop a new model for study FRDA in *S. pombe*. Studies on the *S. pombe* orthologue *pfh1* will contribut to our understanding of the function of frataxin.

FRDA is the most common inherited autosomal recessive disease in white people causing degeneration in the central and peripheral nervous system, cardiomyopathy, skeletal abnormalities and increased risk of diabetes mellitus [386, 387, 388, 389]. FRDA is caused by decreased expression of a highly conserved nuclear-encoded mitochondrial protein known as frataxin. The genetic mutation found in 98% of Friedreich ataxia chromosomes is the unstable hyperexpansion of a GAA triplet repeat in the first intron of the gene [387, 35]. The expanded GAA repeat, by adopting an abnormal triple helical structure, impairs frataxin transcription. Longer repeats cause a more profound frataxin deficiency and are associated with earlier onset and increased severity of the disease. This disease is thought to be the consequence of a mitochondrial defect related to iron metabolism.

Frataxin is a mitochondrial iron binding protein and performs its primary function in the FeS protein assembly [390]. Yeast cells deficient in the frataxin homologue ($\Delta$*YFH1*) become unable to carry out oxidative phosphorylation, lose mitochondrial DNA [391], accumulate iron in mitochondria [392], suffer iron depletion in the cytosol, show unregulated high expression of high affinity iron uptake, exhibit heme deficiency [393] and have an increased sensitivity to oxidative stress. Loss of respiratory competence in $\Delta$*YFH1* is iron-dependent. Additional properties of these cells include a deficiency of FeS cluster containing proteins and impaired iron efflux out of mitochondria. YFH1 interacts in an iron-dependent manner with ISU1 in vivo and its depletion is associated with a defect in the synthesis of the transient ISC on ISUs1 [394]. In vitro studies indicate that YFH1 can bind iron and deliver bound iron to ISU1 for assembly

of the ISC [395]. Thus, frataxin can be regarded as an iron donor for ISC formation on ISU1. Evidence of oxidative stress, mitochondrial dysfunction [396], deficiency of multiple FeS cluster containing proteins and iron deposits are also found in the human disease and in mouse models [397, 398]. Oxidative damage of mitochondria is thought to play a key role in the pathogenesis of the disease. Therefore, a possible therapeutic strategy should be directed to an antioxidant protection against mitochondrial damage, than could be easily test it in a yeast model. Despite the great amount of data obtained with these different model systems, the analysis of the proteome of frataxin-deficient cells has not been addressed. During this thesis we analysed the proteome of frataxin-deficient cells in our model of FRDA in *S. pombe*.

# Chapter 2

# RESULTS

The results chapter will be divided in four sections, three of them as manuscripts (in preparation; sections 2.2-2.4):
2.1 Identification of genes essential for ROS homeostasis: screen of a mutant library.
In the laboratory, we received a collection of *S. pombe* deletion mutants, and I participated in the original screens to identify genes participating in wild-type tolerance to oxidative stress. The main results of the screens, briefly described in sections 2.1.1 and 2.1.2, were published in two papers (Appendix A.1 and A.2). During the course of the genetic screen, we isolated the mutant strain $\Delta$*fep1*, lacking the master regulator of iron starvation genes, as sensitive to oxidative stress. The characterization of this mutants, together with two other deletion strains also sensitive to ROS, is described in section 2.1.3. The isolation of some iron homeostasis genes in the screens for oxidative stress survival prompted us to investigate the iron regulon in *S. pombe*, so that we could define the cross-talk between metals and oxidative stress. The results of these studies constitute the focus of the other three sections, and are provided as manuscripts (in preparation).

2.2 Cross-induction of iron depletion regulon by oxidative stress.
We have tried to dissect the cross-induction of stress and iron genes by iron starvation and $H_2O_2$ signals, respectively.

2.3 Characterization of Grx4.
We ended up studying Grx4, which during the course of this PhD was shown to be essential for iron sensing at least in *S. cerevisiae*.

2.4 New model of Friedreich's ataxia: *S. pombe* $\Delta$*pfh1*.
We noticed that the *S. pombe* genome included a gene homolog of frataxin, known to be mutated in patients of FriedreichÕs ataxia. We have included in this chapter the characterization of this mutants, which defects are associated to iron homeostasis.

Finally, in Appendix A ( A.3 and A.4) are include two papers in which I was contributing in experimental work during this PhD.

Promoter-driven splicing regulation in fission yeast. Moldón A, Malapeira J, Gabrielli N, Gogol M, Gómez-Escoda B, Ivanova T, Seidel C, Ayté J. Nature. 2008 Oct 16;455(7215):997-1000. Epub 2008 Sep 24.

Lifespan extension by calorie restriction relies on the Sty1 MAP kinase stress pathway. Zuin A, Carmona M, Morales-Ivorra I, Gabrielli N, Vivancos AP, Ayté J, Hidalgo E. EMBO J. 2010 Mar 3;29(5):981-91. Epub 2010 Jan 14.

## 2.1. Identification of genes essential for ROS homeostasis: screen of a mutant library

*S. pombe* genome has been fully sequenced [310]. Its genetic material is organised in three chromosomes, containing 5036 genes scattered around 14 megabases (Mb) of total DNA making it the smallest free-living eukaryote [310]. Its genome contains few duplicated genes, making it susceptible to powerful genetic tools such as gene disruption and phenotypic analyses together with more sophisticated functional screens [308, 311, 19]. This further makes it an atractive model species for basic studies in cell biology. To identify genes required for survival upon both exogenous and endogenous oxidative stress, we tested an *S. pombe* collection of viable open reading frame deletion mutants by global mapping of those genes that are required for growth on respiratory-proficient media, hydrogen-peroxide-containing fermentable media and caffeine. As a consequence of the screening that we performed, we publish two paper [311, 19], in which I participated as a first co-author.

### 2.1.1. Mitochondrial Dysfunction Increases Oxidative Stress and Decreases Chronological Life Span in Fission Yeast

ROS homeostasis plays an important role in chronological aging processes and some associated diseases [399, 400, 401, 402, 403]. ROS originate mainly

from the mitochondria due to incomplete reduction of oxygen at several sites along the electron transfer chain [404]. We analyzed the effect of aerobic metabolism on oxidative damage in *S. pombe* by global mapping of those genes that are required for growth on both respiratory-proficient media and $H_2O_2$-containing fermentable media. During this work I, together with Isabel Calvo, conducted the high-throughput sensitivity screen. Out of a collection of approximately 2,700 haploid yeast deletion mutants, 51 were sensitive to both conditions and 19 of these were related to mitochondrial function. Twelve deletion mutants lacked components of the electron transport chain. Mitochondrial mutants displayed reduced oxygen consumption and increased intracellular ROS levels. I tuned-up and performed the assay for measuring ROS levels by a fluorescent dye (DCFH-DA, 2',5'-dichloridofluorescein diacetate). Another observation that was made during this work was that inhibition of the electron transfer chain causes a reduction of the life span. In this case, I was responsible for performing the quantification of the metabolic activity and viability of the mutants using propidium iodide and phloxine B by FACS analysis. The growth defects of these mutants can be alleviated by the addition of antioxidants, which points to intrinsic oxidative stress as the origin of the phenotypes observed. These respiration-deficient mutants display elevated steady-state levels of ROS, probably due to enhanced electron leakage from their defective transport chains, which compromises the viability of chronologically-aged cells. Finally, we conclude that individual mitochondrial dysfunctions have often been described as the cause of diseases or aging, and our global characterization emphasizes the primacy of oxidative stress in the etiology of such processes. I participated as a first co-author in this study which was publish in PLoS One. 2008 Jul 30;3(7):e2842. Mitochondrial dysfunction increases oxidative stress and decreases chronological life span in fission yeast. Zuin A, Gabrielli N, Calvo IA, García-Santamarina S, Hoe KL, Kim DU, Park HO, Hayles J, Ayté J, Hidalgo E. (Appendix A.1).

### 2.1.2. Genome-Wide Screen of Genes Required for Caffeine Tolerance in Fission Yeast

All eukaryotic cell types suffer cytotoxic effects upon an excess of caffeine, but the manner in which caffeine triggers these pleiotropic effects is still largely unknown. We decided to study how cells become tolerant to a toxic dose of this drug to gain insights into the molecular targets of caffeine, and to examine in depth its relationship with oxidative stress pathways. We searched for *S. pombe*

mutants with inhibited growth on caffeine-containing agar plates. A collection of 2,700 haploid mutant cells were screened, of which 98 were sensitive to caffeine. The genes mutated in these sensitive clones were involved in a number of cellular roles including the $H_2O_2$-induced Pap1 and Sty1 stress pathways, the integrity and calcineurin pathways, cell morphology and chromatin remodeling. We have investigated the role of oxidative stress pathways in sensing and promoting survival to caffeine. The Pap1 and the Sty1 pathways are both required for normal tolerance to caffeine, but only the Sty1 pathway is activated by the drug. Cells lacking Pap1 are sensitive to caffeine due to the decreased basal expression of the efflux pump Hba2. Indeed, $\Delta$*hba2* cells are sensitive to caffeine, and constitutive activation of the Pap1 pathway enhances resistance to caffeine in an Hba2-dependent manner. We can conclude from our caffeine-sensitive, genome-wide screen of an *S. pombe* deletion collection, we have demonstrated the importance of some oxidative stress pathway components on wild-type tolerance to the drug. I participated as a first co-author in this study. This work was published in PLoS One. 2009 Aug 12;4(8):e6619. Genome-wide screen of genes required for caffeine tolerance in fission yeast. Calvo IA, Gabrielli N, Iglesias-Baena I, García-Santamarina S, Hoe KL, Kim DU, Sansó M, Zuin A, Pérez P, Ayté J, Hidalgo E. (Appendix A.2).

### 2.1.3. Characterization of genes involved in iron homeostasis that are sensitive to oxidative stress: *fep1*, *pcl1* and *sib2*

Among the genes essential both for wild-type tolerance to $H_2O_2$ and to respiratory-prone conditions (see section 2.1.1), we isolated *fep1*, an essential regulator of iron homeostasis.

Iron is a vital element, an indispensable cofactor for a wide variety of redox enzymes involved in metabolic processes such as the tricarboxilyc acid cycle, the cellular respiratory chain or oxygen transport. Paradoxically, iron levels must be accurately regulated due to its ability to react with ROS causing oxidative stress and cellular damage that can contribute to different diseases. As described above, we performed an high-throughput sensitivity screen to identify new genes involved in the regulation of intrinsic oxidative stress. We identified *fep1* gene as one of the genes that are required for growth on both respiratory-proficient media and hydrogen-peroxide-containing fermentable media by global mapping. Moreover, we determine than in the absence of *fep1* and *sib2* genes the cells are sensitive to $H_2O_2$ in our screening.

As described in the introduction (section 1.4.4) the iron starvation response in *S. pombe* is regulated by Grx4, which controls the Fep1 and Php4 represors. Briefly, the inactivation of Fep1 and activation of Php4 transcriptional repressors mediate the cellular response to iron deficiency (Fig. **). Thus, when iron is limiting Fep1 is released from promoters of genes involved in iron uptake such as *fio* or *sib2* , while Php4 accumulates at the nucleus and represses transcription of genes coding for iron-consuming or iron-storage proteins such as *pcl1*. Sib2 is a Fep1, whereas Pcl1 is a Php4. Due to the obvious relevance of maintaining iron homeostasis in the regulation of intrinsic oxidative stress, we decided to select some gene deletions involved in iron homeostasis present in the collection of mutants and spotted serial dilution in $H_2O_2$, deferoxamine and iron agar plates. The deletion of the *fep1* and *sib2* genes was sensitive to oxidative stress confirming our prior result from the screening, We also found that the mutant containing the *pcl1* deleted gene was sensitive to $H_2O_2$ (Figure 2.1). These results were verified in liquid cultures (Figure 2.1B). The major delay in growth curves upon $H_2O_2$ was found in *fep1* deletion (Figure 2.1C).

**$\Delta$*fep1* cells are sensitive to oxidative stress**

Deletion of the *fep1* gene presents sensitivity to $H_2O_2$. We were therefore interested to see if this was due to the amount of iron in this particular strain. We tested the *fep1* deleted strain in agar plates as well as in liquid cultures with $H_2O_2$; sensitivity was observed in both (Figure 2.2) . The $\Delta$*fep1* strain presented the major dose-dependent delay in growth compared to a WT strain in liquid cultures. In the absence of Fep1, there is lack of transcriptional repression of genes encoding components of the high-affinity Fe uptake machinery [368]. Conveniently, this disruption rendered the mutant strain unable to grow aerobically in medium containing phleomycin [368]. Phleomycin is an antibiotic that acts as an iron-dependent free radical generator. It intersperses between DNA strands which causes free radical generation and leads to DNA oxidation and breakage that confers iron-dependent toxicity. In our hands, when we tested viability to phleomycin we saw a complete inhibition of growth in $\Delta$*fep1* cells. We also tested viability against excess of iron in agar-plates supplemented with 2 mM of $FeCl_3$ and observed defective growth (Figure 2.2); the colonies became small and brown. Furthermore, we tested the $\Delta$*fep1* strain in agar plates with a chelator agent (deferroxamine, DX), observing slight but reproducible resistance to iron starvation (Figure 2.2). The deleted strain also showed a slightly better growth

in liquid culture upon an iron chelator (dipyridyl, Dip) than WT (Figure 2.2). Finally we measured the total amount of iron in a deleted strain showing an increase of 4 fold (Figure 2.2). All this data suggests that the sensitivity to $H_2O_2$ of the *fep1* deleted strain is due to a deleterious effect of the increase of iron that causes an increase in the thermodynamics of the Fenton reaction.

### $\Delta$*pcl1* cells are sensitive to oxidative stress

The deletion of the putative vacuolar Fe importer, Pcl1, rendered cells sensitive to $H_2O_2$ compared to the WT strain, either in liquid cultures or in agar-plate growth with $H_2O_2$ (Figure 2.3). Consistent with these findings, we observed that cells lacking *pcl1* were sensitive to Fe-containing plates. We measured the total iron in the $\Delta$*pcl1* strain and found that it was no significant difference in iron content compared to the WT strain (Figure 2.3). We also tested the viability in agar-plates and liquid culture under the condition of iron starvation, and found that the phenotype of a *pcl1* deleted mutant was like a WT strain. From this data we conclude that in $\Delta$*pcl1* cells the iron is not properly stored in vacuoles and is available to react with $H_2O_2$ generating cell damage via Fenton reaction.

### $\Delta$*sib2* cells are sensitive to oxidative stress

The *sib2* gene is an l-ornithine N(5)-oxygenase that participate in ferrichrome biosynthesis. We observed that the deletion of this gene exhibited a severe growth defect on iron-poor media and at the same time presented sensitivity to $H_2O_2$ (Figure 2.4). We didn't observe growth arrest upon iron starvation conditions in liquid cultures (Figure 2.4). Siderophores have a dual role incorporating iron from the environment and as well most fungi use siderophores as intracellular iron storage compounds. This duality could explain the double sensitivity to iron starvation and oxidative stress: while $\Delta$*sib2* cells would have problems to enhance iron uptake upon iron deprivation conditions, they shoud also display higher basal levels of available iron to react with $H_2O_2$ and induce cell damage.

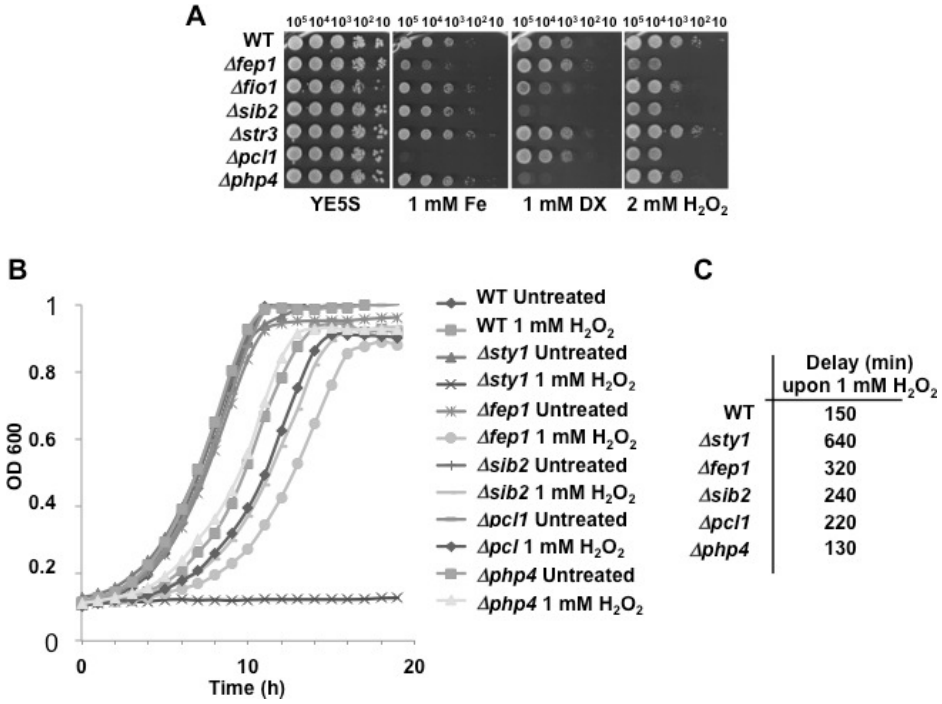


Figure 2.1: (A) Cells lacking *fep1*, *sib2* and *pcl1* showed sensitivity to $H_2O_2$. Strains 972 (WT), NG1 (Δ*fep1*), NG5 (Δ*fio1*) NG8 (Δ*sib2*), NG9 (Δ*str3*), NG18 (Δ*pcl1*) and NG40 (Δ*php4*) were grown in YE5S to a final $OD_{600}$ of 0.5, and serial dilutions from $10^5$ to 10 cells were spotted in YE5S plates containing or not $H_2O_2$, deferroxamine (Dx) and iron (Fe) at the indicated concentrations and were incubated at 30°C for 2-3 days. (B) Growth curves of the strain described in A were grown in YE5S liquid medium treated or not with $H_2O_2$ and $OD_{600}$ was recorded at the indicated times for each culture during 35h. (C) Cells lacking *fep1*, *sib2* and *pcl1* also present a delay in liquid growth upon $H_2O_2$.

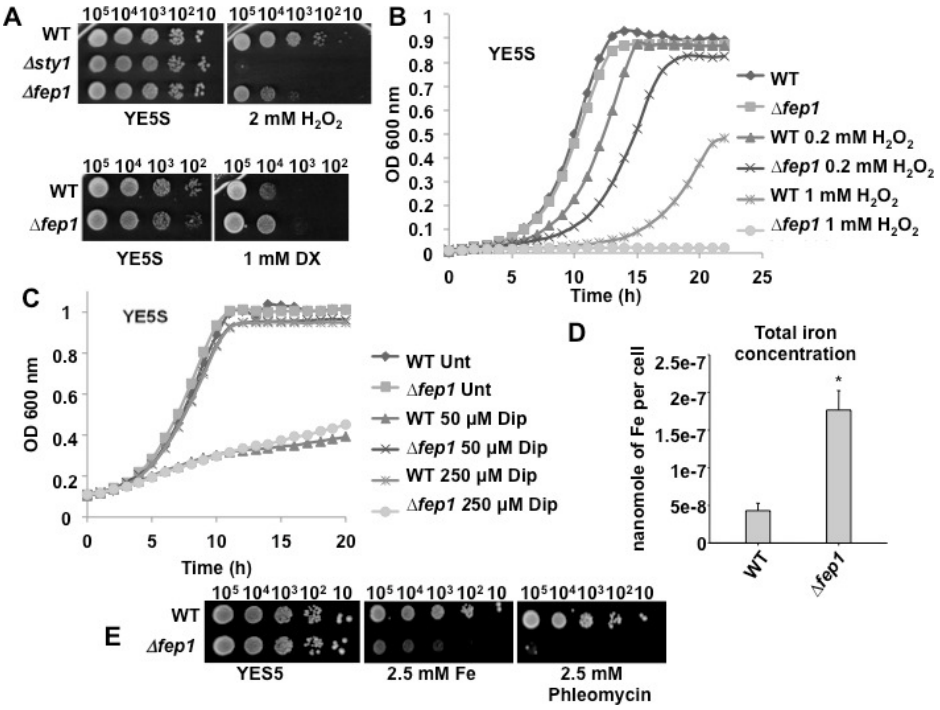


Figure 2.2: (A) Cells lacking *fep1* showed sensitivity to $H_2O_2$. Strains 972 (WT), NG1 ($\Delta$*fep1*) and AV18 ($\Delta$*sty1*) were grown and spotted as in Fig. 2.1 A. (B) Growth curves of 972 (WT) and NG1 ($\Delta$*fep1*) were grown as described in Fig. 2.1B. (C) Cells lacking *fep1* showed slightly improved growth upon iron starvation compared with a wild type strain. Growth curves of strains used in Fig 2.2B were grown as described in Fig. 2.1B. and treated or not with 2, 2' dipyridyl at the indicated concentration. (D) Cells lacking *fep1* present an increase in total amount of iron. Quantification of total iron was done as specified in Materials and Methods. (E) Cells lacking *fep1* present a defect in growth upon iron repletion. Survival spots were performed for the strain cells used in Fig. 2.2 B, with or without phleomycin, as was described in Fig. 2.1B.

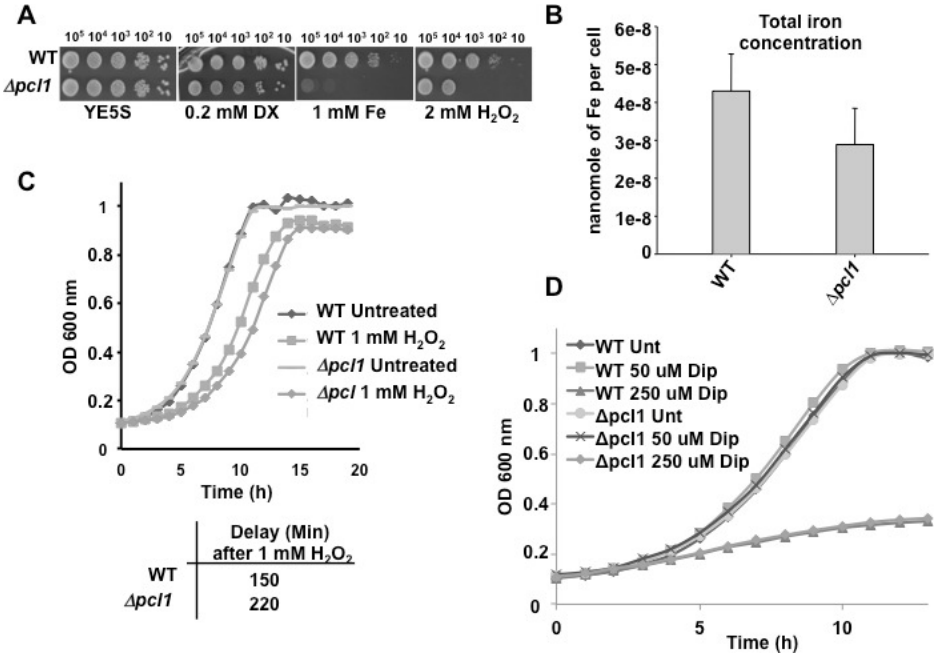


Figure 2.3: (A) Cells lacking *pcl1* showed sensitivity to $H_2O_2$ and high levels of iron. Strains 972 (WT) and NG18 ($\Delta$*pcl1*) were grown and spotted as in Fig. 2.1 A. (B) Cells lacking *pcl1* did not exhibit significant difference in total amount of iron compared with a wild type strain. Quantification of total iron was done with the strains used in Fig.2.3 A as specified in Materials and Methods. (C) Cells lacking *pcl1* showed a defect in growth upon $H_2O_2$. Growth curves of strains used in Fig 2.3 A were grown as described in Fig. 2.1B. (D) Cells lacking *pcl1* did not show resistance to iron starvation. Growth curves of 972 (WT) and NG18 ($\Delta$*pcl1*) were grown and treated as described in Fig. 2.2 B and C.

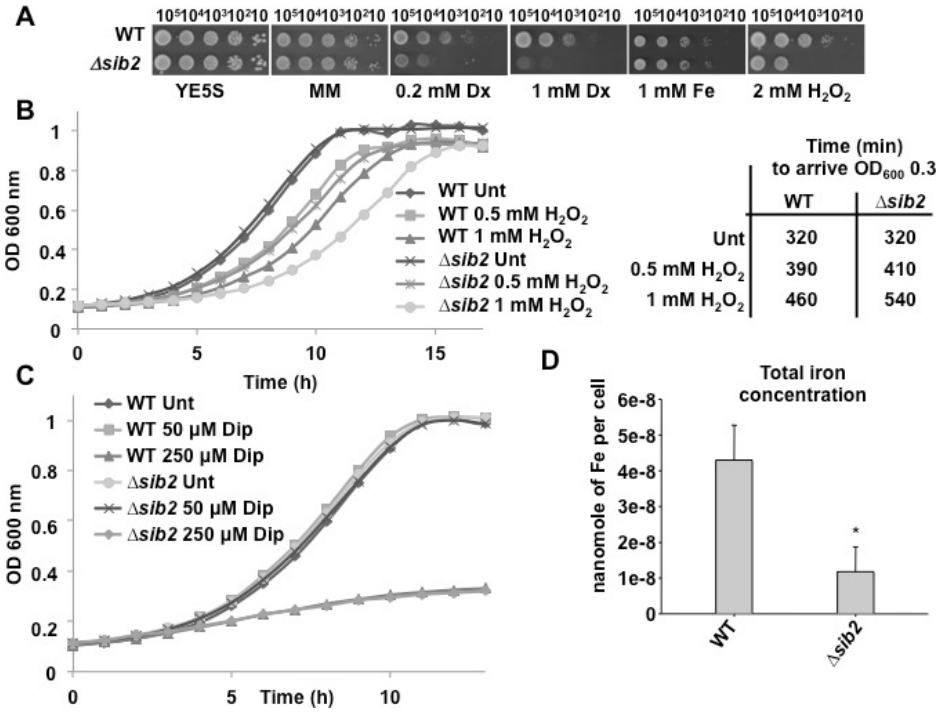


Figure 2.4: (A) Cells lacking *sib2* showed sensitivity to $H_2O_2$ and iron starvation. Strains 972 (WT) and NG8 ($\Delta$*sib2*) were grown and spotted as in Fig. 2.1 A, including MM plates. (B) Cells lacking *sib2* showed a delay in growth upon $H_2O_2$. Growth curves of strains used in Fig 2.4 A were grown as described in Fig. 2.1B. (C) Cells lacking *sib2* are not affected upon iron starvation in liquid cultures. Growth curves of the strains used in Fig. 2.4 A were grown and treated as described in Fig. 2.2 C. (D) Cells lacking *sib2* showed a significant decrease in total amount of iron compared with a wild type strain. Quantification of total iron was done with the strains used in Fig.2.4 A as specified in Materials and Methods.

## 2.2. Cross-induction of iron depletion regulon by oxidative stress

# $H_2O_2$-dependent activation of the antioxidant gene expression program triggers the iron-starvation response in fission yeast

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Running title: catalase is the link between the iron and h2o2 pathways

Keywords: h2o2 responses/iron homeostasis/Sty1/Fep1/Php4/fission yeast

**ABSTRACT**

Iron is probably the most important micronutrient used by microorganisms. But this metal is a double-edged sword for the cell due to its ability to mediate Fenton reactions in combination with hydrogen peroxide ($H_2O_2$) to generate the toxic hydroxyl radical. That is why iron import is tightly regulated to ensure availability of the metal but avoid toxic concentrations. We aim to investigate whether pathways responding to $H_2O_2$ or to iron starvation would regulate each other to prevent the synergistic toxicity of reactive oxygen species and reduced metals, using fission yeast as a model system. We have determined that $H_2O_2$, unexpectedly, triggers the transcriptional iron-starvation response, including enhanced iron import and decreased iron consumption. The MAP kinase Sty1 and its transcription factor Atf1, mediators of the cellular response to $H_2O_2$, are required for this cross-talk. However, they do not seem to directly exert transcription control of the iron-dependent genes. Our experiments support the idea that the $H_2O_2$-, Sty1-, Atf1-dependent over-expression of some antioxidant proteins, and in particular of the heme-containing catalase, causes a strong iron consumption which triggers the accidental induction of the whole iron-starvation pathway.

**INTRODUCTION**

Iron is an essential element for life. Many proteins involved in diverse biological processes, including mitochondrial and cytoplasmic metabolism (aconitase, sulfite reductase, succinate dehydrogenase...), DNA synthesis (ribonucleotide reductase), or stress protection (catalase) do incorporate iron as an essential cofactor. These polypeptides can directly co-ordinate the metal or incorporate it in the form of iron-sulfur clusters or heme groups. Since soluble iron is not easily available in aerobic environments due to its oxidation and insolubilization in the presence of oxygen at neutral pH, siderophores and highly efficient iron acquisition systems are expressed by mostly all cell types to chelate extracellular ferric ion and facilitate its reduction and internalization (for reviews, see (Andrews et al., 2003; Bleackley and Macgillivray, 2011; Grass, 2006).

However, the expression of these import systems is tightly controlled, since the cells have to face the paradox of the need of iron with the capacity of this metal to induce destructive reactions. Thus, an excess of iron can induce damage to proteins, lipids and DNA mainly as a consequence of its interaction with $H_2O_2$ and subsequent generation of the toxic hydroxyl radical through the Fenton reaction. Thus, only under situations of metal scarcity many cell types transcriptionally up-regulate the expression of enzymes involved in synthesis of siderophores and membrane transporters to fulfill the cellular demands.

Different cell types differ greatly in the particular mechanisms employed to regulate the pools of available iron, but in general iron depletion induces diverse cellular responses not only meant to exacerbate iron uptake, but also to decrease iron consumption by cellular constituents (for reviews on iron responses in bacteria, yeasts and mammalian cells, see (Ehrensberger and Bird, 2011; Hentze et al., 2010; Lee and Helmann, 2007; Pantopoulos et al., 2012; Vergara and Thiele, 2008). Thus, this down-regulation often affects the expression of iron-containing proteins, while the intracellular storage of the metal in vacuoles or by ferritin and ferritin-like molecules is

also inhibited. At least in bacteria, and also as a general theme, $H_2O_2$ also elicits modulation of iron homeostasis, with the final consequences of uptake repression and enhanced sequestration and incorporation into metallo-enzymes (Cornelis et al., 2011; Faulkner and Helmann, 2011; Zheng et al., 1999). However, opposite and unexpected cross-talk was described in mammalian cells, with $H_2O_2$ triggering the IRP-dependent activation of the iron starvation response (Pantopoulos and Hentze, 1995).

In *Schizosaccharomyces pombe*, the iron starvation response has started to be unraveled (Labbe et al., 2007). Briefly, the inactivation of Fep1 and activation of Php4 transcriptional repressors mediate the cellular response to iron deficiency (Mercier et al., 2006). Thus, when iron is limiting Fep1 is released from promoters of genes involved in iron uptake (Jbel et al., 2009), while Php4 accumulates at the nucleus and represses transcription of genes coding for iron-consuming or iron-storage proteins (Mercier and Labbe, 2009). Upstream of these transcriptional repressors is the glutaredoxin Grx4, which seems to be the real sensor of iron deprivation, probably through an iron-sulfur center (Gabrielli et al., 2012).

Regarding $H_2O_2$-dependent responses, the fission yeast MAP kinase Sty1 pathway is devoted to trigger a wide gene expression program in response to peroxides and other life-threatening environmental situations (Sanso et al., 2011a; Vivancos et al., 2006). Upon $H_2O_2$ stress, Sty1 becomes phosphorylated, accumulates at the nucleus and phosphorylates the transcription factor Atf1, which together with Pcr1 triggers the recruitment to stress promoters of the Gcn5-containing SAGA complex and of RNA polymerase II (Pol II) (for a review, see (Sanso et al., 2011a). Once the pathway becomes activated, the cell shifts from a growth-related program to a stress survival one, with almost 500 genes being up-regulated by more than two-fold upon 0.5 mM $H_2O_2$ (Chen et al., 2008). Whether this wide change in the gene expression program includes the regulation of iron homeostasis is unknown.

In an attempt to rule out or confirm a cross-talk between the iron starvation and the $H_2O_2$-dependent pathways in fission yeast, we have analyzed the expression of peroxide-responding genes upon iron deprivation and vice versa. Opposite to what we expected, we have found out that oxidative stress triggers genes enhancing iron uptake and decreasing intracellular iron storage and consumption. This late induction of iron starvation genes, which should enhance available iron levels and damage induced by the Fenton reaction, is dependent on the Sty1- and Atf1-dependent induction of the catalase-coding gene. We suspect that newly synthesized heme-containing catalase deprives all the intracellular available iron and mimics an iron-starvation situation. Concomitantly, we have determined that part of the response to iron deprivation is to transcriptionally repress the expression of the catalase gene in a Php4-dependent manner.

## RESULTS

### $H_2O_2$ induces a late iron starvation response in a Sty1- and Atf1-dependent manner

We decided to test whether ROS and iron levels do regulate each other and, thus, whether the iron starvation and $H_2O_2$ responses cross-talk using fission yeast as a model system. Genes such as those coding for the glutathione peroxidase Gpx1, glicerol-3P-dehydrogenase Gpd1, heat shock protein Hsp9 or catalase Ctt1 are up-regulated in response to extracellular $H_2O_2$ after activation of the MAP kinase Sty1 and the transcription factor Atf1 (Fig. 1A, left panel). In parallel, iron starvation conditions both inactivate the repressor Fep1 to induce transcription of genes coding for proteins mediating iron uptake (Fio1, Str3, Sib2...), as well as activate the repressor Php4, which halts transcription of genes promoting iron storage at the vacuole (Pcl1) or coding for metallo-proteins such as succinate dehydrogenase Sdh4 (Fig. 1A, right panel). As shown in Fig. 1B, addition of an iron chelator to the media, dipyridyl (DIP), does not trigger Sty1 nor Atf1 phosphorylation/activation, neither transcription of the stress genes *gpx1, gpd1* or *hsp9* (Fig. 1C). On the contrary, we observed that the genes up- and down-regulated in response to the chelator also respond to the addition of $H_2O_2$ to the growth media, with late kinetics (Fig. 2A). This iron starvation response is specifically triggered by high doses of $H_2O_2$, and other signals triggering the Sy1-Atf1 pathway such as osmotic stress or heat shock do not induce transcription of *fio1* (Fig. 2B). Therefore, only peroxide stress seems to fully engage the iron starvation response, and that induction is Sty1- and Atf1-dependent (Fig. 2C).

### Activation of the stress gene expression program by $H_2O_2$ is required for the late activation of the iron starvation response

Transcriptional activation of stress genes by the Sty1-Atf1 pathway relies on the recruitment of Atf1 to stress promoters (Reiter et al., 2008; Sanso et al., 2008) to facilitate the binding of Pol II

(Sanso et al., 2011b). We could not detect binding of Atf1 to iron starvation genes such as *fio1* or *str3* (data not shown). Since the Sty1 and Atf1 proteins themselves do not seem to be required for the transcriptional regulation of the iron starvation genes, but in Δ*sty1* or Δ*atf1* cells such regulation is fully eliminated (Fig. 2C), we tested whether the stress gene expression program triggered by Sty1 and Atf1 in response to $H_2O_2$ could secondarily cause the induction of the iron starvation response. Cells lacking the histone acetyl transferase Gcn5 are severely defective in the activation of stress genes, since this protein, once recruited to stress promoters by activated Atf1, can facilitate Pol II progression along stress genes (Sanso et al., 2011b). As expected, the induction of the iron starvation response is also severely affected in this strain background (Fig. 2D).

**Over-expression of catalase as part of the $H_2O_2$-dependent stress response depletes the intracellular available iron**

Since transcriptional activation of the stress genes by $H_2O_2$ is essential for the activation of the iron starvation response (Fig. 2D), we speculated that one or several of the stress gene products would trigger an iron deprivation situation by sequestering most of the intracellular available iron. One of the genes most highly expressed by $H_2O_2$, *ctt1,* encodes the heme-containing catalase. Indeed, the gene is up-regulated in a Sty1- and Atf1- dependent manner by any environmental situation tested, but reaches its maximum fold-induction, 35-fold, only upon high doses of peroxides (Chen et al., 2003). We tested our hypothesis by analyzing whether cells lacking *ctt1* would induce the iron starvation response. As previously reported, cell lacking the catalase gene were very sensitive to $H_2O_2$ (Fig. 3A) (Mutoh et al., 1999). As expected, induction of genes such as *fio1* or *sib2,* or down-regulation of *pcl1* by $H_2O_2$ was abolished in Δ*ctt1* cells (Fig. 3B). Similarly, cells over-expressing catalase from an inducible promoter were resistant to $H_2O_2$ on plates (Fig. 3C), and displayed basal activation of the iron starvation response (Fig. 3D).

**The expression of catalase is down-regulated by Php4 in response to iron starvation**

Catalase is highly over-expressed upon treatment of cells with extracellular $H_2O_2$, and our finding suggests that at least upon peroxide exposure the protein sequesters most of the available iron and induces an iron deficiency response. We rationalized that even in the absence of $H_2O_2$ stress, expression of the catalase gene could be a target of iron starvation- and Php4-dependent down-regulation, as previously reported for other metallo-proteins such as aconitase, succinate dehydrogenase or sulfite reductase. Previous analysis of the transcriptome of cells subjected to the iron chelator DIP demonstrated than *ctt1* is among the genes down-regulated by this stress in a Php4-dependent manner (Table 1). We further show here by Northern blot that the basal levels of *ctt1* are repressed by DIP (Fig. 4A), that the Php4 repressor is recruited to the *ctt1* promoter as it is to the previously characterized Php4 target *pcl1* promoter (Fig. 4B), and the presence of RNA polimerase II (Pol II) at both promoters is decreased after exposure to the chelator in a Php4-dependent manner (Fig. 4C).

**DISCUSSION**

It is widely accepted that part of the toxicity exerted by $H_2O_2$ derives from its reactivity with reduced iron, and that at least in bacteria there is a cross-talk between the peroxide-induced responses and the iron starvation ones. Using very different molecular events, the final outcome in diverse microbial model systems is that part of the $H_2O_2$ responses includes down-regulation of iron uptake and stimulation of intracellular iron sequestration (Faulkner and Helmann, 2011). Contrary to that, we have found in *S. pombe* that the over-expression of catalase as part of the $H_2O_2$-dependent response causes a transient iron starvation, leading to the colateral activation of another gene expression program enhancing the levels of available iron. This is unlikely to be an adaptive response, but rather an unexpected effect of the $H_2O_2$ gene response.

Such accidental induction of the iron starvation response by oxidative stress has already been observed in both bacteria and mammals. Indeed, in *Escherichia coli* the Fur repressor, which inactivation occurs in response to iron deprivation, also suffers metal catalyzed oxidation by $H_2O_2$ (Varghese et al., 2007), which is compensated with activation of the *fur* gene directly by the $H_2O_2$ sensor OxyR (Zheng et al., 1999). Similarly, the sensor of iron deprivation in mammalian cells, IRP, was described to be also activated by peroxides (Pantopoulos and Hentze, 1995), but it has also been proposed an IRP-independent mechanism to enhance iron sequestration by ferritin upon $H_2O_2$ stress (Caltagirone et al., 2001). In the case of fission yeast, a similar compensatory effect could arise from the $H_2O_2$–dependent metal-catalyzed oxidation and degradation of the main iron importer Fio1, which protein levels dramatically decrease upon peroxide stress (Supplementary Fig. 1).

It is important to remark again the importance of catalase as an iron reservoir: its expression levels seem to modulate the free available iron pool. Thus, cells lacking *ctt1, sty1* or *atf1* are slightly but reproducibly more resistant to the presence of chelators than wild-type cells

(Supplementary Fig. 2). Furthermore, expression of *ctt1* is strongly down-regulated by Php4 after addition of chelators (Fig. 4A). In fact, with the exception of the *pcl1* gene (coding for a vacuolar iron importer) most of the Php4-repressible genes seem to be coding for metallo-enzymes or for mitochondrial proteins.

## MATERIALS AND METHODS

### Growth conditions and yeast strains

Cells were grown in rich medium (YE) or synthetic minimal medium (MM) as described previously (Alfa et al., 1993). Origins and genotypes of strains used in this study are outlined in Supplementary Table 1.

### Plasmids

The *ctt1* coding sequences were PCR amplified from a *S. pombe* cDNA library using primers specific for the *ctt1*-coding gene. Plasmid p418.41x (*pctt1.41x*) was obtained by digestion of pREP.41x (Maundrell, 1993) with *XhoI* and *BglII* and ligation with *ctt1* ORF flanked with *XhoI* and *BglII* sites. The new clone obtained from PCR-amplified DNA fragments was confirmed by sequencing.

### Preparation of trichloroacetic acid (TCA) extracts and immunoblot analysis

Yeast cells were grown in YE medium and were treated or not with 1 mM of $H_2O_2$ during 5 minutes, or with 250 μM 2,2´-dipyridyl (DIP, Fluka) during 90 minutes. Preparation of *S. pombe* TCA protein extract to detect Atf1 and Sty1 proteins were done as described before (Zuin et al., 2010) using polyclonal antibodies against Atf1 or Sty1, or commercial monoclonat anti-HA antiserum (12CA5).

### RNA analysis

Yeast cells were grown in YE medium and were treated or not with 1 mM of $H_2O_2$ or with 250 μM DIP or 100 μM iron ($FeCl_3.6H_2O$, Sigma) during the indicated time. Total RNA from exponentially growing *S. pombe* cells was extracted, processed and transferred to a

membrane as previously reported (Castillo et al., 2002). Membranes were hybridized with [α-$^{32}$P] dCTP-labeled *hsp9, fio1, gpd1, ctt1, srx1, str3, sib2, php4, pcl1, fep1 and act1* or *tfb2* (as loading controls) probes. Ribosomal RNA (rRNA) was used as a loading control.

**Solid sensitivity assay**

In order to analyze sensitivity to $H_2O_2$ on plates, *S. pombe* strains were grown and spotted as described (Calvo et al., 2009). Serial diluted cells were spotted into MM or YE5S plates containing or not the indicated concentrations of $H_2O_2$ or the iron chelator deferroxamine mesylate (Dx) (Sigma). The spots were allowed to dry, and the plates were incubated at 30°C during 2 to 3 days under aerobic conditions.

**Chromatin immunoprecipitation**

For immunoprecipitation of wild type, Δ*php4* and HA-tagged Atf1, Php4 and Fep1 proteins linked to DNA promoter regions, cells were grown in liquid MM to an $OD_{600}$ of 0.5, chromatin isolation and immunoprecipitation was performed as described previously (Sanso et al., 2011b). But, 50 µl of the soluble chromatin were kept as input, while the rest was immunoprecipitated with 1 µl of anti-HA antiserum (12CA5) or anti-Pol II antiserum (Ab847, Abcam). At the same time, 10 µl of protein G-Sepharose beads (Amersham) were added and incubation proceded rotating overnight at 4°C. Recovered DNA was analyzed by Real Time PCR (Light Cycler 480, Roche) with specific primers and SyberGreen Taq mixture (Roche). The specific primers, amplifying promoter regions, corresponded to the following positions with respect to the translation initiation sites: -855 to -755 of the *fio1* gene; -702 to -602 of the *str3* gene;-281 to -180 of the *pcl1* gene; -506 to -403 of the *ctt1* gene; -291 to -190 of the *hsp9* gene; -359 to -258 of the

*srx1* gene. Control primers, spanning an intergenic region of *S. pombe* chromosome I (position 465226 to 465326) were also used. Results were expressed as a percentage of the input. The error bars (SEM) were calculated from biological duplicates.

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**Table 1. Genes expressed at least 4-fold in iron-repleted cells in a Php4-dependent manner**

| Gene name | Function[a] | Fold changes |
|---|---|---|
| | | WT (+Fe vs -Fe)[b] |
| *pcl1* | Ferrous iron transporter | 16.8 |
| *cyc1*[c,d] | Cytochrome c (putative) | 15.9 |
| *SPBC29A3.21* | Sequence orphan | 14.2 |
| *sdh4* | Mitochondrial inner membrane import complex anchor subunit | 13.5 |
| *sdh2*[e] | Succinate dehydrogenase, iron-sulfur protein subunit | 12.4 |
| *SPAP14E8.05c* | UPF0136 family protein | 10.5 |
| *SPAC20G8.04c*[e] | Electron transfer flavoprotein-ubiquinone oxidoreductase | 8.0 |
| *SPAC17G8.08c* | Conserved protein (broad species distribution) | 7.6 |
| *sdh3* | Succinate dehydrogenase (ubiquinone) | 7.3 |
| *ctt1*[d] | Catalase | 7.1 |
| *sdh1* | Succinate dehydrogenase (ubiquinone) | 5.6 |
| *rip1*[e] | Ubiquinol-cytochrome-c reductase complex subunit 5 | 5.1 |
| *SPAC694.04c* | Conserved eukaryotic protein | 4.9 |
| *glt1*[e] | Glutamate synthase | 4.4 |
| *amt1* | Ammonium transporter | 4.0 |

[a] Gene annotation from PomBase (http://www.pombase.org)
[b] Microarray data of genes expressed more than 4-fold induction in a rich iron containing medium *versus* an iron depleted one, according to (Mercier et al., 2008). All these genes are expressed more than 2-fold in a Δ*php4* strain than in wild-type cells under iron deprivation (Mercier et al., 2008).
[c] Essential genes, according to (Kim et al., 2010)
[d] Binds heme group from PomBase (http://www.pombase.org)
[e] Iron sulfur protein from PomBase (http://www.pombase.org)
[f] No data (ND)

**FIGURES**

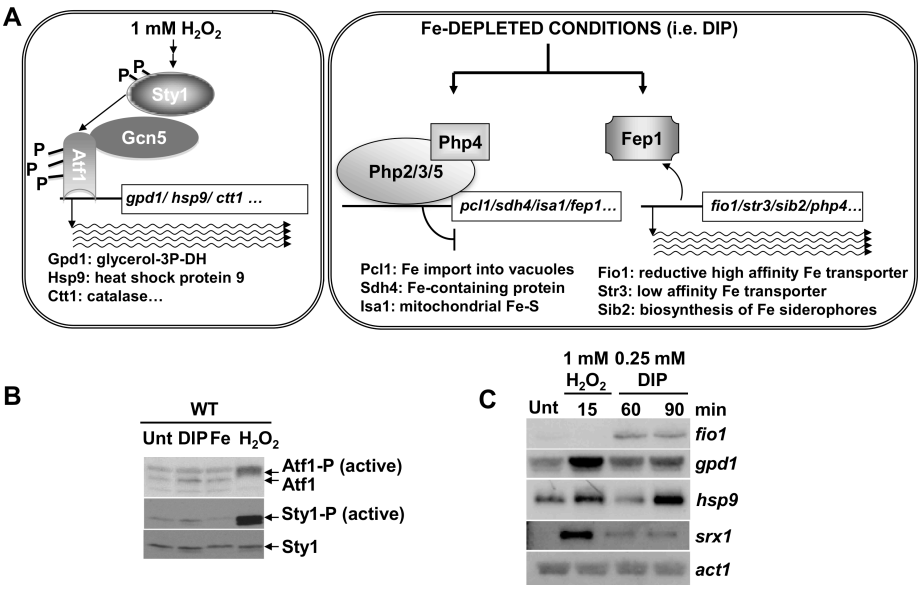



**Fig. 1. The $H_2O_2$-, Sty1-dependent response is not regulated by iron starvation.** (**A**). Scheme depicting the $H_2O_2$ and iron starvation regulons in *S. pombe.* On the left: $H_2O_2$ induces phosphorylation of the MAP kinase Sty1, which activates the transcription factor Atf1; activated Atf1 triggers trasncription of anti-stress genes in a Gcn5-dependent manner. On the right: iron starvation mediates down-regulation of genes coding for iron storage and iron-containing proteins (in a Php4 repressor-dependent manner), and up-regulation of genes coding for iron uptake (in a Fep1 repressor-dependent manner). (**B**). Phosphorylation/activation of Sty1 and Atf1 do not occur upon iron starvation. TCA protein extracts were obtain from 972 (WT) strain from cultures growing in YE treated or not with 250 µM DIP during 60 min, 1 mM $H_2O_2$ for 5 min, or with 100 µM of $FeCl_3.6H_2O$ (Fe) during 90 min. Protein extracts were analyzed by Western blot with anti-Atf1, commercial anti-phosphorylated Sty1 anti-Sty1 antibodies. (**C**). The transcriptional response to $H_2O_2$ stress is not engaged upon iron starvation. Total RNA from strains 972 (WT) was obtained

from cultures growing in YE treated or not with DIP or $H_2O_2$ during the time and concentration indicated in the figure. Northern blot analysis was performed using probes of the stress genes *gpd1, srx1* and *fio1.* The actin gene (*act1)* was analyzed as a loading control.

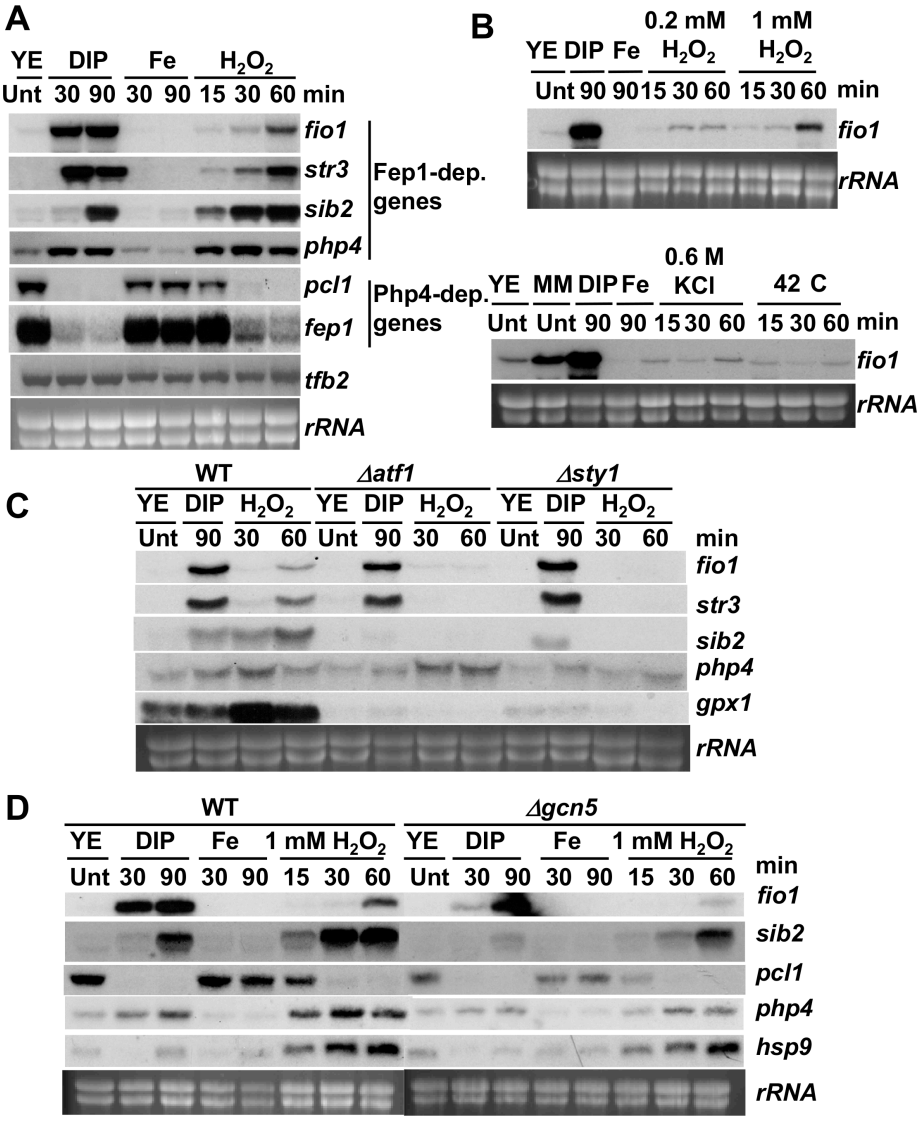



**Fig. 2. The iron-starvation response is activated by $H_2O_2$.** (**A**) and (**B**) Total RNA from strain 972 (WT) was obtained from cultures growing in YE treated or not with 250 μM DIP, 100 μM $FeCl_3.6H_2O$ (Fe), 1 mM $H_2O_2$, 0.2 mM $H_2O_2$, osmotic stress (0.6 M KCl) or with heat shock (at

42ºC) for the times indicated. Northern blot analysis was performed using probes of the iron starvation genes indicated. The *tfb2* gene and ribosomal RNA (*rRNA)* are shown as loading controls. (**C**). The $H_2O_2$-driven activation of the iron starvation gene response relies on Sty1 and Atf1. Same as in B, but using RNA from strains 972 (WT), AV15 (Δ*atf1*),and AV18 (Δ*sty1*). (**D**) Defective transcriptional activation of stress genes in cells lacking Gcn5 impairs $H_2O_2$–dependent induction of the iron starvation genes. Same as in B, but using RNA from strains 972 (WT) and MS112 (Δ*gcn5*).

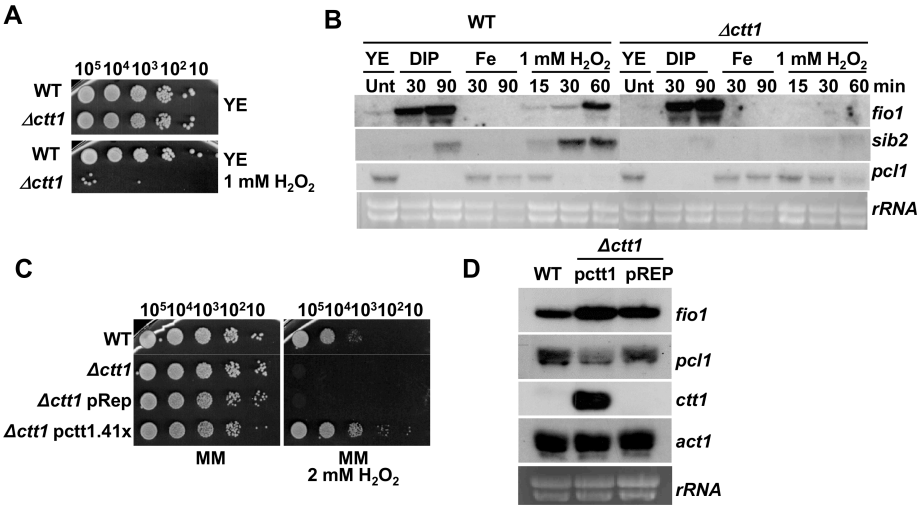



**Fig. 3. Over-expression of catalase after $H_2O_2$ stress causes activation of the iron regulon.** (**A**) Cells lacking *ctt1* are sensitive to oxidative stress. Strains 972 (WT) and NG76 (Δ*ctt1*) were grown in YE5S to a final $OD_{600}$ of 0.5, and serial dilutions from $10^5$ to 10 cells were spotted onto YE5S containing or not 1 mM $H_2O_2$. (**B**) $H_2O_2$ does not trigger the iron starvation response in cells lacking *ctt1*. Northern blot analysis as in Figure 1B, but with RNA from strains 972 (WT) and NG76 (Δ*ctt1*). (**C**) Expression of catalase from a plasmid confers resistance to $H_2O_2$. Minimal media cultures of strains 972 (WT). NG76 (Δ*ctt1*), EP160 (Δ*ctt1*) transformed with an empty vector (*pRep)*

and EP160 (Δ*ctt1*) transformed with the plasmid p418.41x (*pctt1)* were spotted on plates containing or not 2 mM $H_2O_2$ as described in A. (**D**) Cells over-expressing catalase from an inducible promoter display de-repression of the iron regulon. Total RNA strains from 972 (WT), EP160 (Δ*ctt1*) transformed with an empty vector (*pRep*) and EP160 (Δ*ctt1*) transformed with the plasmid p418.41x (*pctt1)* strains was obtained from cultures growing in MM, and analyzed as described in Figure 1B.

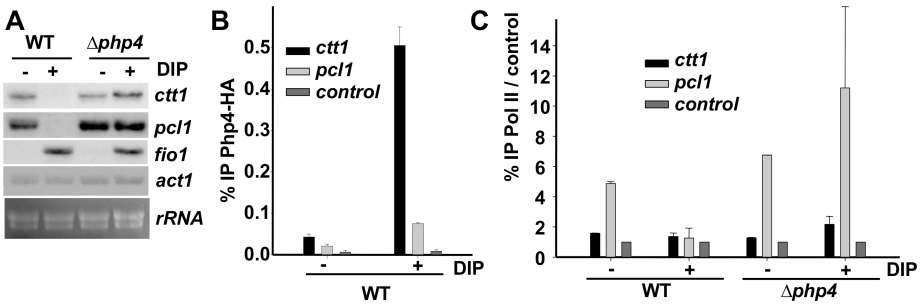
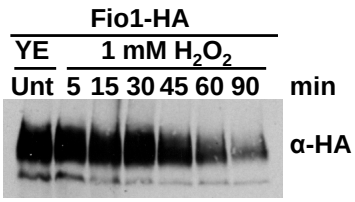
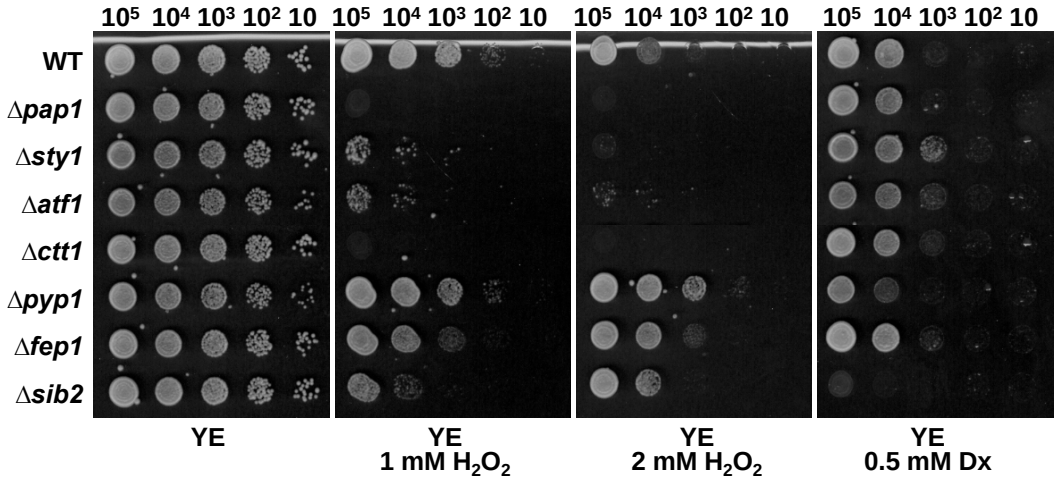



**Fig. 4. Expression of *ctt1* is down-regulated upon iron starvation in a Php4-dependent manner.** (**A**) The mRNA of *ctt1* is down-regulated by Php4 after the addition of DIP. Total RNA from 972 (WT) and NG40 (Δ*php4*) strains was obtained from cultures growing in YE treated or not with 250 μM DIP during 90 min, and analyzed as described in Figure 1B. (**B**) Php4 is recruited to *ctt1* promoter upon iron starvation. Cultures of strain NG123 (Php4-HA) were treated (+) or not (−) with 250 μM DIP during 60 min. ChIP experiments using anti-HA antibodies, coupled to quantification by real-time PCR, were performed using primers covering only promoter regions (*ctt1 and pcl1* promoters). Primers of an intergenic region were used as a negative control (control). Error bars (SEM) for all ChIP experiments were calculated from biological duplicates (**C**) The levels of Pol II decrease at the *ctt1* promoter after the addition of DIP. Cultures of strains 972 (WT) and NG40 (Δ*php4*) were treated and analyzed as in B, using commercial polyclonal antibodies against the large subunit of Pol II (Ab817, Abcam).




**Supplementary Fig. 1. The stability of the high affinity iron importer Fio1 decreases upon $H_2O_2$.** TCA protein extracts were obtained from YE cultures of strain NG90 (Fio1-HA) treated or not with 1 mM $H_2O_2$ at the indicated times. Protein extracts were analyzed by Western blot with anti-HA antibody.

**Gabrielli et al. Supp. Fig. 1**




**Supplementary Fig. 2. Cells lacking Sty1, Atf1 or Ctt1 are slightly more resistant to iron starvation than wild-type cells.** Cultures of trains 972 (WT), AV25 (Δ*pap1*), AV18 (Δ*sty1*), AV18 (Δ*atf1*), NG76 (Δ*ctt1*), EP48 (Δ*pyp1*), NG1 (Δ*fep1*) and NG8 (Δ*sib2*) were grown in YE5S to a final $OD_{600}$ of 0.5, and serial dilutions from $10^5$ to 10 cells were spotted onto YE containing or not 1 or 2 mM $H_2O_2$,or 0.5 mM of the iron chelator dexferroxamine (Dx).

**Gabrielli et al. Supp. Fig. 2**

**Supplementary Table 1. Strains used in this study**

| Strain | Genotype | Origin |
|---|---|---|
| 972 | *h⁻* | (Leupold, 1970) |
| AV15 | *h⁻ atf1::kanMX6* | (Zuin et al., 2005) |
| AV18 | *h⁻ sty1::kanMX6* | (Zuin et al., 2005) |
| MS177 | *h⁻ atf1-HÁ::kanMX6* | This work |
| MS112 | *h⁻ gcn5::kanMX6* | (Sanso et al., 2011) |
| EP160 | *h⁻ ctt1::ura4 leu1-32* | This work |
| CN513 | *h⁻ ctt1::ura4 leu1-32 ade6-M216* | (Nakagawa et al., 1998) |
| NG90 | *h⁻ fio1-HÁ::natMX6* | This work |
| AV25 | *h⁻ pap1::kanMX6* | (Zuin et al., 2005) |
| NG76 | *h⁻ ctt1::ura4* | This work |
| EP48 | *h⁺ pyp1::natMX6* | This work |
| NG1 | *h⁻ fep1::kanMX6* | This work |
| NG8 | *h⁻ sib2::kanMX6* | This work |
| NG40 | *h⁻ php4::kanMX6* | This work |
| NG123 | *h⁻ php4-HA::kanMX6* | This work |

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## 2.3. Characterization of Grx4

# The glutaredoxin Grx4 is a FeS-containing protein involved in iron sensing

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**ABSTRACT**

Iron is an essential cofactor in many cellular processes, but it is also toxic at high levels. Cells have developed tight systems to control, first, iron trafficking towards proteins, and, second, iron homeostasis, so that available iron is limited both at the level of extracellular import and intracellular sequestration. In *Schizosaccharomyces pombe*, the repressors Php4 and Fep1 mediate the transcriptional response to iron deprivation, with the final outcomes of enhanced iron import and decreased iron consumption. We describe here the characterization of Grx4, which is essential for the activation of the transcriptional iron starvation response, and that in budding yeast has been proposed to have an essential role in iron delivery to iron-containing proteins. Purification of recombinant Grx4 reveals that the protein, which contains monothiol glutaredoxin and thioredoxin domains, incorporates a glutathione-containing iron-sulfur cluster. The cysteine residue of the glutaredoxin domain is essential for iron-sulfur cluster assembly, as well as for Grx4 cellular functions. Our results suggest that the iron-sulfur cluster of Grx4 is essential for iron sensing. Our data also challenges the hypothesis that Grx4 participates in iron sulfur cluster biogenesis.

Keywords: iron homeostasis/FeS center/glutaredoxin/Grx4/Fep1/Php4

**INTRODUCTION**

Since iron is essential but also toxic, its uptake from the extracellular environment and its intracellular availability from a "disposable iron pool" are tightly regulated in all cell types. That is why there are reported transcriptional responses to iron starvation, but not to iron excess. These responses are triggered in very distinct ways in each organism, but the final gene expression programs are quite similar in essence: they are meant to increase iron import and decrease iron storage and usage.

In *Schizosaccharomyces pombe*, the repressors Fep1 and Php4 mediate the transcriptional response to iron depletion (Mercier et al., 2006) (Fig. 1). When iron is not limiting, Fep1 represses the expression of several genes which mediate iron uptake and/or increase the intracellular available iron pool, such as those coding for the reductive high-affinity transporter Fio1 (Pelletier et al., 2002), the non-reductive importer Str3 (Pelletier et al., 2003) or the ferrichrome sinthetase Sib2 (Mercier and Labbe, 2010). Fep1 is released from promoters under iron deprivation (Jbel et al., 2009), but is localization stays nuclear (Pelletier et al., 2005). Php4, on the contrary, has Crm1-dependent cytosolic localization under basal conditions. When iron is scarce, it accumulates in the nucleus and represses transcription of genes transcribed by the Pho2/3/5 complex, acting as a transcriptional repressor (Mercier and Labbe, 2009). These more than 80 repressed genes, according to microarray analysis (Mercier et al., 2008), include those coding for the vacuole importer Pcl1, the iron-sulfur cluster (ISC)-containing protein Sdh4 and the ISC assembly protein Isa1 (Mercier et al., 2006). It is worth pointing out that Fep1 also represses the *php4* gene under basal conditions (Mercier et al., 2006), whereas Php4 blocks *fep1* expression under iron depleted conditions (Mercier et al., 2008), even though the biological significance of this cross-regulation is unknown. Therefore, in fission yeast the up- and down-regulation of gene expression in response to iron depletion is accomplished through the

inactivation of a transcriptional repressor, Fep1, and the activation of another one, Php4. To date, no post-transcriptional regulation of mRNA stability in response to iron deprivation has been described in this yeast.

It has been recently reported that a common iron sensor activates Php4 and blocks Fep1 activity upon iron deprivation: the glutaredoxin Grx4 (Jbel et al., 2011; Kim et al., 2011; Mercier and Labbe, 2009). This glutaredoxin was first described to be essential for growth (Chung et al., 2005), although cells devoid of Grx4 can grow under semi-anaerobic conditions (Mercier and Labbe, 2009). It is important to point out that the *Saccharomyces cerevisiae* ortholog, Grx4, has been described to participate not only in iron sensing, but also in iron traffic to iron-containing proteins (Muhlenhoff et al., 2010). In fission yeast, Grx4 is a repressor of Php4 activity under basal conditions, since deletion of *grx4* triggers constitutive nuclear localization of Php4 and repression of genes such as *pcl1* under iron-rich conditions (Mercier and Labbe, 2009). Recently, the Php4 and Grx4 domains involved in this interaction have been described (Vachon et al., 2012). Fep1 is also regulated by Grx4: in the absence of this glutaredoxin the expression of Fep1-dependent genes such as *fio1* can never be induced (Jbel et al., 2011; Kim et al., 2011). Grx4 contains an N-terminal thioredoxin (Trx) domain and a glutaredoxin (Grx) domain, each one of which contains a unique cysteine residue. The role of their thiol groups in the protein's function as a iron sensor is been studied with conditional mutants or tagged versions of Grx4.

Since cells lacking Fep1 or Php4 do not share the same extreme phenotypes of cells devoid of Grx4, we decided to study the participation of this Grx in both iron trafficking and iron starvation response. To do so, we generated new tools such as real knock out strains or Grx4 mutants integrated at the chromosomal *grx4* locus, to unambiguously determine its participation in both processes. We demonstrate here that Grx4 is a ISC-containing protein, which is essential for both iron delivery and iron sensing functions.

## RESULTS

### Fission yeast Grx4 is required for sensing iron starvation

In *Saccharomyces cerevisiae*, Grx3/Grx4 has recently been reported to regulate two important processes of cell survival and adaptation: delivery of iron to proteins and activation of the transcription factor Aft1, activator of a transcriptional response to iron starvation (for a review, see (Li and Outten, 2012)). To test whether the same dual function applies to the *S. pombe* homolog Grx4 (Fig. 1), we generated a Δ*grx4* strain by selection under anaerobic conditions, and tested first the effect of such gene deletion in growth. As shown in Fig. 2A and Fig. 2B, the lack of Grx4 jeopardizes the cell's capacity to grow in the presence of oxygen and in glucose-limited media (MM), probably because more iron-containing proteins are required for growth under respiratory-prone conditions. As a reporter of an ISC-containing protein, we measured total aconitase activity in wild-type and Δ*grx4* cell extracts. In wild-type cells, the total aconitase activity was reduced by half upon the addition of the iron chelator dipyridyl (DIP) (Fig. 2C). The aconitase activity in cells lacking Grx4 was more than 2-fold lower than in wild-type cells (Fig. 2C).

We then tested whether Grx4 is also essential for the induction of the Fep1- and Php4-dependent changes of gene expression upon iron deprivation by Northern blot. As shown in Fig. 2D, in the presence of iron chelators such as DIP wild-type cells up-regulate transcription of *fio1, str3, sib2* and *php4* in a Fep1-dependent manner, whereas they down-regulate the expression of *pcl1*, *isa1* and *fep1*, among others, in a Php4-dependent manner. The inactivation and activation, respectively, of the Fep1 and Php4 transcriptional repressors upon DIP are dependent on Grx4, since Δ*grx4* cells can not activate genes such as *fio1, str3* or *sib2* and constitutively express genes such as *pcl1, isa1* or *fep1* (Fig. 2E).

According to these preliminary results, Grx4 seems to be involved not only in sensing iron deprivation but also in other functions probably linked to iron trafficking: lack of either Grx4, Php4

or Fep1 alone induce changes in the tolerance to iron chelators (Δ*php4* and Δ*grx4* being more sensitive and Δ*fep1* slightly more resistant to desferroxamine than wild-type cells; Fig. 2F), but the absence of Php4 or Fep1 alone does not affect growth under respiratory conditions, like depletion of Grx4 does (Fig. 2A).

**The glutaredoxin Grx4 is an ISC-containing protein**

Mammalian Grx2, a nuclear-mitochondrial Grx, was the first Trx-fold protein reported to contain a ISC (Lillig et al., 2005). The apo-protein had Grx activity, but not the dimeric iron-containing form. The cluster was lost upon aerobic conditions, enhancing protein activity as a Grx. More recently, the redundant Grx3 and Grx4 glutaredoxins of *S. cerevisiae* have also been reported to be ISC-containing proteins, to use two glutathione (GSH) moieties to hold the cluster and to require the ISC for both their functions as iron supplier and as a sensor of iron starvation (Muhlenhoff et al., 2010; Ojeda et al., 2006; Pujol-Carrion et al., 2006). We over-expressed a Tev-cleavable GST-(Tev site)-HA-Grx4 fusion protein in *Escherichia coli*, and noticed than the cell pellet and the early supernatants had brownish color when compared with bacteria over-expressing GST alone. The color disappeared during protein purification. We hypothesized that Grx4 can assemble an oxygen-sensitive ISC, and attempted to reconstitute the metallocenters under anaerobic conditions. We incubated recombinant apo-HA-Grx4 with iron, inorganic sulfide and GSH in the presence of the *E. coli* ISC catalyzer IscS (Berndt et al., 2007). As observed by UV-visible spectroscopy, three shoulders in the 390-650 nm regions could be detected after ISC reconstitution (Fig. 3A); the sample was colorless again after oxygen exposure. It is worth pointing out that reconstitution of the cluster in untagged Grx4 yielded the same spectrum (data not shown). GSH was required for such reconstitution, which suggests that the tripeptide coordinates the ISC. Indead, the levels of total GSH are significantly lower in cells lacking Grx4 than in a wild-type background (Fig. 3B), as previously reported for *S. cerevisiae* cells lacking

Grx3/4 (Muhlenhoff et al., 2010). It is worth mentioning that the total levels of iron in cells lacking Grx4 are also lower than in wild-type cells (Fig. 3C).

**The ISC of Grx4 is essential for both iron delivery and iron sensing**

As shown before for other monothiol Grxs, Grx4 has one cysteine residue in the Trx and another one in the Grx domains (Fig. 4A). We substituted the endogenous *grx4* gene by mutant versions with a Cys-to-Ser substitution in either the Trx or the Grx domains, to maintain protein levels. Fission yeast cells expressing Grx4.C35S behaved very similar to wild-type cells regarding both aerobic growth (Fig. 4BC) and activation of the transcriptional iron starvation response (Fig. 4D). On the contrary, Grx4.C172S was unable to fulfill any function of the Grx, since cells expressing this mutant form display very similar phenotypes to Δ*grx4* cells (Fig. 4BCD). It is important to point out that the *grx4.C172S* strain was isolated under semi-anaerobic conditions.

We next over-expressed Tev-cleavable GST-(Tev site)-HA-Grx4.C35S and C172S fusion proteins in *E. coli*, and noticed again than the cell pellets for the wild-type and Grx4.C35S tagged proteins were clearly brownish, while pellets of cells over-expressing the Grx4.C172S fusion protein were colorless and similar those of bacteria over-expressing GST alone. Again, we attempted to reconstitute the metallocenters under anaerobic conditions. As shown in Fig. 4E, reconstitution of recombinant Grx4-C35S yielded a protein with similar visible spectra to wild-type Fe-Grx4. However, the presence of Cys172 was required for cluster assembly *in vitro*, since recombination could not be observed for mutant Grx4.C172S (Fig. 4E). These results suggest that the ISC of Grx4 is essential for both functions: iron sensing and aerobic growth.

**The interaction of Grx4 with Php4, but not with Fep1, is partially disturbed upon iron starvation**

The experiments described above suggest that under iron-rich conditions Fe-Grx4 could maintain the iron starvation response off by maintaining the Php4 repressor inactive and allowing Fep1 to bind to DNA. We first confirmed with strains expressing the fusion proteins Grx4-GFP, Php4-GFP and Fep1-GFP (all expressed from their own promoters, and displaying a wild-type transcriptional response to iron starvation; data not shown) than Grx4 is localized at both the cytosol and the nucleus, Fep1 is constitutively nuclear, and Php4 shifts from the cytosol to the nucleus upon iron deprivation (Fig. 5A). To discard an effect of the GFP tag on protein localization, we also performed immuno-fluorescence localization with polyclonal antibodies against Grx4, or with monoclonal antibodies against HA, recognizing endogenously tagged Fep1-HA Php4-HA (this small tag did not affect the transcriptional response to iron starvation of these transcriptional repressors; data not shown). As shown in Fig. 5B, Grx4 seems to present a dual cytoplasmic and nuclear localization, while Fep1-HA is constitutively nuclear; only the localization of Php4-HA changes from iron rich to iron deprived conditions (Fig. 5B).

It has been recently described using over-expressed Grx4 fused to different tags that the Grx is constitutively bound to Php4 and to Fep1 under both iron-rich and iron-starved conditions (Jbel et al., 2011; Vachon et al., 2012). We decided to verify it by using our Grx4-GFP chimeras, which are expressed under the control of the *grx4* endogenous promoter. We immuno-precipitated Grx4-GFP, and used commercial antibodies against the Myc-tag to check the *in vivo* binding to Php4-Myc or Fep1-Myc. It is worth pointing out that the strains expressing each one of the tagged proteins displayed wild-type transcriptional responses to iron deprivation (data not shown). As shown in Fig. 5C, a clear co-immuno-precipitation between Grx4-GFP and Fep1-Myc was detected both before and after DIP stress. On the contrary, the association between Grx4-GFP and Php4-Myc was significantly disturbed under iron deprived conditions (Fig. 5D).

**The transcription factors Php4 and Fep1 mediate RNA polymerase recruitment to DNA depending on iron availability**

Since both Php4 and Fep1, are transcriptional repressors, we decided to verify whether their absence from promoters is concomitant to RNA polymerase II (Pol II) recruitment, and whether Grx4 can also be detected by chromatin-immuno precipitation (ChIP) on these iron-regulated promoters.

The transcriptional repressor Php4, known to be activated upon iron starvation, was recruited to promoters of genes such as *isa1* and *pcl1* upon DIP treatment. The absence of Grx4 blocked this gene repression (Fig. 6A). Fep1, on the contrary, was bound to *fio1* and *str3* promoters prior to stress and it was released from DNA after DIP treatment, in a Grx4-dependent manner (Fig. 7A). Since Grx4 is found constitutively bound to Fep1, and it is also associated to Php4 (although weakly) under iron deprived conditions, we expected to detect the Grx at DNA when the repressors were found, without success (data not shown).

In both cases, binding of Php4 or Fep1 to promoters decreased Pol II binding to promoters, as determined using a strain expressing a HA-tagged Rpb1, the large Pol II subunit (Fig. 6B and Fig. 7B). Thus, upon iron rich conditions, Pol II was found to be strongly associated to the Php4-dependent promoters *isa1* and *pcl1,* while it was released from these genes after DIP treatment; repression was constitutive in a strain lacking Grx4 (Fig. 6B and Fig. 6C). Similarly, Pol II was recruited upon DIP treatment to Fep1-repressed genes, whereas such recruitment was abolished in cells lacking Grx4 (Fig. 7B and Fig. 7C).

**DISCUSSION**

Regulation of the intracellular iron available pools largely depends on the activation of iron uptake and inhibition of iron storage and consumption upon iron starvation. In *S. pombe*, we show here that this process fully depends on the glutaredoxin Grx4, an ISC-containing protein distributed at both the cytosol and the nucleus before and after iron starvation. Grx4 seems to bind to the transcriptional repressors Fep1 and Php4, which display nuclear and cytoplasmic localization, respectively, when iron is not limiting. Addition of chelators to the cell cultures triggers an iron starvation response, by probably changing (but not fully eliminating) the ISC architecture on Grx4. Under these circumstances, the binding of activated Grx4 to Php4 is significantly disturbed, and Php4 is then imported to the nucleus and represses iron usage genes; activated Grx4 is still bound to Fep1, but a conformational change may occur in the repressor so that binding to promoters is affected, and transcription of iron uptake genes induced.

It is important to point out that for this study we have constructed full deletion of the *grx4* gene and inserted the cysteine mutations at the *grx4* locus, since previous studies used conditional knock outs and tagged and over-expressed versions of the mutants (Jbel et al., 2011; Kim et al., 2011; Mercier and Labbe, 2009). Our study unambiguously demonstrates that Grx4 is an ISC-containing protein, that cysteine 172 is required for cluster assembly, and that the cluster is essential for the function of Grx4 as an iron sensor, since cells expressing only Grx4.C172S display a transcriptional program to iron deprivation identical to that of cells lacking Grx4.

The transcriptional profile of the *grx4.C172S* mutant, however, also provides clues about the mechanism by which Grx4 senses iron scarcity. We first speculated that loss of the ISC could be the activating event. However, in that case cells expressing a constitutive apo-Grx4 protein (such as Grx4.C172S) would display a fully active iron starvation response, including up-regulation of iron uptake coding genes and down-regulation of genes coding for ISC-containing

proteins. As shown in Fig. 4D, that is not the case: cells expressing Grx4.C172S display constitutive repression of Php4-dependent genes, as expected, but do not show up regulation of the Fep1-dependent iron import genes; in fact, they cannot activate them in response to iron deprivation. We believe that the ISC in Grx4 is required for binding of the Grx to Php4 and Fep1, and that is why the *grx4.C172S* mutant allows nuclear import of Php4, and is unable to relieve Fep1 from DNA. In wild-type cells, the ISC of Grx4 may change from a conformation to another, disturbing interaction with Php4 and forcing a protein change in Fep1 hindering its DNA binding domain. Further experiments to demonstrate this hypothesis are ongoing.

The presence of an ISC in a cytosolic Grx is not a novel finding, since the orthologs of Grx4 in *S. cerevisiae*, the redundant Grx3 and Grx4 proteins, were reported to assemble an oxygen-sensitive ISC of the type [2Fe-2S], which bridges a Grx homodimer *in vitro* (Li et al., 2009). The cluster was more stable if reconstitution was done in the presence of Fra2, a protein originally shown at the genetic level to be required to transduce an iron starvation signal to the yeast transcriptional activator Aft1 (Kumanovics et al., 2008; Li et al., 2011; Li et al., 2009). We have found a Fra2 homolog in *S. pombe*, *SPAC8C9.11,* and have analyzed its transcriptional response to iron deprivation (Supplementary Fig. 1). Cells lacking the *S. pombe* Fra2 protein are able to repress Php4-dependent genes upon iron deprivation as wild-type cells, but cannot induce iron uptake genes. Whether this protein participates in the assembly of the ISC of Grx4, or whether both proteins form a heterodimer to sense iron deprivation, is still to be determined.

Cells lacking Grx4 display a severe growth defect in the presence of oxygen that is not shared by cells lacking Php4 or Fep1 (Fig. 2A). This fact, combined with the impaired aconitase activity of $\Delta$*grx4* extracts (Fig. 2C), prompted us to speculate that this glutaredoxin has an essential role in iron delivery towards iron-containing proteins, as it has been recently proposed for the *S. cerevisiae* homolog Grx4 (Muhlenhoff et al., 2010). However, the transcriptional profile of cells lacking Grx4 under normal (iron-rich) conditions differs from that of wild-type cells in that

all the Php4-dependent genes are constitutively repressed (Fig. 2E). As indicated in the introduction, Php4 represses in wild-type cells upon iron depleted conditions many genes coding for ISC-containing proteins, many of which are essential for respiratory growth (Mercier et al., 2008). Among them, the gene/s coding for aconitase are severely repressed under normal growth conditions in cells lacking Grx4, so that the protein levels, and not only the protein activity, are diminished in strain Δ*grx4* (data not shown). In view of these facts, we suggest that Grx4 has only a role in iron sensing and transduction of the iron scarcity signal towards Php4 and Fep1, but is not required for iron trafficking. However, we have not been able to fully suppress the Δ*grx4* aerobic growth defects by further deletion of the *php4* gene (data not shown). Therefore, further experiments will be required to dismiss or confirm whether Grx4 directly participates in iron delivery, as suggested before for yeast and human glutaredoxins (Muhlenhoff et al., 2010).

## MATERIALS AND METHODS

### Growth conditions and yeast strains

Cells were grown in rich medium (YE) or synthetic minimal medium (MM) as described previously (Alfa et al., 1993). Origins and genotypes of strains used in this study are outlined in Supplementary Table 1.

### Solid sensitivity assay

For survival on solid plates, *S. pombe* strains were grown, diluted and spotted in YE or MM medium agar plates as described previously (Calvo et al., 2009). The spots were allowed to dry, and the plates were incubated at 30ºC during 2 to 3 days under aerobic or anaerobic conditions (Forma Anaerobic, System Thermo electron corporation).

### Growth Curves

Yeast cells were grown in YE from an initial $OD_{600}$ of 0.1 as described (Calvo et al., 2009).

### RNA analysis

Total RNA from exponentially growing *S. pombe* cells in YE was extracted, processed and transferred to a membrane as previously reported (Castillo et al., 2002). Membranes were hybridized with [α-32P] dCTP-labeled *fio1, str3, sib2, php4, pcl1, fep1* and *isa1* probes. Ribosomal *rRNAs* were used as loading controls.

### Enzymatic activity assay for aconitase

In order to measure aconitase activity for wild type and Δ*grx4* cells, *S. pombe* was grown in YE under aerobic conditions treated or not 2,2´-dipyiridyl (DIP; Fluka) or they were grown

overnight under anaerobic conditions and shifted for 3 hours to aerobic conditions, to reach an $OD_{600}$ of 0.5. Aconitase activity was assayed as described (Hausladen and Fridovich, 1996) following method 2. Aconitase activity was measured spectrophotometrically at 340 nm using citrate as the substrate of aconitase, and the isocitrate formed is then converted to α-ketoglutarate by NADP+-dependent isocitrate dehygrogenase. Yeast cells were washed once with PBS buffer pH 7.4. Total protein extracts were prepared by homogenization with glass beads in Tris buffer (50 mM Tris-HCl pH 7.6, 1 mM cysteine, 1 mM citrate, 0.5 mM MnCl2). Insoluble material was removed by centrifugation during 10 minutes at 16,000 g at 4ºC. Supernatants were collected and 10 µl of the total extract were mixed with 90 µl of reaction buffer [50 mM Tris-HCl, pH 7.4, 30 mM sodium citrate, 0.5 mM $MnCl_2$, 0.2 mM NADP+, isocitrate dehygrogenase (2 units/ml, Fluka)]. Absorbance at 340 nm (ε 340=6.22 mM-1 cm-1) was recorded by a UV-visible spectrophotometer (UV-1700 Pharma Spec Shimadzu). Data was obtained from three independent experiments and are expressed as mean ± SEM.

**Quantitative determination of glutathione (GSH) and glutathione disulfide (GSSG) levels**

GSH and GSSG levels were quantitatively measured as previously reported (Rahman et al., 2006), with some minor modifications. 50 ml of exponentially growing *S. pombe* cells were pelleted, washed twice with cold 1x PBS and lysed by vortexing with glass beads in 300 µl of extraction buffer (0.1 M potassium phosphate buffer pH 7.5, 5 mM EDTA, 0.1% Triton X-100 and 1.3% sulfosalicylic acid). Cell lysates were then pelleted and supernatants were used for measuring total glutathione (GSH + GSSG) and for measuring GSSG. Total glutathione could be directly measured from the supernatant, but to measure GSSG, GSH was first quickly blocked by incubating 100 µl of the supernatant with 2 µl of 2-vinylpyridine

for 1 hour at room temperature in a fume hood. Reaction was neutralized by incubating 10 min with 6 µl of 1:6 triethanolamine. GSH and GSSG standards were prepared in 0.1 M potassium phosphate buffer pH 7.5 and 5 mM EDTA with concentrations ranging from 26.4 nM/ml to 0.4125 nM/ml. GSSG standards were subjected to the same treatment as supernatants from cell lysates. 20 µl of both, standards and properly diluted supernatants (to fit in the standard curve), were loaded into a 96-well plate. 120 µl of a 1:1 solution of 0.67 mg/ml 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) (Sigma-Aldrich) and 13.3 µl/ml of glutathione reductase (Sigma-Aldrich) (250 units/ml) were added to each well and, after incubating 30 sec, 60 µl of 0.67 mg/ml of β-NADPH were also added. Absorbance was immediately read at 412 nm in a Powerwave HT340 microplate reader (BioTek, Winooski, VT) with readings every 30 seconds during 5 min. The change in absorbance per min was calculated for each sample and the actual total GSH and GSSG concentrations in the samples were extrapolated from the standard curve. GSH concentration was obtained using the formula $GSH_{total}$= GSH + 2 x GSSG. Concentration is expressed as mM per cell, considering that the half volume of a *S. pombe* cell is ~ 3.14 x $10^{-8}$ µl.

**Colorimetric assay for iron quantification**

Yeast cells were grown in YE anaerobic liquid cultures (50 ml). Cells were washed twice with PBS buffer pH 7.4 and treated as described (Tamarit et al., 2006) using ferrozine (Fluka). Absorbance of iron-chelator complex was recorded at $OD_{565}$ nm in a UV-visible Ultraspec 2100-pro (Amersham Biosciences) spectrophotometer. The accuracy of the assay was improved by subtracting non-specific absorbance recorded at $OD_{680}$. The number of cells were calculated from $OD_{600}$ ($OD_{600}$ 0.5 = 1x$10^7$ cells/ml). Standard curves were prepared from 10-40 nmoles of $FeCl_3$ dissolved in 3% nitric acid. All chemicals (except ferrozine from Fluka) were purchased from Sigma and resuspended in ultrapure

water obtained from a Millipore Milli-Q Advantage. Data was obtained from three independent experiments and are expressed as mean ± SEM.

**Plasmids to over-express Grx4 proteins in *E. coli* and bacteria growth conditions**

The *grx4* coding sequence was PCR-amplified from an *S. pombe* cDNA library using primers specific for the *grx4*-coding gene. Plasmid p389 (pGEX-2T-Tev-GST-*grx4*) was obtained by digestion of pGEX-2T-Tev (that encode a TEV protease cleavage site; kindly provided by Xavi Gomis-Rueth) with *BamHI* and *SmaI* and ligation with *grx4* ORF flanked with the same sites. The plasmid p400 (pGEX-2T-Tev-GST-HA-*grx4*) was obtained by digestion of plasmid p389 with *BamHI* and ligation with annealed primers coding for the HA tag, flanked with *BglII* and *BamHI* sites. The plasmids p400.C35S (pGEX2T-Tev-GST-HA-*grx4.C35S*) and p400.C172S (pGEX-2T-Tev-GST-HA-*grx4.C172S*) were generated by site directed mutagenesis using QuikChange XL (Agilent Tecnologies) following manufacturer instructions. The mutagenized plasmids obtained were confirmed by sequencing. Bacteria strain FB810 (Benson et al., 1994) transformed with pGEX-2T-TEV derivatives were inoculated into LB Broth Base with 100 µg/ml ampicilin and incubated at 37ºC for 16 hours with vigorous shaking. The overnight cultures were diluted 80-fold in 400 ml of fresh medium and incubated at 37ºC until the culture reached an optical density $OD_{600}$ of 0.6. Isopropyl-β-thio-D-galactoside (IPTG) was then added to a final concentration of 1 mM and shaking continued at 18ºC for 18 h. The plasmid expressing His tagged IscS from *E. coli* was a gift from Dr. C.H. Lillig, and was expressed and purified as described (Berndt et al., 2007).

**Purification of recombinant Grx4, Grx4.C35S and Grx4.C172S proteins from *E. coli* cells**

Cells froms 400 ml cultures were harvested and pellets were resuspended in 10 ml of STET extration buffer [50 mM Tris-HCl, pH 8.0, 150 mM sodium chloride (NaCl), 1 mM EDTA pH 8. 0,1% Triton X-100] and broken by sonication. Debris and unbroken cells were removed by centrifugation. Supernatants containing our GST-HA-tagged fusion proteins were incubated with GSH-Sepharose 4B beads (GE Healthcare) for 1 h at 4ºC. The beads were washed three times with NET-N (20 mM Tris-HCl pH 8.0, 0.5 mM EDTA pH 8.0, 100 mM NaCl, 0.5% NP-40) and once with TEV cleavage buffer (50 mM Tris-HCl pH 8.0 and 0.5 mM EDTA pH 8.0). The GST-HA tagged fusion proteins was release from the beads by elution in TEV cleavage buffer with 1 mM fresh DTT and 10 µg/ml of TEV protease (Invitrogen, Carlsbad, CA) overnight at 4ºC. Once recombinant proteins were released, NaCl was added to a final concentration of 100 mM and stored at -80ºC. Relative protein concentrations were determined by electrophoretic separation on 10% denaturing polyacrylamide gels and Coomassie staining with standards of bovim serum albumin (Sigma).

**FeS cluster reconstitution assay**

Cluster reassembly was performed under anaerobic conditions in Forma Anaerobic System (Thermo Electron Corporation) at room temperature. Grx4 WT or mutant proteins (100-200 µM) from *E. coli* were incubated in 50 mM sodium phosphate buffer (pH 8.0) containing 200 mM NaCl, 0.01 molar equivalents of *E. coli* IscS, 2 molar equivalents of $Fe(NH_4)_2(SO_4)_2$, 2.5 molar equivalents of L-Cys, 1 mM GSH , 5 mM dithiotreitol (DTT), 10 µM pyridoxal phosphate. After 2 hours, the mixture was desalted using Microspin S-200 HR columns (GE Healthcare). UV-VIS spectra were recorded under anaerobic conditions using a Shimadzu UV-1700 sphectrophotometer (PharmaSpec).

**Fluorescence microscopy**

Fluorescence microscopy and image capture was performed as previously described (Vivancos et al., 2004).

**Immuno-fluorescence assay**

10 ml of cells grown in minimal media were grown till an $OD_{600}$ of 0.3 and harvested. Cells were fixed with 1-2% of formaldehyde during 20 min at 25ºC in a water bath. Pelleted cells were washed twice in cold PBS 1%. Cells were resuspended in 1.2 ml of preincubation buffer (20 mM citric acid, 20 mM $Na_2HPO_4$, 40 mM EDTA pH 8.0). Then, 30 mM of β-mercaptoethanol was added and incubation proceeded for 10 min at 30ºC in a water bath. Cells were collected by centrifugation for 1min at 8,000 rpm, and pellets were incubated in 0.6 ml sorbitol-Tris buffer (1 M sorbitol, 50 mM Tris-HCl pH 7.4) with 10 mM β-mercaptoethanol and 2.25 mg of zymolyase 20T (ICN biochemicals) for 40 min in a water bath at 30ºC. Cell wall digestion was confirmed by adding 1% SDS to 2 µl of cells and observing lysis at the microscope. Cells were pelleted at 6,000 rpm for 1 min, and pellets were washed three times with 1 ml of cold PEMS (100 mM PIPES, 1 mM EGTA, 1 mM $MgSO_4$, 1.2 sorbitol, pH 6.5-6.9). Then, cells were incubated in 1 ml of cold PEMS with 1% Triton X-100 during 1 min at room temperature to permeabilize nuclear membranes. Cells were pelleted at 6,000 rpm for 1 min, and washed three times with 1 ml of PEM (100 mM PIPES pH 6.9, 1 mM EGTA, 1 mM $MgSO_4$). Then, cells were resuspended in 1 ml of PEMBAL (100 mM PIPES, 1 mM EGTA, 1 mM $MgSO_4$, 3% BSA, 0.1% NaN3, 100 mM lysine hydrochloride) and incubated rocking during 20 min at room temperature. Cells were pelleted again and resuspended in 100 µl of PEMBAL plus 1/200 dilution of polyclonal anti-Grx4 or monoclonal anti-HA (12CA5) antibodies. Incubation proceeded rocking overnight at room temperature. Cells were pelleted, washed three times in PEMBAL, rocking during 10

min at room temperature each time. Pelleted cells were then resuspended in 50 µl of PEMBAL plus 1(500 dilution of secondary antibody [Cy2 AffiniPure Donkey anti-rabbit IgG (H+L) (ref. 711-225-152, Jackson) or Alexa Fluor 555 goat anti-mouse IgG (ref. A21424, Invitrogen)], and incubated by rocking 5-7 h at room temperature in the darkness. Cells were pelleted and washed three times in PEMBAL, rocking them during 10 min at room temperature each time. Cells were resuspended cells in 100 µl of PEMBAL, and analyzed directly by fluorescence microscopy.

**Co-immunoprecipitation analysis**

Cells from 100 ml of MM medium cultures at an $OD_{600}$ of 0.5 ($1\times10^7$ cells) were treated as described (Calvo et al., 2012). Samples were separated by 8% SDS-polyacrylamide gel electrophoresis (PAGE) and detected by immunoblotting with monoclonal anti-Myc (9E10) or polyclonal anti-GFP antiserum (Calvo et al., 2012).

**Chromatin immuno-precipitation**

For the *in vivo* detection of Pol II or HA-tagged Php4 or Fep1 onto promoter regions, cells were grown in liquid MM to an $OD_{600}$ of 0.5, and chromatin isolation and immunoprecipitation was performed as described previously (Sanso et al., 2011), with small modifications. Thus, 50 µl of the soluble chromatin were kept as input, while the rest was immuno-precipitated with 1 µl of anti-HA antiserum (12CA5) or anti-Pol II antiserum (Ab847, Abcam). At the same time, 10 µl of protein G-Sepharose beads (Amersham) was added and incubation proceeded rotating overnight at 4°C. Recovered DNA was analyzed by Real Time PCR (Light Cycler 480, Roche) with specific primers and SyberGreen Taq mixture (Roche). The specific primers, amplifying promoter regions, corresponded to the following positions with respect to the translation initiation sites: -855 to -755 of the *fio1* gene; -702 to

-602 of the *str3* gene; -281 to -180 of the *pcl1* gene; -270 to -170 of the *isa1* gene. Control primers, spanning an intergenic region of *S. pombe* chromosome I (position 465226 to 465326) were also used. Results were expressed as a percentage of the input. The error bars (SEM) were calculated from biological duplicates.

**ACKNOWLEDGEMENTS**

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**FIGURES**

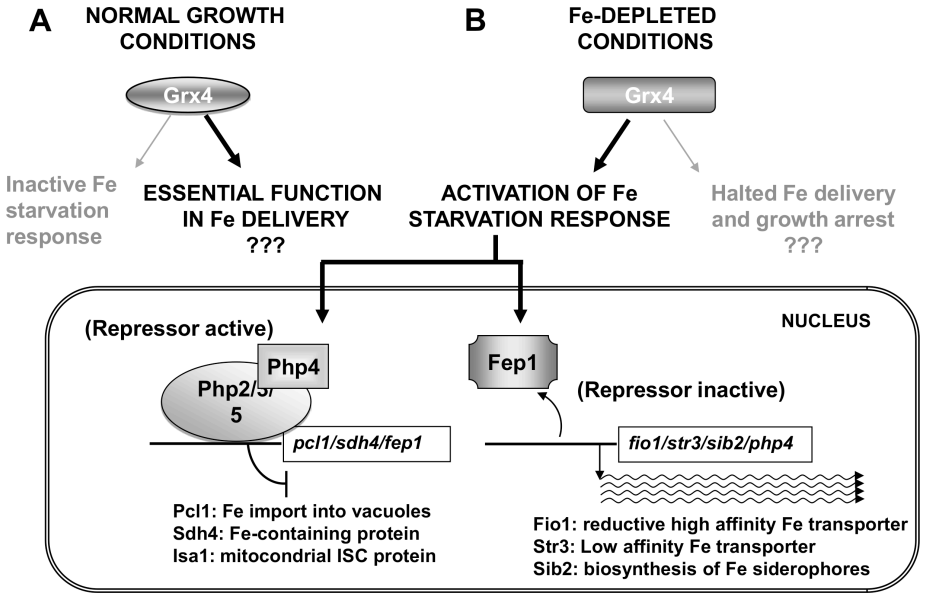



**Fig. 1. Scheme of Grx4 functions: iron delivery and iron starvation sensor.** (**A**) Illustration of a putative role of Grx4 in iron delivery under normal growth condition in *S. pombe.* (**B**) Scheme of the iron regulon response under iron starvation conditions in *S. pombe.* Grx4 as an iron sensor interacts with the repressors Fep1 and Php4, which mediate the transcriptional response to iron depletion. Fep1-dependent genes code for proteins promoting iron uptake (such as *fio1*, *str3*, *sib2*), while Php4-dependent genes code for iron-containing proteins (*sdh4* and *isa1*) and for a vacuole iron importer (*pcl1*).

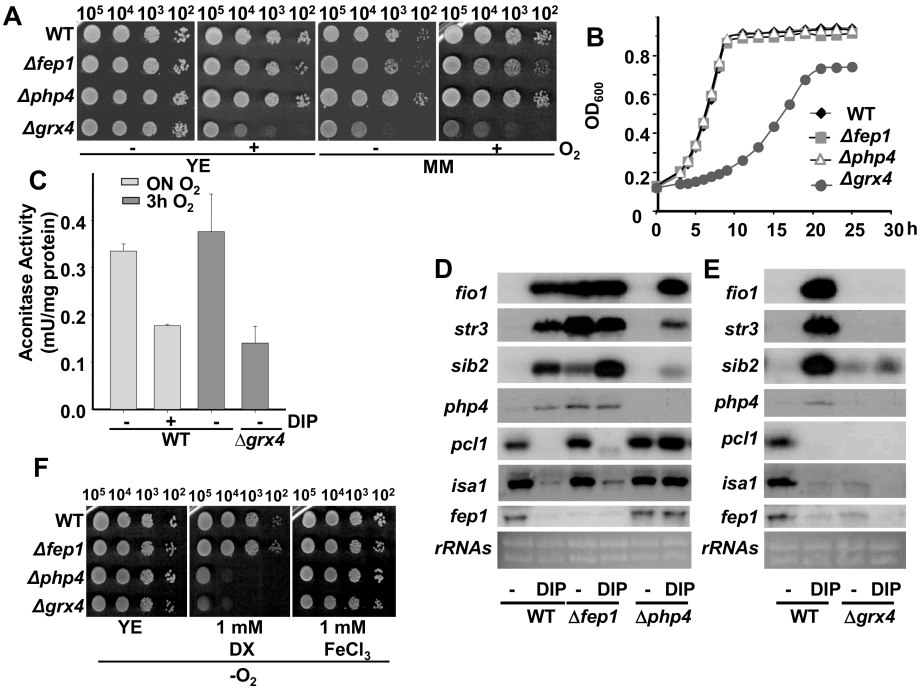



**Fig. 2. Fission yeast Grx4 is required for sensing Fe starvation.** (**A**) Cells lacking Grx4 displays severely compromised aerobic growth. Strains 972 (WT), NG1 (Δ*fep1*), NG40 (Δ*php4*) and NG81 (Δ*grx4*) were grown in YE to a final $OD_{600}$ of 0.5, and serial dilutions were spotted in duplicate into YE or MM plates and incubated at 30°C for 2-3 days under aerobic or anaerobic conditions. (**B**) Growth curves of 972 (WT), NG1 (Δ*fep1*), NG40 (Δ*php4*) and NG81 (Δ*grx4*) strains, grown under semi-aerobic conditions in YE media; $OD_{600}$ was recorded at the indicated times for each culture during 35h. (**C**) Total aconitase activity is lower in Δ*grx4* than in wild-type cells. Aconitase activity of extracts from strains 972 (WT) and NG81 (Δ*grx4*) was determined as indicated in Materials and Methods. (**D**) and (**E**) Php4, Fep1 and Grx4 are essential for the induction of iron starvation response. Total RNA from strains 972 (WT), NG1 (Δ*fep1*), NG40 (Δ*php4*) and NG81 (Δ*grx4*) was obtained from cultures growing in YE treated or not with 0.25 mM

DIP during 90 min, and analyzed by Northern blot with the probes indicated. *rRNA* was used as a loading control. (**F**) Cells lacking Grx4 and Php4 display severely compromised growth in iron starvation conditions. Serial dilutions from cultures of strains 972 (WT), NG1 (Δ*fep1*), NG40 (Δ*php4*) and NG81 (Δ*grx4*), growing anaerobically till an $OD_{600}$ of 0.5, were spotted on plates containing or not 1 mM deferroxamine or 1 mM $FeCl_2$; the plates were grown under anaerobic conditions.

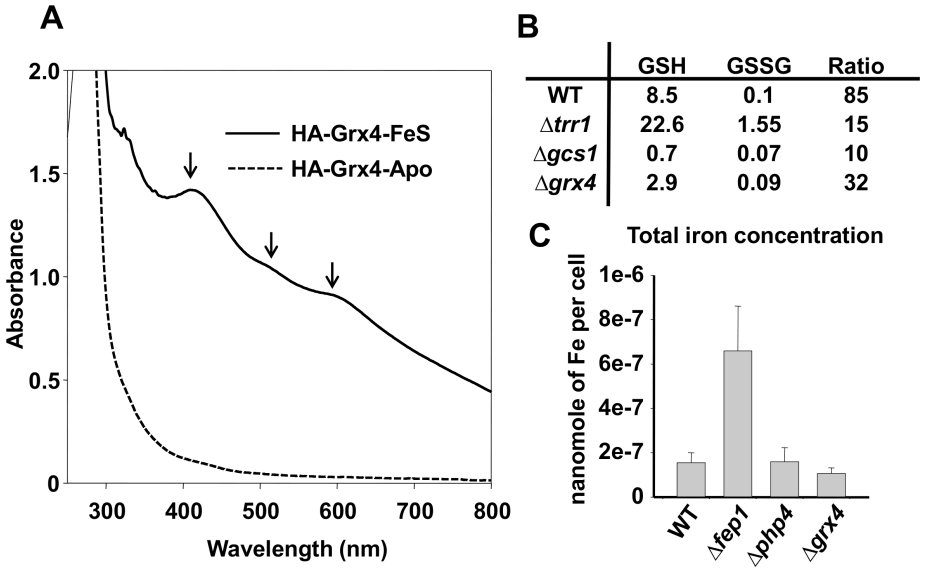



**Fig. 3. The glutaredoxin Grx4 is an ISC-containing protein.** (**A**) Reconstitution of the ISC of Grx4. UV-visible absortion spectra of reconstituted Fe-HA-Grx4 protein. The three maxima of its visible spectrum are indicated with arrows. The dashed line indicates the spectrum of the apo-protein, obtained in the absence of added iron. (**B**) Levels of GSH are lower in Δ*grx4* than in wild-type cells. The GSH levels of 972 (WT) and NG81 (Δ*grx4*) strains were determined from cells grown anaerobically in MM, as described in Materials and Methods. (**C**) The total iron

concentration of strain Δ*grx4* strain is higher than in a wild type strain. Total iron of strains 972 (WT), NG1 (Δ*fep1*), NG40 (Δ*php4*) and NG81 (Δ*grx4*) strains was determined as described in Materials and Methods.

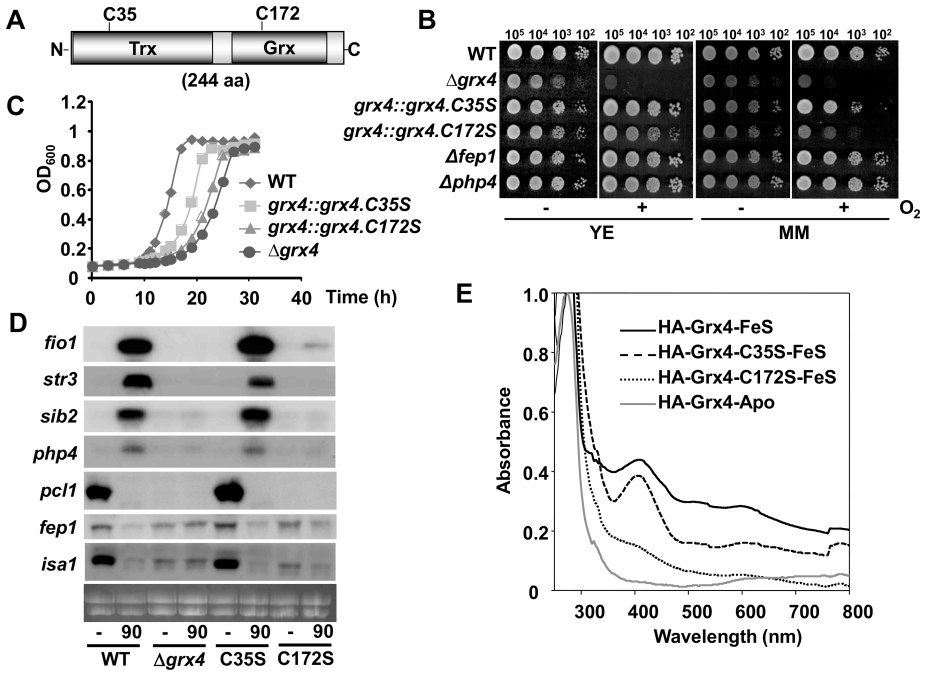



**Fig. 4. The ISC of Grx4 is essential for both Fe delivery and Fe sensing.** (**A**) Scheme of the 244 aa-long Grx4 protein, showing the conserved cysteine-containing Trx and Grx domains. (**B**) Survival spots of cultures from strains 972 (WT), NG1 (Δ*fep1*), NG40 (Δ*php4*), NG81 (Δ*grx4*), NG86.C35S (*grx4::grx4.C35S*) and NG86.C172S (*grx4::grx4.C172S*), as described in Fig. 2A. (**C**) Growth curves of 972 (WT), NG81 (Δ*grx4*), NG86.C35S (*grx4::grx4.C35S*) and NG86.C172S (*grx4::grx4.C172S*) strains were recorded as described in Fig. 2B. (**D**) Total RNA from strains

972 (WT), NG81 (Δ*grx4*), NG86.C35S (*grx4::grx4.C35S*) and NG86.C172S (*grx4::grx4.C172S*) strains were processed as described in Fig. 2D. (**E**) Reconstitution of the ISC in HA-Grx4, HA-Grx4.C35S and HA-Grx4.C172S recombinant proteins. UV-visible absorption spectra of wild-type and mutant Grx4 after ISC reconstitution as described in Materials and Methods.

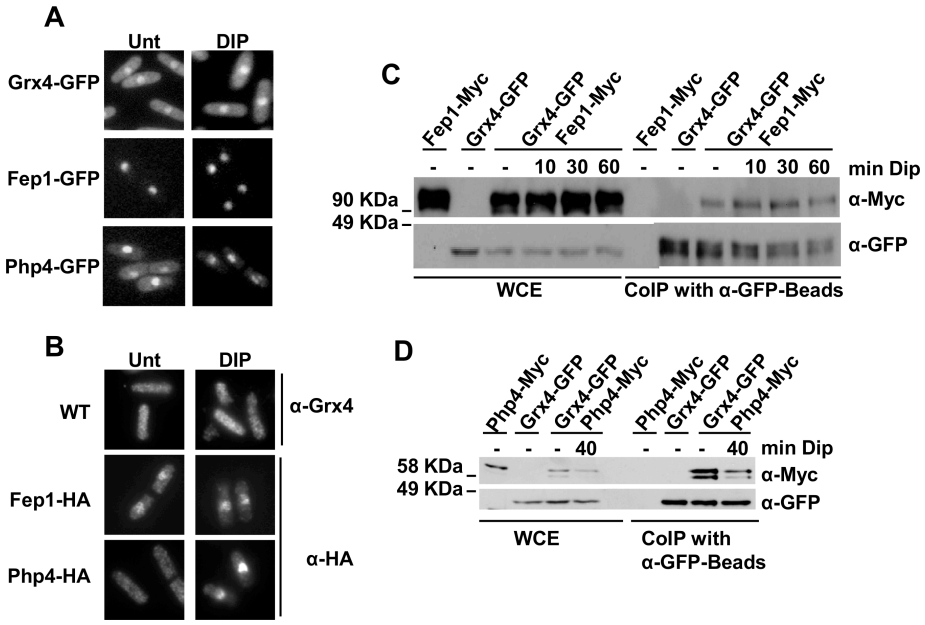



**Fig. 5. Cellular localization and interaction between Grx4, Fep1 and Php4.** (**A**) Fep1 has constitutively nuclear localization and Grx4 and Php4 have cytoplasmic and nuclear localization. Cellular localization of GFP-tagged Grx4, Php4 and Fep1 was determined by fluorescence microscopy from strains NG115 (*grx4-GFP*), NG105 (*fep1-GFP*) and NG70 (*php4-GFP*); the three tags were introduced at the respective gene loci. (**B**) Cellular localization of untagged Grx4, Php4-HA and Fep1-HA was determined by inmuno-fluorescence from strains 972 (WT), NG64 (*fep1-HA*) and NG123 (*php4-HA*) as described in Materials and Methods. The tags were

introduced at the gene loci. (**C**) and (**D**) The interaction of Grx4 with Php4, but not with Fep1, is disturbed upon Fe starvation. (**C**) Strains NG107 (*php4-Myc*), NG115 (*grx4-GFP*) and NG120 (*grx4-GFP php4-Myc*) were treated or not with 0.25 mM DIP during the indicated times. Native extracts were obtained, and total protein extracts were immunoprecipitated with GFP-trap beads. The resulting immuno-precipitates were analyzed by SDS–PAGE and blotted with anti-Myc or anti-GFP antibodies. As a loading control, whole-cell extracts were loaded (WCE). (**D**) Strains NG108 (*fep1-Myc*), NG115 (*grx4-GFP*) and NG109 (*grx4-GFP fep1-Myc*) were treated as described in C.

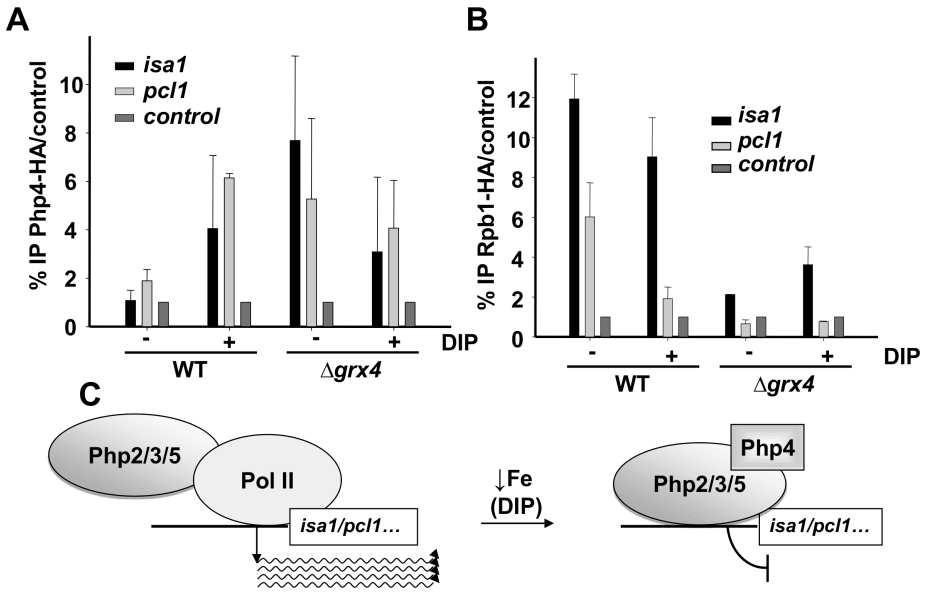



**Fig. 6. The transcription repressor Fep1 inhibits Pol II recruitment to promoters during normal iron-rich conditions.** (**A**) Fep1 is released from promoters upon iron starvation. Strains NG64 (*fep1-HA*) and NG126 (*Δgrx4 fep1-HA*) were treated or not with 0.25 mM DIP for 60 min.

ChIP of Fep1-HA using anti-HA antibody was performed coupled to quantification by real-time PCR, using primers covering the promoter regions of *str3* and *fio1* genes. The percentage of immuno-precipitation (%IP) is indicated relative to control primers, corresponding to an intergenic region. Error bars (SEM) for all ChIP experiments were calculated from biological duplicates. (**B**) The levels of Pol II increase at *fep1*-dependent promoter genes upon iron starvation. Strains NG129 (*rpb1-HA*) and NG135 (*Δgrx4 rpb1-HA*) were treated and analyzed as described in A.

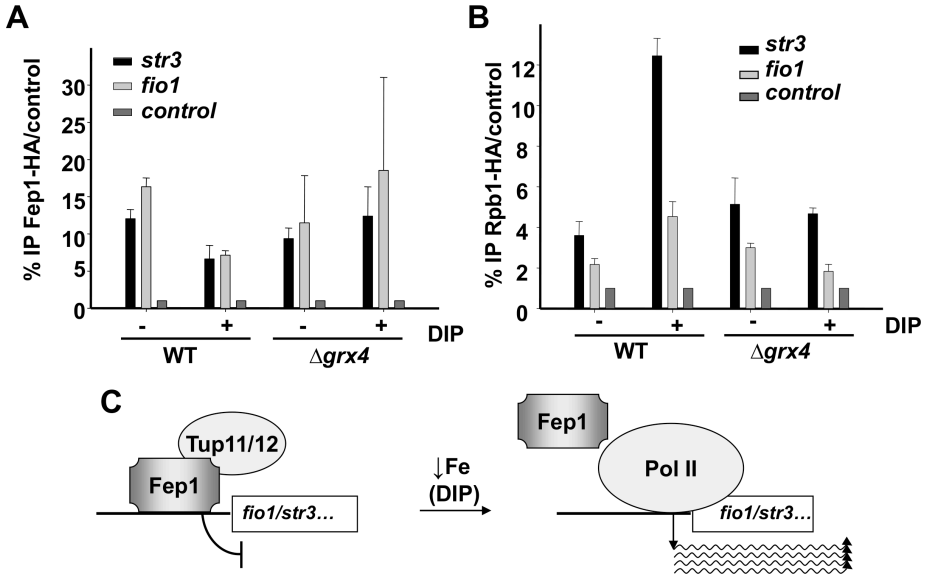
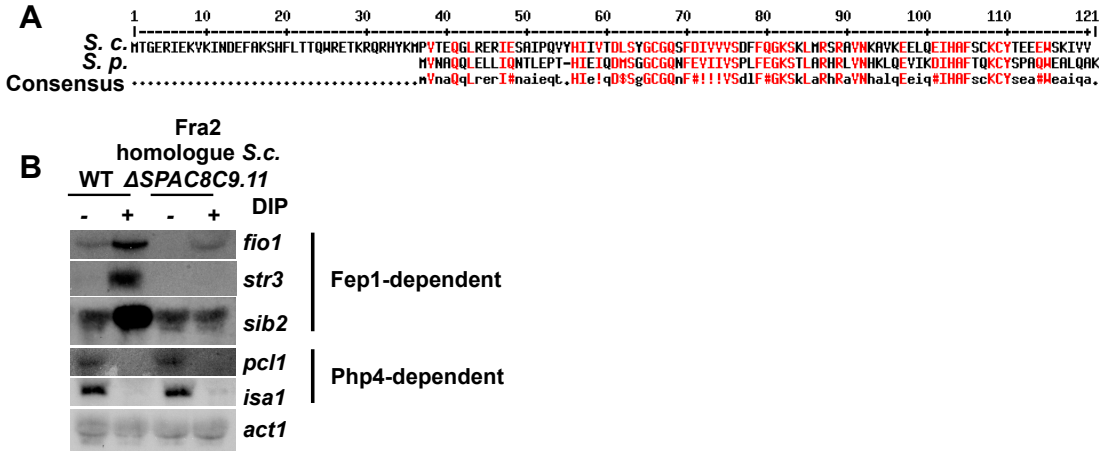



**Fig. 7. The transcription repressor Php4 inhibits Pol II recruitment to promoters under iron deprivation.** (**A**) Php4 is recruited to promoters upon iron starvation. Strains NG123 (*php4-HA*) and NG131 (*Δgrx4 php4-HA*) were treated and analyzed as described in Fig. 6A, using primers covering the promoter regions of *isa1* and *pcl1* genes. (**B**) The levels of Pol II decrease at *php4*-dependent promoters upon iron starvation. Strains NG129 (*rpb1-HA*) and NG135 (*Δgrx4 rpb1-HA*) were treated as described in Fig. 6A.




**Supplementary Fig. 1**. **Identification and characterization of *SPAC8C9.11*, the *S. pombe* homolog of *S. cerevisiae* Fra2.** (**A**) Amino acid alignment of Fra2 of *S. cerevisiae* (*S. c.*) and *SPAC8C9.11* of *S. pombe* (*S. p.*). In red upper case are represented the consensus sequence of aa that are conserved in both organisms. Multiple sequence alignment with hierarchical clustering (F. CORPET, 1988). (**B**) Cells lacking *S. pombe SPAC8C9.11* are able to repress Php4-dependent genes upon iron deprivation as wild-type cells, but cannot induce Fep1-dependent iron uptake genes. Total RNA from strains 972 (WT) and NG101 (Δ*SPAC8C9.11*) was obtained from cultures growing anaerobically in YE and treated or not with 0.25 mM DIP for 90 min. The RNA was analyzed by Northern blot using the probes indicated in the figure. *act1* was used as a loading control.

**Gabrielli et al. Supplementary Fig. 1**

**Supplementary Table 1. Strains used in this study**

| Strain | Genotype | Origin |
|---|---|---|
| 972 | *h⁻* | (Leupold, 1970) |
| NG86 | *h⁻ grx4::ura leu1-32* | This work |
| NG40 | *h⁻ php4::kanMX6* | This work |
| NG1 | *h⁻ fep1::kanMX6* | This work |
| NG86 | *h⁻ grx4::ura leu1-32* | This work |
| NG86.C35S | *h⁻ grx4::grx4.C35S leu1-32* | This work |
| NG86.C172S | *h⁻ grx4::grx4.C172S leu1-32* | This work |
| NG25 | *h⁺ trr1::ura* | (Calvo et al., 2009) |
| NG81 | *h⁻ grx4::natMX6* | This work |
| NG77 | *h⁻ gcs1::kanMX6* | This work |
| NG123 | *h⁻ php4-HA::kanMX6* | This work |
| NG64 | *h⁻ fep1-HA::kanMX6* | This work |
| NG129 | *h⁻ rpb1-HÁ::kanMX6* | This work |
| NG131 | *h⁻ grx4::natMX6 php4-HA::kanMX6* | This work |
| NG135 | *h⁻ grx4::natMX6 rpb1-HÁ::kanMX6* | This work |
| NG115 | *h⁺ grx4-GFP::natMX6* | This work |
| NG107 | *h⁺ php4-Myc::kanMX6* | This work |
| NG108 | *h⁻ fep1-Myc::hygMX6* | This work |
| NG109 | *h⁻ fep1-Myc::hygMX6 grx4-GFP::kanMX6* | This work |
| NG120 | *h⁺ grx4-GFP::natMX6 php4-Myc::kanMX6* | This work |
| NG101 | *h⁻ SPAC9C8.11::kanMX6* | This work |

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## 2.4. New model of Friedreich's ataxia: *S. pombe* $\Delta$*pfh1*

# Cells lacking Pfh1, a fission yeast homolog of mammalian frataxin, display constitutive activation of the iron starvation response

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Gabrielli N, Ayté J, Hidalgo E. Cells lacking pfh1, a fission yeast homolog of mammalian frataxin protein, display constitutive activation of the iron starvation response. J Biol Chem. 2012 Dec 14;287(51):43042–51. DOI: 10.1074/jbc.M112.421735

# Chapter 3

# MATERIALS AND METHODS

## 3.1. Experimental Procedures

### 3.1.1. Growth conditions and yeast strains

Cells were grown in rich medium (YE5S) or synthetic minimal medium (MM) as described previously [405]. Origins and genotypes of strains used in this study are outlined in Table 3.2

### 3.1.2. Liquid sensitivity assay

To measure cellular growth we used an assay based on automatic measurements of optical densities (OD) of small (100 $\mu$l) liquid cell cultures, which allowed us to plot comparable growth curves for each treatment. Cells were grown in YE5S media to an $OD_{600}$ of 0.3 at 30°C under continuous shaking in Erlenmeyer flasks, or in anaerobic conditions in a flasks filled to the top without shaking . Then, we diluted the cultures in MM or YE5S medium to an $OD_{600}$ of 0.1 or 0.3 and cultures were treated with different agents (0.2, 0.5, 1 mM $H_2O_2$ or 50, 250 $\mu$M DIP (dipyridyl, Fluka?) or 50, 300 $\mu$M $FeCl_3$ (Sigma)). Then, we placed 100-$\mu$L samples into 96-well non-coated polystyrene microplates (in triplicate) with an adhesive plate seal. We used Power Wave microplate scanning spectrophotometer (Bio-Tek) to obtain the growth curves. The $OD_{600}$ was automatically recorded using Gen5 software. The software was set as follows: OD was measured at 600 nm, incubation temperature was kept at 30°, the microplates were subjected to continuous shaking and the readings were done every

10 min during a 25 h period.

### 3.1.3. Solid sensitivity assay

*S. pombe* strains were grown in liquid MM or YE5S medium to an an optical density ($OD_{600}$) of $\sim$0.5 at 600 nanometers. Cells were then diluted in the same media than they were grown. Two microliters of cells with the indicated number of cells were spotted into minimal or rich media plates containing or not the indicated concentrations of $H_2O_2$, deferroxamine mesylate (Sigma) or iron ($FeCl_3$. $6H_2O$, Sigma). The spots were allowed to dry, and the plates were incubated at 30°C during 2 to 3 days in aerobic or anaerobic conditions (Forma Anaerobic, System Thermo electron corporation).

### 3.1.4. Transformation of *S. pombe*

Cell culture were grown in YE5S until $OD_{600}$) of $\sim$0.5. Cells ($1\times10^7$ cells/mL) were washed with water and resuspended in lithium acetate (LiAc-TE: 0.1 M litium acetate, 10 mM Tris pH 7.5, 1mM EDTA). $2x10^6$ cells are mixed with 4 $\mu$g of carrier DNA (SIGMA Herring Sperm DNA (D-7290)) and 1 $\mu$g of the transformant DNA. After incubating the cells for 10 minutes at room temperature, 260 $\mu$L of PEG/LiAc-TE (LiAc-TE, 40% PolyEthilenGlycol) are added and incubated for 1 hour at 30°C. 43 $\mu$l dimethyl sulfoxide are added and cells are heat shocked at 42°C for 5 minutes. After 1 wash with YE5S, cells are plated on selective media.

### 3.1.5. High-throughput sensitivity screen

Genome-wide *S. pombe* haploid deletion collection covers more than 2,700 genes. *S. pombe* diploid deletion mutants were systematically constructed with targeted mutagenesis at each ORF, and haploid deletion strains for non-essential genes were isolated. The wild-type strains of the collection are 666 (*h+ ade6-M210 ura4-D18 leu1-32*) and 668 (*h+ ade6-M216 ura4-D18 leu1-32*). More information is provided at the Bioneer web page (http://pombe.bioneer.co.kr/introduction/ResearchPurpose.jsp). The haploid deletion collection was screened as described elsewhere [19]. The collection was first grown in liquid YE5S medium, and then spread with a manual replicator on three types of solid agar

plates: YE5S medium, MM medium, YE5S medium with 5 mM $H_2O_2$ and YE5S medium with 10 mM caffeine. The plates were incubated at 30°C for 3-4 days.

### 3.1.6. Colorimetric assay for iron quantification

Yeast cells were grown in anaerobic liquid cultures in flasks filled to the top (50 mL) with YE5S rich medium [405] until exponential phase ($OD_{600}$ ∼0.5). Cells were washed two times with PBS buffer pH 7.4. Either standarts or yeast cells were resuspended in 3% nitric acid (400$\mu$L) and incubated during 16 h at 100°C. Digest cells were centrifuged 5 min at 12000 g. Supernatant of digest cells and standarts were mixed with 160 $\mu$L (38 mg/mL) of sodium ascorbate, 320 $\mu$L (1.7 mg/mL) of ferrozine (iron chelator, Fluka) and 126 $\mu$L of ammonium acetate solution (saturated ammonium acetate solution diluted 1/3). After 5 min, specific absorbance of iron-chelator complex was recorded at $OD_{565}$ nm in a UV-visible Ultraspec 2100-pro (Amersham Biosciences) spectrophotometer. The accuracy of the assay was improved by subtracting nonspecific absorbance recorded at $OD_{680}$. The number of cells were calculated from $OD_{600}$ ($OD_{600}$ 0. 5 ≅ 1x$10^7$ cells/mL). Standart curve were prepared from 10-40 nmoles of $FeCl_3$ dissolved in 3% nitric acid. All chemicals (except ferrozine from Fluka) were purchased from Sigma and resuspended in ultrapure water obtained from a Millipore Milli-Q Advantage.

### 3.1.7. Bacteria growth conditions

Bacteria strain FB810 [406] transformed with pGEX-2T-TEV derivatives were inoculated into LB Broth Base with 100 $\mu$g/ml ampicilin and incubated at 37°C for ∼ 16 hours (h) with vigorous shaking. The overnight (ON) culture were diluted 80-fold in 400 mL of fresh medium and incubated at 37°C until the culture reached an optical density (OD) of ∼ 0.6 at 600 nm. Isopropyl-$\beta$-thio-D-galactoside (IPTG) was then added to a final concentration of 1 mM and shaking continued at 18°C for 18 h.

### 3.1.8. Purification of recombinant HA-Grx4, Ha-Grx4.C35S and HA-sGrx4.C172S proteins from *E. coli* cells

Cells from 400 mL of culture were harvested and pellets were resuspended in 10 mL of STET extration buffer (50 mM Tris-HCl, pH 8.0, 150 mM sodium chlo-

ride (NaCl), 1 mM EDTA pH 8. 0,1% Triton X-100) and broken by sonication. Debris and unbroken cells were removed by centrifugation. Supernatants containg our GST-HA tagged fusion proteins were incubated with GSH-Sepharose 4B beads (GE Healthcare) for 1 h at 4°C. The beads were washed tree times with NET-N (20 mM Tris-HCl pH 8.0, 0.5 mM EDTA pH 8.0, 100 mM NaCl, 0.5% NP-40) and once with TEV cleavage buffer (50 mM Tris-HCl pH 8.0 and 0.5 mM EDTA pH 8.0). The GST-HA tagged fusion proteins was release from the beads by elution in TEV cleavage buffer with 1 mM fresh DTT and 10 $\mu$g/mL of TEV protease (Invitrogen, Carlsbad, CA) overnight at 4°C. Once, recombinant proteins were released, NaCl were added to 100 mM final concentration and storage at -80°C. Protein concentration were quantified by electrophoretic separation on 10% denaturing polyacrylamide gels and Coomassie staining.

### 3.1.9. FeS cluster reconstitution assay

Cluster reassembly was performed in anaerobic conditions in Forma Anaerobic System (Thermo Electron Corporation) at room temperature. Grx4 WT or mutant proteins (100-200 $\mu$M) from *E. coli* were incubated in 50 mM sodium phosphate buffer (pH 8.0) containing 200 mM NaCl, 0.01 molar equivalents of *E. coli* IscS, 2 molar equivalents of $Fe(NH_4)_2(SO_4)_2$, 2.5 molar equivalents of L-Cys, 1 mM GSH, 5 mM dithiotreitol (DTT), 10 $\mu$M pyridoxal phosphate. After 2 hours, the mixture was desalted using Microspin S-200 HR columns (GE Healthcare). UV-VIS spectra were recorded using a Shimadzu UV-1700 sphectrophotometer (PharmaSpec).

### 3.1.10. RNA analysis

Cells were grown in minimal or rich media to a final $OD_{600}$ of 0.5, them they were either untreated or treated for the indicated times with the different agents such as 0.2 or 1 mM of $H_2O_2$, 250 uM 2,2'- Bipyridyl (Fluka). Yeast cultures (50 ml) were centrifuged at 500 g. for 3 min, washed with $H_2O$ and cell pellets were immediately frozen in dry ice. Each sample was resuspended in 0.4 ml of AE buffer (50mM sodium acetate pH 5.3, 10mM EDTA pH 8.0). Sodium dodecyl sulphate (SDS) was then added to a final concentration of 1%, and proteins and DNA were extracted by adding 0.4 ml of acidic phenol, and incubation at 65°C for 5 min. Samples were cooled down in ice, 0.4 ml of chloroform was added, and the aqueous phase was separated by centrifugation at 10,000 g for

2 min at 4°C. After phenol chloroform extraction, RNA was precipitated with ethanol 100% plus 0.1 M of sodium acetate pH 5.3 and resuspended in DEPC water. RNA concentration was determined by $OD_{260}$ and equal amounts (20 $\mu$g) were loaded in formaldehyde agarose gels [407] and transferred by capilarity procedure to GeneScreen Plus membranes (NEN Life Science Products). Hybridization and washes were performed as recommended by the manufacturer. The blots were hybridized with the indicated specific probe which contained the open reading frame of the encoding genes: fio1, pcl1, fep1, php4, sib2, str3. Probe was labelled with $\alpha$-32PdCTP using a random primer system from Roche. Equal loading was confirmed by ribosomal RNAs, *tfb2* or *act1* probes as a loading controls.

### 3.1.11. Preparation of *S. pombe* TCA extracts and immunoblot analysis

For in vivo redox state analysis of different proteins such as Grx4, Pap1, Trx1, Tpx1 *S. pombe* cultures (5 mL) at an $OD_{600}$ of 0.5 were pelleted just after the addition of 100% trichloroacetic acid (TCA) to a final concentration of 10% and washed in 20% TCA. The pellets were lysed by vortexing after the addition of glass beads and 12.5% TCA. Cell lysates were pelleted, washed in acetone, and dried. Pellets were resuspended in 50 $\mu$L of a solution containing 1% SDS, 100 mM Tris-HCl (pH 8.0), and 1 mM EDTA. For non reduced samples, alkylation of free thiols was accomplished by resuspension of the dry pellets in 50$\mu$L of a solution containing 75 mM iodoacetamide, 1% SDS, 100 mM Tris-HCl (pH 8), 1 mM EDTA, and it they were incubated at 25°C for 15 min. Samples for Pap1 protein were then dephosphorylated to avoid broad bands after electrophoresis by diluting them 5-fold with calf intestinal phosphatase (CIP) buffer to a final concentration of 80 mM Tris- HCl (pH 9.5), 0.08 mM EDTA, and adding 0.04 units/$\mu$L of CIP (Roche) for 60 min at 37°C. The alkylated dephosphorylated or not samples were electrophoretically separated by nonreducing (or reducing by 1mM of dithiothreitol (DTT, Sigma), when it was indicated) SDS-PAGE. Immunoblotting was performed using monoclonal anti-HA antiserum (12CA5), or monoclonal anti-Myc antiserum (9E10), or monoclonal anti-tubulin (Tub2; Sigma, as loading control), or a polyclonal anti-Pap1 antibody, polyclonal anti-Tpx1, anti-Trx1, anti-Sty1 [126], anti-Atf1 [334] and anti-Grx4 antibodies. All those antiserums raised against an *E. coli* fusion proteins of GST-Tpx1, GST-Trx1, GST-Sty1, GST-Atf1, HA-Grx4 following Standard rabbit immunization

procedures.

### 3.1.12. Fluorescence microscopy

Fluorescence microscopy and image capture was performed as previously described [318].

### 3.1.13. Immuno-fluorescence assay

Harvest, 10 mL of cells in MM at exponentially growth ($OD_{600}$ 0.3). Fix cells in 1-2% of formaldehide during 20 min at 25C in a water bath. Centrifuge cells and wash twice in cold PBS 1%. Resuspend cells in 1.2 mL of preincubation buffer (20 mM citric acid, 20 mM $Na_2HPO_4$, 40 mM EDTA pH 8.0), add 30 mM of $\beta$-mercaptoethanol and incubate during 10 min at 30°C in a water bath. Centrifuge cells 1min at 8000 rpm. resuspend cells in 0.6 mL sorbitol-Tris buffer and add 10 mM $\beta$-mercaptoethanol and 2.25 mg of zymolyase 20T (ICN biochemicals) for 40 min in a water bath at 30C (Check digestion microscopically (cells lose refractive halo) and by adding 1% SDS to the slide (to induce lysis). Centrifuge 1 min at 6000 rpm. Wash cells three times with 1 mL of cold PEMS (100 mM PIPES, 1 mM EGTA, 1 mM $MgSO_4$, 1.2 sorbitol. pH 6.5-6.9). Resuspend cells in 1 mL of cold PEMS and 1% Triton X-100 during 1 min (to permeabilizes nuclear membranes) at RT. Centrifuge 1 min at 6000 rpm. Wash cells three times with 1 mL of PEM (100 mM PIPES pH 6.9, 1 mM EGTA, 1 mM $MgSO_4$). Resuspend cells in 1 mL of PEMBAL (100 mM PIPES, 1 mM EGTA, 1 mM $MgSO_4$, 3% BSA, 0.1% $NaN_3$, 100 mM Lysine hydrochloride) and incubate them rocking during 20 min at RT. Resuspend cells in 100 $\mu$L of PEMBAL plus antibody (1/200 dilution) polyclonal $\alpha$-Grx4 or monoclonal $\alpha$-HA (12CA5). Incubate rocking ON at RT. Wash cells three times in PEMBAL, rocking them during 10 min at RT each time. Resuspend cells in 50 $\mu$M of PEMBAL plus secondary antibody (1/500 dilute) Cy2 AffiniPure Donkey $\alpha$-rabbit IgG (H+L) (ref. 711-225-152, Jackson) or Alexa Fluor 555 goat $\alpha$-mouse IgG (ref. A21424, Invitrogen). Incubate rocking 5-7 hs at RT in darkness. Wash cells three times in PEMBAL, rocking them during 10 min at RT each time. Resuspend cells in 100 $\mu$L of PEMBAL. Treat each hole of the slide with 7 $\mu$L of PLL (1/20 dilute), vaccum supernadant and spread 25 $\mu$L of cells solution per hole. Vaccum supernatant and add 5 $\mu$L of 50% glycerol and a coverslip.

### 3.1.14. Co-immunoprecipitation analysis

Cells from 100 mL of YE medium cultures at an $OD_{600}$ of 0.5 ($1\times10^7$ cells) were pelleted and re-suspended in lysis buffer (10mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.5% NP40), and lysed with two 60-s pulses in a cryogenic grinder (6770 Freezer/Mill; SPEX SamplePrep). Lysates were centrifuged for 5min at 6,000 g and supernatants transferred to fresh microtubes. Grx4-GFP was immunoprecipitated from cleared supernatants by adding 10 $\mu$L of GFP-Trap beads (Chromotek) for 1 h at 4°C. Immunoprecipitates were washed twice with dilution buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA). Proteins were released from immunocomplexes by boiling for 5 min in sodium dodecyl sulfate (SDS) loading buffer. Samples were separated by 8% SDS-polyacrylamide gel electrophoresis (PAGE) and detected by immunoblotting with monoclonal anti-Myc or anti-GFP antiserum, raised against a fusion protein GST-GFP purified from *E. coli*, following standard rabbit immunization procedures.

### 3.1.15. Chromatin immunoprecipitation

For immunoprecipitation of HA-tagged Atf1, Php4, Fep1 and Rpb1 proteins linked to DNA promoter regions, cells were grown in liquid MM to an $OD_{600}$ of 0.5 and formaldehyde (1% vol/vol) was added for 20 min at 25°C. Cross-linking was stopped by adding 125 mM glycine. After 5 min, cells were collected by centrifugation and washed twice with cold PBS. Pellets were resuspended in 250 $\mu$L breaking buffer (0.1 M Tris-HCl (pH 8.0), 20% glycerol, and 1 mM PMSF(phenylmethanesulfonylfluoride)) and lysed with glass beads in a bead beater (Biospect Products). Pellets were collected, washed twice with lysis buffer (50 mM HEPES-NaOH (pH 7.5), 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF), and resuspended in 250 $\mu$L lysis buffer. Lysates were then sonicated in a Bioruptor (Diagenode) sonicator with eight 30-s high-sonication pulses at 4°C and 1-min pauses between pulses, yielding chromatin fragments with an average size of 500 bp. Lysis buffer was added up to 1 mL, and samples were centrifuged at 16,000 g for 30 min at 4°C. Fifty $\mu$L from the soluble chromatin samples were kept as inputs, and the remaining was immunoprecipitated with 1 $\mu$L monoclonal anti-HA (12CA5) antiserum and 10 $\mu$L protein A-Sepharose beads (Amersham?) incubated overnight rotating at 4°C. Immunocomplexes were washed once in lysis

buffer, twice in lysis buffer containing 0.5 M NaCl, twice in washing buffer (10 mM Tris-HCl (pH 8.0), 0.25 M LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA, and 1 mM PMSF), and finally once in TE (10 mM Tris-HCl (pH 8.0), 1 mM EDTA). Beads were pelleted, and DNA was eluted in 100 $\mu$L elution buffer (50 mM Tris-HCl (pH 7.5), 10 mM EDTA (pH 8.0), 1% SDS) during 20 min at 65°C. Beads were re-pelleted, the supernatants were transferred to new tubes, and any remaining DNA was eluted from the beads by washing it once in 150 $\mu$L TE (0.67% SDS). Corresponding elution supernatants were pooled, and formaldehyde cross-linking of both the 50 $\mu$L of soluble chromatin and immunoprecipitated chromatin was reversed by overnight incubation at 65°C. DNA was cleaned up by incubation for 2 h at 37°C with 0.3 mg/ml proteinase K and 0.04 mg/ml glycogen and was then purified by phenol-chloroform extraction and precipitated with ethanol and NaCl. DNA was resuspended in 100 $\mu$L TE. Recovered DNA was analyzed by Real Time PCR (Light Cycler 480, Roche) with specific primers: (see Table IX) and SyberGreen Taq mixture (Roche). Results were expressed as a percentage of the input. The error bars (SEM) were calculated from biological duplicates.

## 3.2. Strains

Strains uesd in Results section 1.

| Name | Genotype | Source |
|---|---|---|
| 972 | $h^-$ | [408] |
| AV18 | $h^-$ *sty1::kanMX6* | [409] |
| NG1 | $h^-$ *fep1::kanMX6* | This work |
| NG40 | $h^-$ *php4::kanMX6* | This work |
| NG8 | $h^-$ *sib2::kanMX6* | This work |
| NG9 | $h^-$ *str3::kanMX6* | This work |
| NG18 | $h^-$ *pcl1::kanMX6* | This work |

Table 3.1: *S. pombe* strains

# Chapter 4

# DISCUSSION

## 4.1. Strains with an increase of the accesible free iron exacerbate the sensitivity to $H_2O_2$

Aerobic organisms are fated to expose to ROS as an inherent part of its metabolism. Chronic damage by endogenous ROS harm all biomolecules. Oxidative stress occurs when an imbalance between ROS generation and detoxification appears causing ROS to exceed the buffering capacity of the antioxidant defense system. Cells lacking detoxifying enzymes (e.g. superoxide dismutases or peroxiredoxins) can display growth defects under aerobic conditions [126, 410]. However, intrinsic oxidative stress can be caused not only by cells devoid of antioxidant activities but also by genetic or drug-mediated disassembly of mitochondrial components, as we have previously reported[19].

In this thesis, we were interested in identifying new genes involved in intrinsic oxidative stress. We have isolated several strains lacking mitochondrial components that showed inhibited respiration and enhanced ROS production using double selection screening. The strains with growth defects on defined medium and in complex medium in the presence of extracellular $H_2O_2$ were selected. This double selection was to distinguish the strains that displayed growth defects in defined medium due to nutritional auxotrophies. Another gene that we identifyed in this global screening was the *fep1* gene, an iron-responsive transcription factor that repress genes that code for iron import such us *fio1*, *fip1*, *str1-3*, *sib1-2* [368].

It is widely accepted that part of the toxicity exerted by $H_2O_2$ derives from

its reactivity with reduced iron by the Fenton reaction. In this work we focused on the relationship between iron homeostasis and oxidative stress pathways. We observed that cells lacking *fep1* showed sensitivity to $H_2O_2$ (Fig 2.2 A). We also showed that this strain has an up-regulated iron transport system (Fig1F in [411]) with a consequent increase in the total iron of 3-fold in comparison with a wild type strain (fig 2.2 D). Furthermore, the *fep1* strain is sensitive to growth in rich iron conditions (Fig 2.2 E). This evidence suggests that the sensitivity to $H_2O_2$ could be explained by the increase in the amount of iron inside this mutant resulting in oxidative damage.

A collection of haploid yeast deletion mutants was screened, from which we also identifyed *pcl1* and *sib2* genes that were sensitive to $H_2O_2$. Pcl1 is an iron transporter involved in iron storage in vacuoles. In this case, the deletion of *pcl1* genes did not increase the total iron concentration (Fig 2.3 B). However, the survival of *pcl1* during exposure to $H_2O_2$ (Fig 2.3 A and C) is compromised. This could be due to the fact that the mutant is not able to store the iron properly thus leaving a pool of free accessible iron which could in turn react with the $H_2O_2$ provoking cellular damage.

Based on the two cases of *fep1* and *pcl1* we are able to conclude that an increase in the total amount of iron or in the free iron within the cells could result in a defect in growth if the cells are threatened with $H_2O_2$ due to an increase in the amount of Fenton reaction and its consequent molecular damage.

Finally, the deletion of *sib2* gene showed sensitivity to iron starvation and $H_2O_2$ (Fig 2.4 A and B). Futhermore, the total iron content was widely diminished. This was a controversial result given our previous hypothesis that more available iron implied increased sensitivity to oxidative stress. Sib2 is an ornithine N5 monooxygenase, an enzyme in charge of siderophore biosynthesis (ferrichrome in the case of *S. pombe* The homologue gene to *sib2* in *Ustilago maydis* (*sid1* gene) also presents defects in growth under iron limiting conditions [412]. Siderophores are small molecules with a high affinity to iron and they are secreted to the enviroment to chelate extracellular iron. This is an alternative mechanism to the reductive system by which a steady supply of iron is obtained by cells. Once the iron is chelated, siderophores enter the cell through specific transporters. Once inside the cell the siderophores could be considered as iron storage compounds[281], not allowing the iron to react. This double function of the siderophores could explain why the *sib2* mutant is sensitive to iron starvation and to $H_2O_2$, due to the cell not having such storage molecules available.

## 4.2. $H_2O_2$-dependent activation of the antioxidant gene expression program triggers the iron-starvation response in fission yeast

$H_2O_2$ is among the most stable ROS and consequently can accumulate to significant levels both within cells and in the environment. Part of the toxicity of $H_2O_2$ is caused by its reactivity with ferrous iron to generate highly reactive hydroxyl radicals. This explains why in several bacterial systems part of the response to $H_2O_2$ insult is to modify metal homeostasis; down-regulating iron uptake and stimulating intracellular iron sequestration, to minimize the damage inflicted on the cell[413].

Contrary to such findings, we have found in *S. pombe* that over-expression of catalase as part of the $H_2O_2$-dependent response causes transient and lagged iron starvation, leading to the colateral activation of another gene expression program that enhances the levels of available iron. This is unlikely to be an adaptive response, but rather an unexpected effect of the $H_2O_2$ gene response. Such accidental induction of the iron starvation response by oxidative stress has already been observed in both bacteria and mammals.

Indeed, in *E. coli*, the Fur repressor, for which inactivation occurs in response to iron deprivation, also suffers metal catalyzed oxidation by $H_2O_2$ [414]. This is compensated with activation of the fur gene directly by the $H_2O_2$ sensor OxyR [415]. Similarly, the sensor of iron deprivation in mammalian cells, IRP, was also described to be activated by peroxides [416]; however, an IRP-independent mechanism to enhance iron sequestration by ferritin upon $H_2O_2$ stress has also been proposed[417].

In the case of fission yeast, a similar compensatory effect could arise from the $H_2O_2$-dependent metal-catalyzed oxidation and degradation of the main iron importer Fio1, for which protein levels dramatically decrease upon peroxide stress (Supplementary Fig. 1 in [418]). It is important to remark again the importance of catalase as an iron reservoir: its expression levels seem to modulate the free available iron pool. Thus, cells lacking *ctt1*, *sty1* or *atf1* are slightly but reproducibly more resistant to the presence of chelators than wild-type cells (Supplementary Fig. 2 in [418]).

Furthermore, expression of *ctt1* is strongly down-regulated by Php4 after addition of chelators (Fig. 4A in [418]). In fact, with the exception of the *pcl1* gene (coding for a vacuolar iron importer) most of the Php4-repressible genes

seem to be coding for metallo-enzymes or for mitochondrial proteins.

## 4.3. The glutaredoxin Grx4 is an FeS-containing protein involved in iron sensing

Regulation of the available pools of intracellular iron largely depends on the activation of iron uptake and inhibition of iron storage and consumption upon iron starvation. In *S. pombe*, we show here that this process fully depends on the glutaredoxin, Grx4, an ISC-containing protein distributed at both the cytosol and the nucleus before and after iron starvation. Grx4 seems to bind to the transcriptional repressors Fep1 and Php4, which display nuclear and cytoplasmic localization, respectively, when iron is not limiting. Addition of chelators to the cell cultures triggers an iron starvation response, by probably changing (but not fully eliminating) the ISC architecture on Grx4. Under these circumstances, the binding of activated Grx4 to Php4 is significantly disturbed, and Php4 is then imported to the nucleus and represses iron usage genes; activated Grx4 is still bound to Fep1, but a conformational change may occur in the repressor so that binding to promoters is affected, and transcription of iron uptake genes induced.

It is important to point out that for this study we have constructed full deletion of the *grx4* gene and inserted the cysteine mutations at the grx4 locus, since previous studies used conditional knock outs and tagged and over-expressed versions of the mutants [382, 419, 379]. Our study unambiguously demonstrates that Grx4 is an ISC-containing protein, that cysteine 172 is required for cluster assembly, and that the cluster is essential for the function of Grx4 as an iron sensor, since cells expressing only Grx4.C172S display a transcriptional program to iron deprivation identical to that of cells lacking Grx4.

The transcriptional profile of the *grx4.C172S* mutant, however, also provides clues about the mechanism by which Grx4 senses iron scarcity. We first speculated that loss of the ISC could be the activating event. However, in that case, cells expressing a constitutive apo-Grx4 protein (such as Grx4.C172S) would display a fully active iron starvation response, including up-regulation of iron uptake coding genes and down-regulation of genes coding for ISC-containing proteins. As shown in Fig. 4D in [411], that is not the case: cells expressing Grx4.C172S display constitutive repression of Php4-dependent genes, as expected, but do not show up-regulation of the Fep1-dependent iron import genes; in fact, they cannot activate them in response to iron deprivation. We believe that

the ISC in Grx4 is required for binding of the Grx to Php4 and Fep1, and that is why the *grx4.C172S* mutant allows nuclear import of Php4 and is unable to relieve Fep1 from DNA. In wild-type cells, the ISC of Grx4 may change from one conformation to another, disturbing interaction with Php4 and forcing a protein change in Fep1 hindering its DNA binding domain. Further experiments to demonstrate this hypothesis are ongoing.

The presence of an ISC in a cytosolic Grx is not a novel finding, since the orthologs of Grx4 in *S. cerevisiae*, the redundant Grx3 and Grx4 proteins, were reported to assemble an oxygen-sensitive ISC of the type [2Fe-2S], which bridges a Grx homodimer in vitro [420]. The cluster was more stable if reconstitution was done in the presence of Fra2, a protein originally shown at the genetic level to be required to transduce an iron starvation signal to the yeast transcriptional activator Aft1 [385, 420, 421]. We have found a Fra2 homolog in *S. pombe*, *SPAC8C9.11*, and have analyzed its transcriptional response to iron deprivation (Supplementary Fig. 1 in [411]). Cells lacking the *S. pombe* Fra2 protein are able to repress Php4-dependent genes upon iron deprivation as wild-type cells, but cannot induce iron uptake genes. Whether this protein participates in the assembly of the ISC of Grx4, or whether both proteins form a heterodimer to sense iron deprivation, is still to be determined.

Cells lacking Grx4 display a severe growth defect in the presence of oxygen that is not shared by cells lacking Php4 or Fep1 (Fig. 2A in [411]). This fact, combined with the impaired aconitase activity of $\Delta$*grx4* extracts (Fig. 2C in [411]), prompted us to speculate that this glutaredoxin has an essential role in iron delivery towards iron-containing proteins, as it has been recently proposed for the *S. cerevisiae* homolog Grx4 [422]. However, the transcriptional profile of cells lacking Grx4 under normal (iron-rich) conditions differs from that of wild-type cells in that all the Php4-dependent genes are constitutively repressed (Fig. 2E in [411]).

As indicated in the introduction, Php4 represses in wild-type cells upon iron depleted conditions many genes coding for ISC-containing proteins, many of which are essential for respiratory growth[228]. Among them, the gene/s coding for aconitase are severely repressed under normal growth conditions in cells lacking Grx4, so that the protein levels, and not only the protein activity, are diminished in strain $\Delta$*grx4* (data not shown). In view of these facts, we suggest that Grx4 has only a role in iron sensing and transduction of the iron scarcity signal towards Php4 and Fep1, but is not required for iron trafficking. However,

we have not been able to fully suppress the Δ*grx4* aerobic growth defects by further deletion of the *php4* gene (data not shown). Therefore, further experiments will be required to dismiss or confirm whether Grx4 directly participates in iron delivery, as suggested before for yeast and human glutaredoxins [422].

## 4.4. Cells lacking Pfh1, a fission yeast homolog of mammalian frataxin, display constitutive activation of the iron starvation response

Friedreich's ataxia is caused by a deficit in frataxin, a small mitochondrial protein that has been conserved during evolution but its function remains controversial. We have here developed a new model system to study the molecular events leading to the disease. In our model organism we verify that cells lacking Pfh1 display all hallmarks of other previously reported model systems (Fig. 2 in [423]). Understanding the function of frataxin is crucial to unraveling the cellular consequences of its depletion and therefore the molecular events leading to the disease. Our new model system may shed light into the essential function of this protein.

Previous studies in *S. cerevisiae* Yfh1, the budding yeast frataxin homolog, have pointed out a role for frataxin in mitochondrial FeS metabolism and a direct participation in ISC biogenesis. Consequently, aconitase activity was reduced in cells lacking the protein, and this inactivation seemed to precede iron accumulation [424, 398]. In particular, yeast frataxin has been suggested to participate in ISC maturation as an iron donor, based on its reported interaction with Isu1 [394, 425]. However, it has also been demonstrated that restricting oxidative damage, either by decreasing ROS production [426] or by diminishing available iron [427], prevents aconitase inactivation. Furthermore, a conditional knockdown of Yfh1 expression has allowed the order of sequential events occurring upon frataxin depletion to be established, indicating that induction of iron import is a primary event leading to the late inactivation of ISC-containing proteins [428]. Our results suggest that the low levels of aconitase activity in cells lacking Pfh1 are a consequence of the earlier activation of Php4 repressor, which in wild-type cells lowers expression of most ISC proteins in an iron starvation-dependent manner. Therefore, our work does not support the idea that frataxin is required for ISC biogenesis, but rather directly or indirectly participates in iron-sensing

and signaling.

As explained above, the fission yeast gene expression program upon iron starvation is coordinated by Grx4, which ultimately is responsible of both up- and down-regulation of several genes. The inactivation of Fep1 and activation of Php4 transcriptional repressors mediate the cellular response to iron deficiency [367]. Briefly, when iron is experimentally depleted by the use of chelators Fep1 is released from promoters of genes involved in iron uptake [378], while Php4 accumulates at the nucleus and represses transcription of genes coding for iron-storage or iron-consuming proteins [379]. Importantly enough, Grx4 is the real sensor of iron deprivation, probably through its ISC [411] (our unpublished results). However, the apo-protein does not mimic an iron starvation response, indicating that the loss of the ISC is not the mechanism by which wild-type Grx4 becomes active (our unpublished results). Similarly, cells devoid of Grx4 only mimic the iron starvation response with regard to gene down-regulation by Php4, but cannot trigger activation of Fep1-dependent genes (Fig. 6A in [423]).

As we have shown here, cells devoid of Pfh1 display all hallmarks of an iron deprivation condition, which can hardly be accomplished by genetic modulation of iron sensing/signaling components. In fact, only double deletion of $\Delta$*grx4* $\Delta$*fep1* display a similar transcriptome and phenotype than $\Delta$*pfh1* cells (Fig. 6AB in [423]). Pfh1 could modulate Grx4 activity by, for instance, stabilizing the inactive, iron-rich conformation via chaperone or scaffold properties, its deficiency leading to the basal accumulation of the iron starvation-induced conformation. However, it is difficult to reconcile this putative chaperone role of Pfh1 on Grx4 activity when the first protein has mitochondrial localization (Fig. 2F in [423]) and Grx4 displays cytoplasmic and nuclear localization.

Our results unambiguously indicate that the absence of *S. pombe* frataxin causes a real iron starvation situation able to trigger the complex up- and down-regulation of the gene expression program. A possible role for frataxin which nicely fits with our results is its participation in the regulation of cellular iron homeostasis from the mitochondria. Maybe Pfh1 depletion triggers accumulation of the metal in this compartment, with the concomitant decrease of available cytosolic iron, and Grx4 activation. Further experiments to confirm this hypothesis are of course required.

Our study also strongly suggests that constitutive repression of many essential ISC-containing proteins in strain $\Delta$*pfh1* contributes to the severe phenotypes observed, since they can be partially suppressed by deletion of *php4* (Fig. 5BC

in [423]). Studies on our new *S. pombe* model system on Friedreich ataxia will hopefully contribute to understanding the function of frataxin and to easily test different therapeutic interventions, which may prevent the onset of the disease.

# Conclusions

1. Strains with an increase of the accessible free iron exacerbate the sensitivity to $H_2O_2$.
2. $H_2O_2$-dependent activation of the antioxidant gene expression program triggers the iron-starvation response in fission yeast.
3. The transient activation of the stress gene expression program by $H_2O_2$ triggers iron-starvation response in fission yeast
4. $H_2O_2$ induces a late iron starvation response in a Sty1- and Atf1-dependent manner.
5. Over-expression of catalase depletes the intracellular available iron from the cell.
6. The expression of catalase is down-regulated by Php4 in response to iron starvation.
7. The glutaredoxin Grx4 is an FeS-containing protein involved in iron sensing.
8. Grx4 is required for sensing iron starvation in fission yeast.
9. The glutaredoxin Grx4 is an ISC-containing protein and its ISC is essential for iron delivery and iron sensing.
10. The interaction of Grx4 with Php4, but not with Fep1, is partially disturbed upon iron starvation.
11. The transcription factors Php4 and Fep1 mediate RNA polymerase recruitment to DNA depending on iron availability.

12. The gene *pfh1* codes for the fission yeast frataxin homolog and its deletion generates a new model system to study Friedreich's ataxia.

13. Cells lacking Pfh1 mimic an iron starvation situation and display increased expression of some Pap1-dependent proteins.

14. Frataxin does not have an essential function in iron-sulfur cluster assembly.

# Appendix A

# PAPERS

The following papers are appended below:

1. Mitochondrial dysfunction increases oxidative stress and decreases chronological life span in fission yeast.
2. Genome-wide screen of genes required for caffeine tolerance in fission yeast.
3. Promoter-driven splicing regulation in fission yeast.
4. Lifespan extension by calorie restriction relies on the Sty1 MAP kinase stress pathway.

## A.1. Mitochondrial dysfunction increases oxidative stress and decreases chronological life span in fission yeast.

Zuin A[1], Gabrielli N[1], Calvo IA[1], García-Santamarina S, Hoe KL, Kim DU, Park HO, Hayles J, Ayté J, Hidalgo E. PLoS One. 2008 Jul 30;3(7):e2842.

[1] These authors contributed equally to this work.

## A.2. Genome-wide screen of genes required for caffeine tolerance in fission yeast.

Calvo IA[1], Gabrielli N[1], Iglesias-Baena I[1], García-Santamarina S, Hoe KL, Kim DU, Sansó M, Zuin A, Pérez P, Ayté J, Hidalgo E. PLoS One. 2009 Aug 12;4(8):e6619.

[1] These authors contributed equally to this work.

## A.3. Promoter-driven splicing regulation in fission yeast.

Moldón A, Malapeira J, Gabrielli N, Gogol M, Gómez-Escoda B, Ivanova T, Seidel C, Ayté J. Nature. 2008 Oct 16;455(7215):997-1000. Epub 2008 Sep 24.

## A.4. Lifespan extension by calorie restriction relies on the Sty1 MAP kinase stress pathway.

Zuin A, Carmona M, Morales-Ivorra I, Gabrielli N, Vivancos AP, Ayté J, Hidalgo E. EMBO J. 2010 Mar 3;29(5):981-91. Epub 2010 Jan 14.

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