# Glutaredoxins Grx3 and Grx4 regulate nuclear localisation of Aft1 and the oxidative stress response in *Saccharomyces cerevisiae*

**Nuria Pujol-Carrion[1], Gemma Belli[1], Enrique Herrero[1], Antoni Nogues[2] and Maria Angeles de la Torre-Ruiz[1,]***

[1]Departament de Ciències Mèdiques Bàsiques, Universitat de Lleida, Lleida 25198, Spain
[2]Servei d'analisis cliniques, Hospital Arnau de Vilanova, Lleida 25192, Spain
*Author for correspondence (e-mail: madelatorre@cmb.udl.es)

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## Summary

**Grx3 and Grx4, two monothiol glutaredoxins of *Saccharomyces cerevisiae*, regulate Aft1 nuclear localisation. We provide evidence of a negative regulation of Aft1 activity by Grx3 and Grx4. The Grx domain of both proteins played an important role in Aft1 translocation to the cytoplasm. This function was not, however, dependent on the availability of iron. Here we demonstrate that Grx3, Grx4 and Aft1 interact each other both in vivo and in vitro, which suggests the existence of a functional protein complex. Interestingly, each interaction occurred independently on the third member of the complex. The absence of both Grx3 and Grx4 induced a clear enrichment of G1 cells in asynchronous cultures, a slow growth phenotype, the accumulation of intracellular iron and a constitutive activation of the genes regulated by Aft1. The *grx3grx4* double mutant was highly sensitive to the oxidising agents hydrogen peroxide and t-butylhydroperoxide but not to diamide. The phenotypes of the double mutant *grx3grx4* characterised in this study were mainly mediated by the Aft1 function, suggesting that *grx3grx4* could be a suitable cellular model for studying endogenous oxidative stress induced by deregulation of the iron homeostasis. However, our results also suggest that Grx3 and Grx4 might play additional roles in the oxidative stress response through proteins other than Aft1.**

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## Introduction

Cells are exposed to a number of environmental changes and must therefore develop different strategies to respond and adapt to the various resulting stresses. Aerobic metabolism gives rise to reactive oxygen species (ROS), such as superoxide, hydrogen peroxide and hydroxyl ions (Cadenas, 1989), which provoke oxidative stress and cause damage to cells (Aruoma et al., 1991). As a consequence, cells need to develop a series of different mechanisms to protect themselves from the harmful reactive oxygen species (Poyton, 1999). Iron is an essential element for all organisms and appropriate iron homeostasis is required to prevent the impairment of cellular functions caused by excesses or deficiencies of this metal. Iron is also required in a number of essential proteins related to respiratory chain reactions, and it plays an essential role in at least one electron chain reaction (Arredondo and Núñez, 2005). An excess of iron can be very toxic for cells because it generates free radicals that may oxidise and damage DNA, lipids and proteins (Halliwell and Gutteridge, 1991). Iron deficiency, on the other hand, is responsible for several health problems including anaemia (Beard, 2001) and both neuronal (Ortiz et al., 2004) and immunological alterations. In *Saccharomyces cerevisiae*, the transcription factor Aft1 regulates a subset of genes defined as the high-affinity iron-uptake regulon (Yamaguchi-Iwai et al., 1995; Casas et al., 1997). This group comprises genes involved in the uptake, compartmentalisation and use of iron. Aft1 binds to specific promoter regions and induces expression of the iron regulon in conditions of iron depletion (Yamaguchi-Iwai et al., 1996). In a subsequent study, Yamaguchi-Iwai and co-workers (Yamaguchi-Iwai et al., 2002) reported that Aft1 responds to iron availability by changing its intracellular localisation. This means that under iron-replete conditions Aft1 localises to the cytoplasm, but under conditions of iron starvation Aft1 translocates to the nucleus. Even so, the transcriptional activity of Aft1 is determined by its nuclear localisation regardless of the iron intracellular status.

Another physiological effect of the intracellular iron accumulation mediated by Aft1 is cell-cycle arrest. Philpott and co-workers (Philpott et al., 1998) reported that the expression of an *AFT1*-up allele induces iron accumulation, and as a consequence, cells arrest in G1 at START. Constitutive activation of the iron-responsive regulon resulting from constant transcriptional induction driven by Aft1 therefore causes a reduced expression of the G1 cyclins, Cln1 and Cln2. One of the physiological effects derived from this accumulation of iron in cells affects cell-cycle progression in a similar way to that previously described for other environmental stresses, including heat shock and oxidative and nutritional stress (Cross, 1995; Lee et al., 1996).

Aft2 is another transcription factor required for iron

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homeostasis and resistance to oxidative stress in the absence of Aft1 function (Blaiseau et al., 2001). Aft2 also activates transcription of specific genes in response to low iron conditions (Rutherford et al., 2001; Rutherford et al., 2005). A recent study has demonstrated that Aft2 acts in the absence of Aft1 (Courel et al., 2005) and that the transcriptional function of both proteins is iron-dependent. DNA microarray clustering has revealed that both Aft1 and Aft2 share the regulation of a number of iron-responsive genes. However, there is a group of genes related to iron homeostasis whose regulation depends on Aft2 but not on Aft1.

In proteins, cysteine residues are very susceptible to oxidation. Living cells contain regulatory proteins that are involved in maintaining the redox states of oxidised proteins (Rietsch and Beckwith, 1998; Carmel-Harel and Storz, 2000; Grant, 2001). Monothiol glutaredoxins are thiol oxidoreductases, which require the reduced form of glutathione, GSH, as an electron donor to reduce protein-glutathione disulfides (Holmgren, 1989; Holmgren and Aslund, 1995; Grant, 2001; Herrero and Ros, 2002).

In *Saccharomyces cerevisiae*, three different monothiol glutaredoxins, Grx3, Grx4 and Grx5 (Rodríguez-Manzaneque et al., 1999), have been described to date. Grx5 plays a role in the biogenesis of iron/sulphur clusters at the mitochondria and its function has been extensively characterised (Rodríguez-Manzaneque et al., 1999; Rodríguez-Manzaneque et al., 2002; Bellí et al., 2002). Recent reports have demonstrated that Grx3 and Grx4 both localise to the nucleus (Lopreiato et al., 2004; Molina et al., 2004).

In this study we describe a function for Grx3 and Grx4 in the cellular iron homeostasis through the regulation of the nuclear localisation of Aft1. At the time of submission of this manuscript, one study was accepted in press (Ojeda et al., 2006), which contained a number of similarities with respect to the present one. Both studies demonstrate the interaction between Aft1 and the monothiol glutaredoxins Grx3 and Grx4, and also that in the absence of both Grx3 and Grx4, the genes regulated by Aft1 are constitutively induced. Here we analyse the consequences of this regulation in the transcriptional response mediated by Aft1 and hypothesise a possible mechanism by which Grx3 and Grx4 might regulate Aft1 translocation from the nucleus to the cytoplasm. In addition, we also demonstrate a physical nuclear interaction between Grx3, Grx4 and Aft1, which could reflect the functional specific regulation of Aft1 by both monothiol glutaredoxins. The simultaneous absence of both Grx3 and Grx4 proteins had a pronounced effect on cell-cycle progression, the rate of cell growth and sensitivity to oxidising agents. Hence, Grx3 and Grx4 might regulate the oxidative status of the cell by regulating iron homeostasis in iron-rich conditions.

## Results

### Grx3 and Grx4 are required for the cellular response to oxidative stress

Since both Grx3 and Grx4 are monothiol glutaredoxins we wondered whether they each could play a role in reducing oxidised proteins and therefore in the oxidative stress response. In order to answer this question we assayed sensitivity to various oxidant agents in *grx3*, *grx4*, *grx3grx4* and wild-type strains. We took aliquots from each of the cell cultures growing exponentially and spotted them on plates containing different concentrations of hydrogen peroxide, t-butylhydroperoxide and diamide. We obtained very encouraging results, although neither of the single mutants was substantially sensitive to the oxidising agents hydrogen peroxide and t-butylhydroperoxide, however, the double mutant turned out to be very sensitive to both agents compared with wild-type cells (Fig. 1). Interestingly, none of the mutants tested was sensitive to diamide. From this result we deduced that both Grx3 and Grx4 are required for cells to respond to certain types of oxidative stress and that both glutaredoxins perform additive functions in protecting against oxidation.

### Both Grx3 and Grx4 interact in vivo and in vitro with Aft1 in the nucleus

In an attempt to further characterise the function of both Grx3 and Grx4 glutaredoxins, we searched the SGD database and found a possible interaction between Grx3 and Aft1. This interaction turned out to be quite interesting for several reasons: (1) Aft1 is a transcription factor involved in the high affinity system for iron capture, and misregulation of iron inside cells is an important cause of oxidative stress (Gakh et al., 2006); (2) Glutaredoxins are molecules that detoxify oxidised residues; (3) it has recently been reported that Grx3 and Grx4 localises in the nucleus and Aft1 operates in the nucleus by inducing the transcription of a subset of genes required for iron uptake. As a result, Aft1 proved a suitable candidate as a substrate for Grx3 and/or Grx4.

We first constructed a number of plasmids to perform two-hybrid analysis between Grx3 and Aft1, Grx3 and Grx4, and Grx4 and Aft1. We obtained a clear result: the existence of strong in vivo interactions in the nucleus between Grx3 and Aft1, Grx3 and Grx4 and Aft1 and Grx4 (Fig. 2A). We wondered whether Grx3 and Grx4 were precluding the interaction of the other glutaredoxin with the transcriptional factor Aft1. In an attempt to gain a clearer picture of this interaction we therefore performed two-hybrid assays between Grx4 and Aft1 in *grx3* background, between Grx3 and Aft1 in *grx4* mutant cells and between Grx3 and Grx4 in *aft1* background (Fig. 2C). We subsequently observed that: (1) in the absence of Grx3, Grx4 still interacted with Aft, (2) in the

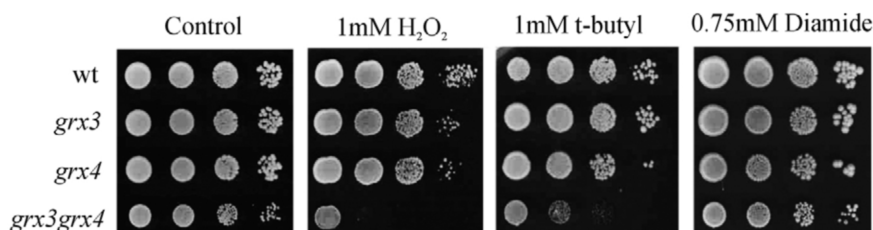


**Fig. 1.** Grx3 and Grx4 are required for survival upon treatment with hydrogen peroxide and t-butylhydroperoxide. Exponentially growing cells from wild-type, *grx3*, *grx4* and *grx3grx4* strains were harvested, serially diluted and spotted onto control SD plates or on SD plates containing 1 mM $H_2O_2$, 1 mM t-butylhidroperoxide or 0.75 mM diamide. Plates were incubated at 30°C for 3 days.

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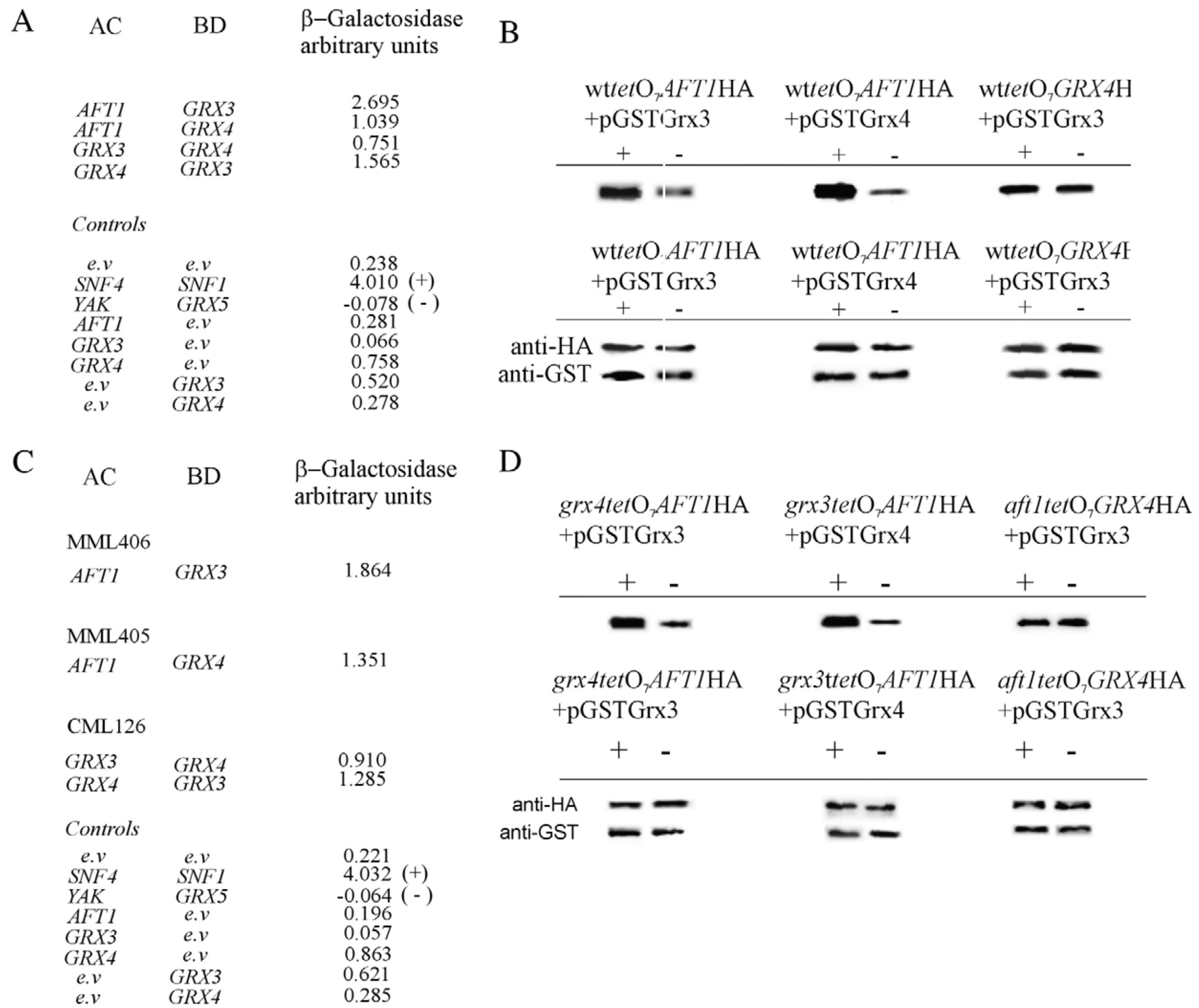


**Fig. 2.** In vivo and in vitro interaction of Grx3 and Aft1, Grx4 and Aft1 and Grx3 and Grx4. (A) Two-hybrid analyses for the following interactions: Grx3 with Aft1, Grx4 with Aft1 and Grx3 with Grx4. Values for interaction between *SNF4* and *SNF1* were used as a strong positive control for nuclear interaction (+). Values obtained from the nuclear interaction between *YAK1* and *GRX5* were used as a positive control for weak nuclear interaction (–). *e.v*, empty vector. (B) Pull-down assays between Grx3 and Aft1, Grx4 and Aft1, Grx3 and Grx4. To detect these interactions, total protein extracts were obtained and subsequently bound to GST beads. In this first step we isolate either Grx3 or Grx4. To detect the second protein component of the complex, we tagged either Aft1 or Grx4 with the HA epitope and detected its presence by western blot with anti-HA antibody. As a loading control, we used anti-GST or anti-HA antibodies in aliquots taken from the same protein extracts. (C) Two-hybrid assay between: Grx3 and Aft1 in the *grx4* mutant MML406; Grx4 and Aft1 in the *grx3* strain MML405; Grx3 and Grx4 in the *aft1* mutant CML126. +, positive control; –, negative control; *e.v*, empty vector. We performed a control for each of the backgrounds assayed, but for simplicity and because the three values were almost identical, the average values are shown. (D) Pull-down assays between Aft1 and Grx3 in the *grx4* mutant; between Aft1 and Grx4 in the *grx3* mutant; and Grx3 and Grx4 in the *aft1* background.

absence of Grx4, Grx3 interacted with Aft1; and (3) Grx3 and Grx4 also interacted in the absence of Aft1 (Fig. 2C). These results suggested that Grx3 and Grx4 both regulate Aft1 and also that Grx3, Grx4 and Aft1 form a complex and each of the three proteins interact with each other independently.

These interactions were confirmed by pull-down assays in all the mutants detailed above (Fig. 2B,D). We used ferrocene to sequester iron because in iron-limiting conditions Aft1 translocates to the nucleus. The addition of ferrocene produced a higher degree of isolation of the Aft1-Grx3 and Aft1-Grx4 complexes, but the complex formed between Grx3 and Grx4 did not vary. This indicated that Grx3 and Grx4 interact with Aft1 in the nucleus and that the greater the presence of Aft1 in the nucleus the greater the interaction with Grx3 and Grx4. This led us to conclude that the interaction between Grx3 and Grx4 with Aft1 was only limited by Aft1 cellular localisation. Another relevant finding was that the physical binding between Grx3 and Grx4 in the nucleus was not dependent on either the availability of iron or the presence of Aft1.

### Grx3 and Grx4 negatively regulate the transcriptional function of Aft1 in the nucleus

In view of the previously mentioned results we decided to investigate the functional significance of the interaction between Grx3, Grx4 and Aft1. Since Aft1 regulates the transcription of the high-affinity iron uptake genes, we decided

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to select two of the genes whose transcriptional control is tightly regulated by Aft1: *FIT3* and *FET3*. *FIT3* encodes a mannoprotein involved in the retention of siderophore iron in the cell wall whose transcription is regulated by Aft1 (Philpott et al., 2002). *FET3* is a ferro-$O_2$-oxidoreductase required for high-affinity iron uptake and is located in the plasma membrane (De Silva et al., 1995; Rutherford et al., 2003).

Under conditions in which there is an excess of iron in the culture medium, neither of the two genes is transcriptionally induced but when iron concentration is a limiting factor in the external medium, Aft1 induces the expression of these genes. We used ferrocene, an iron-chelating agent, in order to mimic an environmental situation in which there was an iron deficiency. We then performed northern blot analysis in different backgrounds: *grx3*, *grx4*, *grx3grx4* and wild-type cells and used *FIT3* and *FET3* as probes. In Fig. 3 we observed how the addition of ferrocene to the culture medium gradually induced a very pronounced expression of these genes in wild-type, *grx3* and *grx4* backgrounds with respect to the basal level. In both single mutants: *grx3* and *grx4,* the constitutive mRNA levels of both *FIT3* and *FET3* genes were higher than those determined in wild-type cells; this indicated a negative regulation of each Grx3 and Grx4 on the Aft1 transcriptional function. However, the most revealing result was that obtained with the double mutant *grx3grx4*: both *FIT3* and *FET3* genes were constitutively induced with similar levels of expression in untreated cells and in cells treated with ferrocene (Fig. 3). These observations were in line with the previously mentioned results in which we observed that the glutaredoxins interacted and regulated Aft1 independently of each other, but that the two acted together in the regulation of Aft1 function in the nucleus. To ascertain whether the transcriptional upregulation detected in the *grx3grx4* double mutant was specifically Aft1 dependent, we constructed the *grx3grx4aft1* triple mutant and observed that in this background neither the *FET3* or *FIT3* transcriptional level was detectable at time 0 (exponentially growing cultures not treated with ferrocene): this was similar to that observed in the case of the *aft1* null mutant (Fig. 3). This led us to conclude that the very high constitutive expression levels of *FIT3* and *FET3* observed in the *grx3grx4* double mutant were due to Aft1 gene regulation and that Grx3 and Grx4 consequently negatively regulate the Aft1 transcriptional function regardless of iron availability.

The existence of a low-affinity system for iron uptake, regulated mainly by Aft2 has already been widely documented. In the absence of Aft1, Aft2 induces the expression of the iron regulon of genes under conditions of iron limitation. Moreover, the expression levels of *FET3* and *FIT3* remarkably increased upon ferrocene treatment in both *aft1* and *grx3grx4aft1* strains, in a similar fashion as in wild-type cells (Fig. 3). These results are in accordance with a model in which Aft2 drives the expression of the iron regulon when iron constitutes a limiting factor in the culture medium and when Aft1 is not functional. It also suggests that Grx3 and Grx4 do not regulate the Aft2 function, but further studies are required to validate this model.

### The absence of Grx3 and Grx4 do not influence the mRNA levels of Aft1

One possible interpretation of the results presented above is that Grx3 and Grx4 could have regulated the transcriptional levels of *AFT1:* this would lead to the increase in Aft1 protein levels and consequently to the transcriptional induction of the genes regulated by Aft1. We decided to perform northern blot analysis using samples from the wild-type, *grx3grx4*, *aft1*, *grx3grx4tet*$O_7$Grx3HA, *grx3grx4tet*$O_7$Grx4HA, *grx3grx4tet*$O_7$Grx3HA+pGSTGrx4 and *tet*$O_7$Grx4HA+pGSTGrx3 strains and using the probe *AFT1*. We observed (Fig. 4) that the mRNA levels in *AFT1* were independent of the presence or absence of Grx3 and Grx4. We therefore conclude that neither Grx3 nor Grx4 regulated the expression of *AFT1*.

### The absence of Grx3 and Grx4 causes a growth and cell-cycle defect as a consequence of Aft1 upregulation

In the course of this study we observed that the double mutant *grx3grx4* presented a marked growth defect. This consisted of a much longer generation time (135 minutes) than that observed in wild-type cells (90 minutes in SD medium growing at 30°C) and also a curious accumulation of G1 cells in exponentially growing cells (Fig. 5). Since Aft1 overexpression also induces a delay in the G1 phase of the cell cycle (see Fig. 5) and we have demonstrated that both proteins negatively regulate Aft1 function, we wondered whether the cell-cycle phenotype observed in the *grx3grx4* double mutant was another consequence of Aft1 misregulation. When we measured the growth rate in the triple mutant *grx3grx4aft1* we

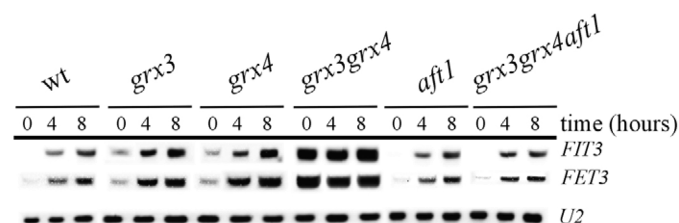


**Fig. 3.** Grx3 and Grx4 negatively regulate the expression of *FIT3* and *FET3* in a manner dependent on Aft1 activity. Cells from the following strains: wild type, *grx3*, *grx4*, *grx3grx4*, *aft1* and *grx3grx4aft1*, were exponentially grown in SD medium plus amino acids, at 30°C, then treated with 2 mM ferrocene. Samples were taken after 4 and 8 hours as indicated. Samples were taken for mRNA isolation and northern blot using *FIT3* and *FET3* as probes, *U2* was detected as a loading control.

**Fig. 4.** *AFT1* RNA levels are not regulated by Grx3 nor Grx4. Northern blot analysis of *AFT1* expression levels in the wild type, *grx3grx4* and *aft1* mutants and under conditions of overexpression of Grx3, Grx4, or both. Overexpression of Grx3 and Grx4 was driven by the *tet*$O_7$ or by the *ADH1* promoter (pGSTGrx3 or pGSTGrx4) as stated. To regulate gene expression under the *tet*$O_7$ promoter, we added (+) or not (–) 20 μg/ml doxycycline to the culture media.

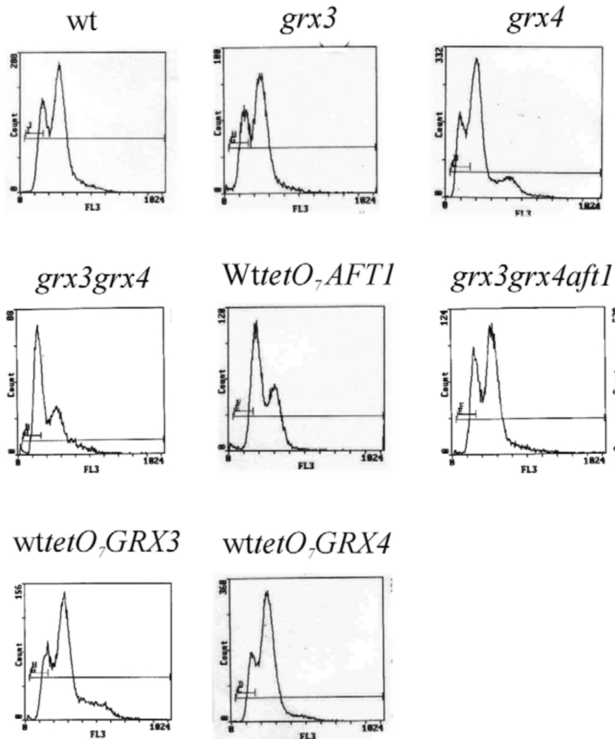


**Fig. 5.** The simultaneous absence of Grx3 and Grx4 induces accumulation of cells in G1. FACS profiles of different strains growing exponentially in SD medium plus amino acids. The strains are: wild type, *grx3*, *grx4*, *grx3grx4*, wild type overexpressing Aft1, *grx3grx4aft1*, wild type overexpressing Grx3 and wild type overexpressing Grx4. All the proteins tested were overexpressed under the *tet*$O_7$ promoter.

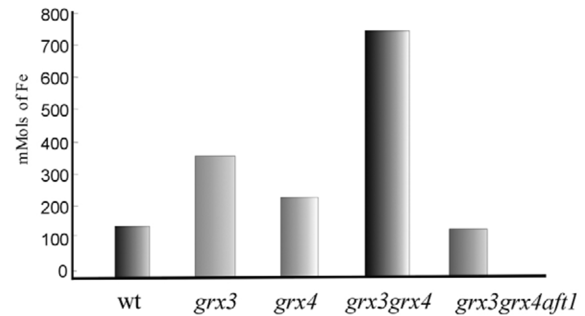


**Fig. 6.** Total iron accumulates in the cell in the absence of Grx3 and Grx4. Total iron concentration was spectrophotometrically determined as described in the Materials and Methods in exponentially growing cultures of wild-type, *grx3*, *grx4*, *grx3grx4* and *grx3grx4aft1* strains. Numerical values represented in the histograms of the figure are averages from three experiments. In all cases standard errors were lower than 10, therefore no error bars are distinguished.

observed that it was similar to that in wild-type cells (90 minutes of generation time in SD medium growing at 30°C). We also observed that the FACS profile of *grx3grx4aft1* was similar to that of wild-type cells in which the 2N DNA content of the population had been enriched in comparison with the 1N content. This was characteristic of the wild-type background used in this study. As expected, the absence of only Grx3 or Grx4 proteins did not affect cell-cycle progression nor did the overexpression of each of both proteins (Fig. 5). These results suggest that both the growth defects and cell-cycle defects observed in the *grx3grx4* double mutant were mediated by Aft1 activity.

## Grx3 and Grx4 are involved in the regulation of iron homeostasis through Aft1

We then decided to investigate whether the Aft1 upregulation observed in this study in the absence of Grx3 and Grx4 would affect the concentration of intracellular iron. We measured total iron concentration in wild-type, *grx3*, *grx4*, *grx3grx4* and *aft1grx3grx4* strains. As shown in Fig. 6, in both *grx3* and *grx4* single mutants, total iron concentration significantly increased with respect to that recorded in wild-type cells, but it was in the double mutant that the highest intracellular iron levels were detected. Interestingly, in the triple *aft1grx3grx4* mutant the intracellular iron concentration was equivalent to that detected in wild-type cells, which clearly indicates that the iron accumulation observed in the absence of both Grx3 and Grx4 is a consequence of Aft1 activity. Therefore, we conclude that one cellular consequence of Aft1 upregulation in the *grx3grx4* mutant is the accumulation of intracellular iron.

It has already been demonstrated that the accumulation of iron inside cells provokes oxidative stress through the Fenton reaction, which releases hydroxyl radical to the cytoplasm. Bearing this in mind, we decided to investigate whether the greatest sensitivity to oxidant agents observed in the *grx3grx4* double mutant was the consequence of the upregulation of the genes governing iron uptake driven by Aft1. To determine this, we first tested sensitivity to various oxidant agents in the following strains: wild type, *grx3grx4*, *aft1* and *grx3grx4aft1*, and observed that the very high sensitivity of the *grx3grx4* double mutant to hydrogen peroxide and t-butyl hydroperoxide was only partly recovered to wild type levels upon *aft1* deletion (Fig. 7A). In order to further characterise whether the abrogation of sensitivity in the *grx3grx4aft1* triple mutant was due to iron accumulation, we decided to treat cells with hydrogen peroxide in the presence of ferrocene. The presence of the iron chelator abrogated most of the sensitivity to the oxidising agent observed in the *grx3grx4* double mutant (Fig. 7B). These data indicated that the phenotype of oxidative stress sensitivity characteristic of *grx3grx4* is at least in part, due to the accumulation of high levels of iron inside cells as a consequence of Aft1 upregulation. However, other specific functions not mediated by Aft1 must also be regulated by Grx3 and Grx4 within the oxidative stress response.

## Grx3 and Grx4 regulate Aft1 compartmentalisation

Some authors have reported that the Aft1 function was determined by its nuclear localisation because the iron regulon of genes dependent on Aft1 was constitutively induced in a mutant of the protein permanently located in the nucleus (Yamaguchi-Iwai et al., 2002). We therefore wondered whether the upregulation of *FET3* and *FIT3* detected in the *grx3grx4* strain was also the consequence of Aft1 translocation to the nucleus. To check this, we constructed a GFP-Aft1 fusion protein in a multicopy plasmid to monitor, in vivo, the cellular localisation of the protein by fluorescence microscopy. In wild-

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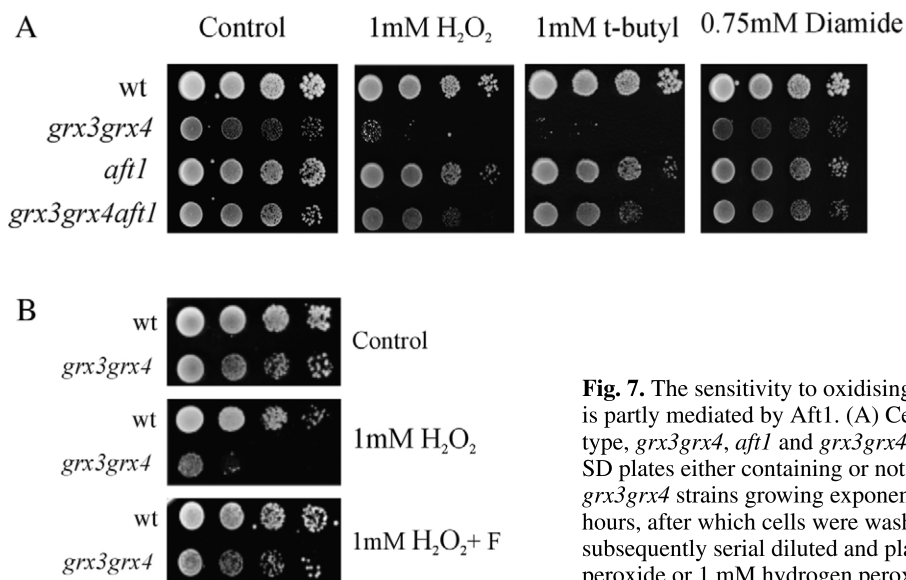


**Fig. 7.** The sensitivity to oxidising agents observed in the *grx3grx4* double mutant is partly mediated by Aft1. (A) Cells from exponentially growing cultures of wild-type, *grx3grx4*, *aft1* and *grx3grx4aft1* strains were serial diluted and spotted onto SD plates either containing or not oxidising agents as in Fig. 1. (B) Wild-type and *grx3grx4* strains growing exponentially were treated with 2 mM ferrocene for 6 hours, after which cells were washed and placed in fresh medium to be subsequently serial diluted and plated on SD plates containing 1 mM hydrogen peroxide or 1 mM hydrogen peroxide plus 150 μM ferrocene (F).

type cells, Aft1 was dispersed throughout the cell (Fig. 8A, panel 1) whereas in the *grx3grx4* mutant, Aft1 was permanently located in the nucleus, as demonstrated by DAPI staining (Fig. 8B, panel 2). This finding is in accordance with the results shown in this study and explains the constitutive upregulation of *FIT3* and *FET3* detected in the *grx3grx4* double mutant.

The next question to investigate was whether Grx3 and Grx4 regulated the nucleus-cytoplasm Aft1 localisation. We designed an in vivo experiment that allowed us to determine how the two monothiol proteins regulated Aft1 nuclear localisation. To do this, we constructed two double conditional mutants. In the first, Grx3 was regulated by the *tet*$O_7$ promoter, so that the addition of doxycycline would inhibit Grx3 expression and the release of doxycycline would induce Grx3 overexpression. The second double mutant was similar to the first, but with the difference that the regulated protein under the *tet*$O_7$ promoter was Grx4. When we checked the transcriptional regulation of *FET3* we observed that there was a very high expression of this gene in samples taken from both mutants when they were exponentially grown in the presence of doxycycline. This was similar to what we observed in samples taken from the double mutant *grx3grx4* (Fig. 8B, lines 2, 5 and 7). Furthermore, under these conditions, Aft1 was located in the nucleus in all three cell populations (not shown). Upon removal of doxycycline, Grx3 protein was overexpressed and in this context Aft1 was partly translocated to the cytoplasm (Fig. 8A, panel 3) whereas expression of *FET3* diminished (Fig. 8B, line 6). We repeated this assay with the second strain, in which the regulatable protein was Grx4, and obtained qualitatively identical results (Fig. 8A, panel 4 and Fig. 8B, line 8). This result indicated that expression of either Grx3 or Grx4 was sufficient to allow the Aft1 translocation from the nucleus to the cytoplasm. We reasoned that if both Grx3 and Grx4 inhibited the importation of Aft1 to the nucleus, the treatment with ferrocene (which induces Aft1 nuclear translocation) in conditions of Grx3 and Grx4 overexpression, should impair Aft1 translocation to the nucleus. To test this hypothesis we simultaneously overexpressed Grx4 and Grx3 in a *grx3grx4* strain. Upon the addition of ferrocene to the culture medium, Aft1 clearly accumulated in the nucleus after 6 hours of treatment (Fig. 9A) correlated to the induction of *FIT3* (Fig. 9B, line 9) and *FET3* (not shown) transcripts, in a similar manner to that observed for wild-type cells (Fig. 9B, line 3). In a *grx3grx4* double mutant, the addition of ferrocene did not produce variations in either patterns of gene expression or Aft1 localisation (as shown in Fig. 9A,B). Moreover, Grx3 and Grx4 protein levels were not affected by ferrocene, as indicated in Fig. 9C, which effectively rules out any possible posttranslational regulation of the two proteins resulting from iron depletion.

These results suggest the possibility that neither Grx3 nor Grx4 regulates Aft1 nuclear import. Our hypothesis, however, is that both proteins negatively regulate Aft1 activity by promoting its nuclear exportation from the nucleus to the cytoplasm. This is further supported by the observation of a more-pronounced inhibition of *FET3* transcription when we simultaneously overexpressed both Grx3 and Grx4 in the *grx3grx4* background (Fig. 8B, line 9).

### Aft1 interacts with both the glutaredoxin and thioredoxin domains

Since both Grx3 and Grx4 present two functional domains, namely Grx, a glutaredoxin-like sequence and Trx, the thioredoxin domain, we decided to investigate the role of these domains in the regulation of Aft1 function. We first performed two-hybrid assays between each of the Grx and Trx domains of Grx3 and Grx4 and Aft1. We found that both Grx and Trx domains from Grx3 or Grx4 physically interacted with Aft1 in the nucleus (Fig. 10A). However, we observed that the full-length Grx3 protein interacted more strongly with Aft1 than each of its single Grx or Trx domains, and that Grx4 increased the binding efficiency between Grx3 and Aft1 (see Fig. 2A,C and Fig. 10A). We conclude not only that Grx3 and Grx4 can independently interact with Aft1 but also that Grx and Trx domains of Grx3 and Grx4 are able to independently bind Aft1

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in the nucleus. We next wondered which of those domains was responsible for the regulation of the Aft1 transcriptional function and according to our model, for the translocation of Aft1 from the nucleus to the cytoplasm. To answer this question we overexpressed each of the pGrx and pTrx domains of Grx3 and Grx4, respectively, in a *grx3grx4* double mutant strain and analysed the levels of *FIT3* and *FET3* mRNAs. We observed that the overexpression of each of the Grx domains from Grx3 and Grx4, significantly diminished the levels of expression of *FIT3* and *FET3* compared with the *grx3grx4* double mutant (Fig. 10B). Following this observation and to investigate which domain was involved in Aft1 translocation from the nucleus to the cytoplasm, we decided to express each of the four domains in the nucleus. For this, we used the following plasmids: pTP17, pTP19, pTP20 and pTP21, which contain a nuclear localisation domain and a strong promoter such as ADH1. The results we obtained clearly indicated that high expression of the Grx domains of both Grx3 and Grx4 in the nucleus induced Aft1 translocation from the nucleus to the cytoplasm in a *grx3grx4* double mutant bearing an Aft1GFP fusion protein (Fig. 10C). However, the overexpression of each of the Trx domains did not cause any variation in the levels of *FIT3* and *FET3* expression compared with that determined in the empty *grx3grx4* double mutant (Fig. 10B) and under these conditions, Aft1 remained localised in the nucleus. We conclude from all these results that both Grx domains from Grx3 and Grx4 are responsible for Aft1 transcriptional function by regulating its translocation from the nucleus to the cytoplasm.

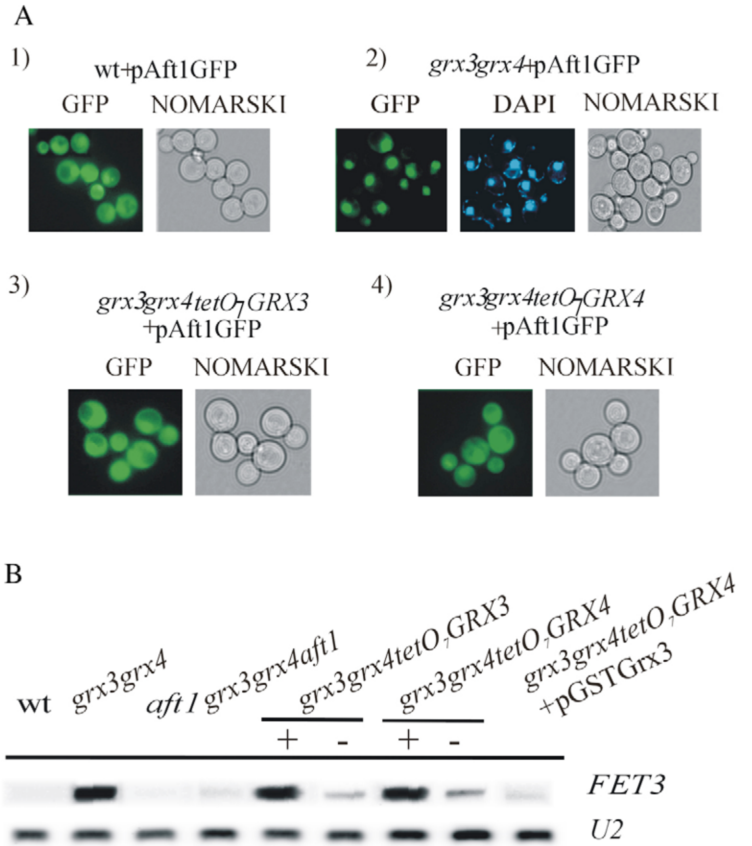


**Fig. 8.** Aft1 nuclear localisation is regulated by both Grx3 and Grx4. (A) GFP-Aft1 fusion protein was visualised by fluorescence microscopy and recorded in different backgrounds. 1. wild type. 2. *grx3grx4* double mutant. 3. *grx3grx4* double mutant overexpressing *GRX3* under the *tet*$O_7$ promoter. 4. *grx3grx4* double mutant overexpressing *GRX4* under the *tet*$O_7$ promoter. (B) Northern blot of *FET3* from mRNA samples collected from the same experiment described in A. To regulate gene expression under the *tet*$O_7$ promoter, we added (+) or not (–) 20 μg/ml doxycycline to the culture media.

## Discussion

In this study we demonstrate that Grx3 and Grx4 regulate the subcellular localisation of Aft1 under conditions in which iron is not a limiting factor. In the absence of both proteins Aft1 localisation is nuclear in iron-rich medium. As discussed below, this function is directly related to the oxidative stress response. Grx3 and Grx4 negatively regulate Aft1 activity. The two proteins have overlapping additive functions, although the absence of either does not significantly affect cell viability under normal conditions or in response to oxidative treatment. Yamaguchi-Iwai et al. (Yamaguchi-Iwai et al., 2002) reported that Aft1 responds to iron concentration by regulating changes in the subcellular localisation of Aft1. Here, we demonstrate that both Grx3 and Grx4 regulate the cellular localisation of Aft1 and that both proteins are required to permit cytoplasmic localisation of Aft1 under iron-rich conditions.

If iron were to signal Aft1 for subcellular compartmentalisation in the absence of both Grx3 and Grx4, we would expect Aft1 to translocate to the cytoplasm. The fact that in the absence of both Grx3 and Grx4 Aft1 is always located in the nucleus, regardless of iron availability, suggests the direct regulation of both Grx3 and Grx4 on Aft1 nuclear localisation. This conclusion is supported by the fact that both monothiol glutaredoxins are always nuclear and also by our evidence of a clear physical interaction between Grx3 and Grx4 and Aft1 in the nucleus. The individual absence of either Grx3 or Grx4 induces a partial translocation of Aft1 from the cytoplasm to the nucleus. As expected, in the *grx3grx4* double mutant, the genes under the control of Aft1 are totally upregulated under all the conditions tested. This observation has been confirmed in a simultaneous study carried out by Ojeda et al. (Ojeda et al., 2006). Furthermore, we demonstrate that this transcriptional upregulation observed in the *grx3grx4* double mutant is totally explained by the Aft1 activity in iron-rich conditions. However, in iron-limiting conditions, upon the addition of ferrocene, our results suggest that in the absence of Aft1, Aft2 activates the transcription of both *FIT3* and *FET3*. This hypothesis derives from the observation that in the *grx3grx4* double mutant the transcription levels of *FIT3* and *FET3* are five times higher than those in both *aft1* and *grx3grx4aft1* mutants upon ferrocene treatment (Fig. 3). In addition, the former model would be in accordance with the recent results published by Courel et al. (Courel et al., 2005) demonstrating that Aft2 acts in the absence of Aft1.

We also demonstrate that the upregulation of the genes dependent on Aft1 activity implies an additional number of cellular consequences: iron accumulates in the *grx3grx4* double

mutant, although the iron concentration is also greater in the two single mutants than in wild-type cells. This high intracellular iron concentration is potentially cytotoxic because it might produce free radicals in the presence of oxygen (Halliwell and Gutteridge, 1988). Apart from this, iron may also induce oxidative stress through the Fenton reaction, which eventually induces the accumulation of ROS (Symons and Gutteridge, 1998; Gutteridge and Halliwell, 2000). This explains why the double mutant is highly sensitive to some oxidative agents such as hydrogen peroxide or t-butylhydroperoxide. Thus, in a double mutant in which iron concentration is high, the addition of hydrogen peroxide would favour the Fenton reaction. The consequence of this would be the generation of endogenous oxidative stress in the double mutant with respect to the wild type. Curiously, the *grx3grx4* double mutant is not sensitive to diamide, an oxidative agent, which, as recently reported (Vilella et al., 2005) mainly oxidises cysteine residues present in cell-surface proteins. This supports the conclusion that iron accumulation in the *grx3grx4* strain activates the Fenton reaction and subsequently causes an accumulation of anion superoxide in the cell. This mechanism resembles the oxidative effect of hydrogen peroxide but not that exerted by diamide. In accordance with our conclusions, Liu et al. (Liu et al., 2005) have recently reported in a genomic study performed with cardiac cells, that the expression of a number of genes responding to oxidative stress was affected by iron.

Oxidative stress induces an accumulation of cells in G1 (Philpott et al., 1998). The double mutant presents a defect in cell growth: its generation time is greater than that estimated for wild-type cells and FACS profiles indicate that the populations of exponentially growing *grx3grx4* cells also accumulate in G1. We have demonstrated that all these effects are the result of Aft1 misregulation, because in the absence of Aft1, cell growth and the FACS profile were restored to wild-type levels.

Recent reports have shown that both Grx3 and Grx4 localise in the nucleus (Molina et al., 2004) and that this localisation is also conserved in other eukaryotic models such as *Schizosaccharomyces pombe* (Chung et al., 2005). Our data demonstrate that iron homeostasis directly depends on Grx3 and Grx4 functions and not vice versa, because Grx3 and Grx4 nuclear localisation do not vary in the absence of Aft1 or under conditions of iron depletion upon treatment with ferrocene (not shown). We have observed that Grx3, Grx4 and Aft1 form a nuclear complex, and that the more Aft1 is localised to the nucleus the greater are the number of molecules that form the complex between the three proteins, Grx3, Grx4 and Aft1. Therefore Aft1 binding to Grx3 and Grx4 is determined by their nuclear localisation. However, binding of Grx3 to Grx4, Grx4 to Aft1 and Grx3 to Aft1 does not depend of the third partner of the complex. Aft1 binds to the glutaredoxin (Grx) and the thioredoxin (Trx) domains of Grx3 and Grx4 proteins. We have also demonstrated that Grx3 and Grx4 also interact each other in vivo and in vitro in the nucleus, and that this interaction does not depend on iron availability or on the presence of Aft1. It is probable that the respective functions of both Grx3 and Grx4 could be determined by the formation of a nuclear heterodimer. However, the biological meaning of the physical interactions between these proteins and the nature of the biochemical structure of the complexes deserves deeper future analyses.

It has been reported that Pse1 positively regulates Aft1 activity by regulating nuclear import of Aft1 independently of the Aft1 regulation resulting from iron starvation (Ueta et al., 2003). Furthermore, glucose exhaustion also positively regulates a set of genes through Aft1 (Haurie et al., 2003). In fact, these authors argue that the Snf1/Snf4 kinase pathway positively regulates the iron uptake pathway at the diauxic shift. Apart from this, only one report describes the negative regulation of Aft1 by Tpk2 (Robertson et al., 2000). In this study the authors demonstrate, by means of a genome-wide transcriptional profiling, that Tpk2, a PKA catalytic subunit, negatively regulates genes involved in iron uptake, whose transcriptional induction depends on Aft1. However, our study demonstrates a genetic, physical and functional interaction between Aft1 and two negative regulatory proteins: Grx3 and Grx4, and also the clear implication of this regulation in the endogenous oxidative status of the cell.

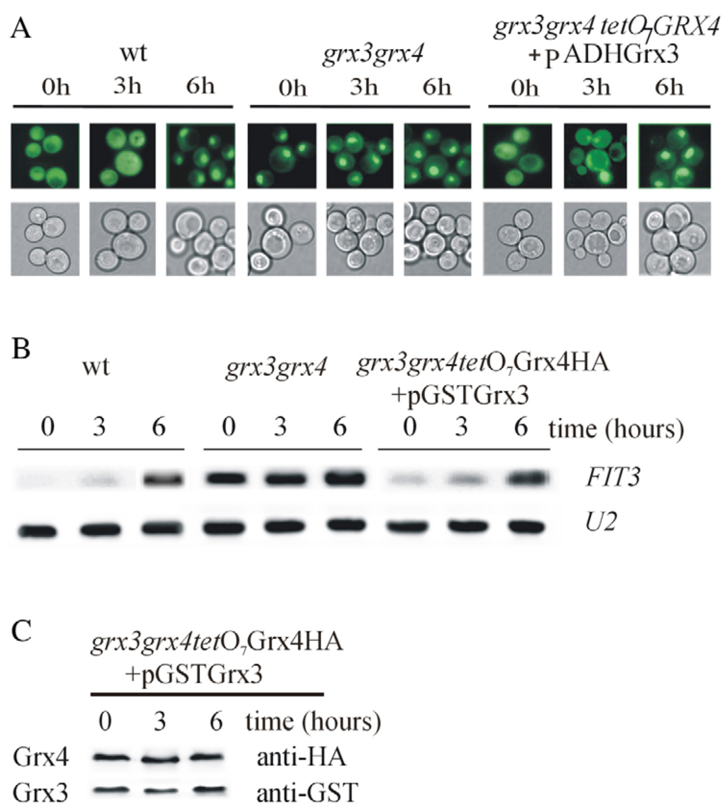


**Fig. 9.** Aft1 nuclear translocation mediated by iron deficit is independent of the Aft1 nuclear localisation mediated by both Grx3 and Grx4. (A) GFPAft1 localisation in the different strains: wild type, *grx3grx4* and *grx3grx4tet*$O_7$*GRX4*+pADH1Grx3, upon the addition of 2 mM ferrocene to exponentially growing cells at 30°C in SD medium. (B) Northern blot of *FIT3* in mRNA samples extracted from aliquots collected from wild type, *grx3grx4* and *grx3grx4tet*$O_7$*GRX4*+pGSTGrx3 upon the addition of 2 mM ferrocene to exponentially growing cells at 30°C in SD medium. (C) Western blot of total protein extracts prepared from the previous experiment. We used anti-HA antibody to detect Grx4 protein and anti-GST antibody to detect Grx3 protein.

One argument supporting the hypothesis that Grx3 and Grx4 regulate the export of Aft1 from the nucleus is that in a *pse1* mutant Aft1 cannot be imported to the nucleus and the absence of iron is unable to induce Aft1 nuclear translocation (Ueta et al., 2003). If Grx3 and Grx4 function were to negatively regulate Aft1 importation into the nucleus from the cytoplasm, we would expect Grx3 or Grx4 overexpression to avoid Aft1 nuclear translocation from the cytoplasm in iron-limiting conditions. However, we observed that this was not the case because Aft1 translocates to the nucleus under iron-limiting conditions, upon the addition of ferrocene, and even under conditions of Grx3 or Grx4 overexpression. Our hypothesis points to a role for Grx3 and Grx4 in Aft1 nuclear exportation.

Grx3 and Grx4 present two functional domains whose cellular localisation has been described (Molina et al., 2004). The glutaredoxin domain has a nuclear-cytoplasmic localisation, whereas the thioredoxin domain targets the protein to the nucleus. We present evidence demonstrating that the glutaredoxin domain of either Grx3 or Grx4 is the one responsible for the regulation of a normal Aft1 transcriptional function in cells growing exponentially in rich medium. Consequently, the Grx domain drives a normal Aft1 translocation from the nucleus to the cytoplasm in iron-rich conditions. However, based on our unpublished results, either a complete Grx3 or Grx4 protein is required to restore cell viability to wild-type levels in response to peroxides, meaning that each of the Trx domains of Grx3 and Grx4 also play an essential role in the cellular response to oxidative stress. Also evident from our results is that Grx3 and Grx4 work cooperatively and additively and although one partly compensates the lack of function of the other, together both work more efficiently in their Aft1 regulatory function.

In summary, in this study we present a model for endogenous oxidative stress induced by the misregulation of iron homeostasis resulting from the absence of Grx3 and Grx4 function. We also characterise a novel function for Grx3 and Grx4 in the cellular defence against oxidative stress and also in the correct compartmentalisation of Aft1 ensuring iron stress detoxification.

A

| AC | BD | β-Galactosidase arbitrary units |
|---|---|---|
| *AFT1* | Dom*Trx(GRX3)* | 1.337 |
| *AFT1* | Dom*Grx(GRX3)* | 1.442 |
| *AFT1* | Dom*Trx(GRX4)* | 1.395 |
| *AFT1* | Dom*Grx(GRX4)* | 1.507 |
| *Controls* | | |
| *e.v* | *e.v* | 0.296 |
| *SNF4* | *SNF1* | 3.810 (+) |
| *YAK* | *GRX5* | -0.040 ( - ) |
| *AFT1* | *e.v* | 0.332 |
| *e.v* | *GRX3* | 0.564 |
| *e.v* | *GRX4* | 0.324 |
| *AFT1* | *GRX3* | 3.789 |
| *AFT1* | *GRX4* | 1.567 |
| *e.v* | Dom*Trx(GRX3)* | 0.532 |
| *e.v* | Dom*Grx(GRX3)* | 0.309 |
| *e.v* | Dom*Trx(GRX4)* | 0.302 |
| *e.v* | Dom*Grx(GRX4)* | 0.243 |

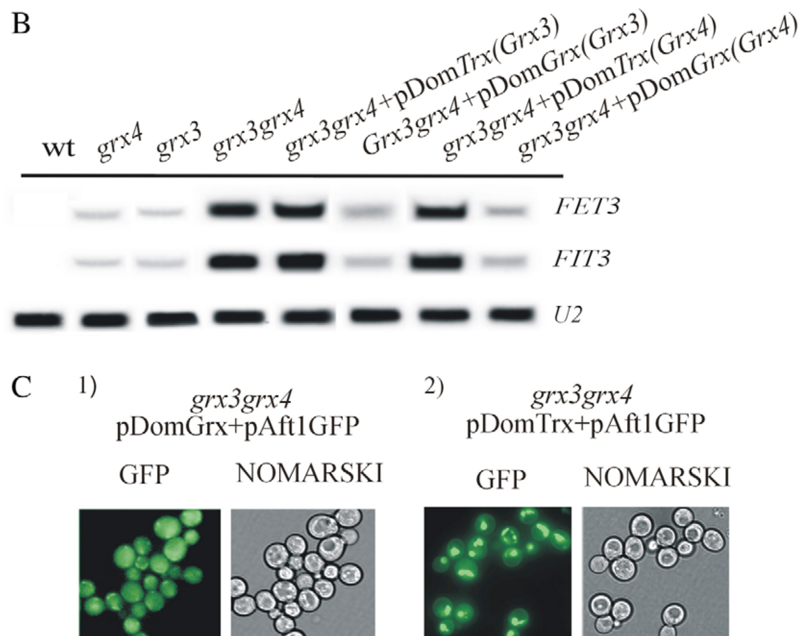


**Fig. 10.** Aft1 interacts with both Grx and Trx domains of Grx3 and Grx4, but the Grx domains of Grx3 or Grx4 are responsible for Aft1 transcriptional function and cellular compartmentalisation. (A) Two-hybrid analysis for the following interactions: Aft1 with the Grx domain of Grx3, Aft1 with the Trx domain of Grx3, Aft1 with the Grx domain of Grx4 and Aft1 with the Trx domain of Grx4, determined as in Fig. 2A and Fig. 2C. (B) Northern blot analysis of *FET3* and *FIT3* expression in samples taken from cultures of the following strains: wild type, *grx3grx4*, *grx3grx4*+pMM491(pDomGrx of Grx3) (Dom, domain), *grx3grx4*+pMM486 (pDomTrx of Grx3), *grx3grx4*+ pMM506 (pDomGrx of Grx4) and grxgrx4+pMM502 (pDomTrx of Grx4). Cells were exponentially grown in SD at 30°C. (C) GFPAft1 localisation in the *grx3grx4* strain co-transformed with pTP20 (pDomGrx, identical results were observed with pTP19) or with pTP17 (pDomTrx, identical results were observed with pTP21) in cultures growing exponentially at 30°C in SD medium.

## Materials and Methods

### Media and growth conditions

Yeasts were grown in SC or SD medium (2% glucose, 0.67% yeast nitrogen base and the required amino acids) (Kaiser et al., 1994). Diamide, hydrogen peroxide and t-butylhydroperoxide were purchased from Sigma. Diamide was dissolved in DMSO. Both peroxides were prepared in sterile distilled water. Ferrocene (Sigma) was added directly to the culture medium.

### Yeast strains and gene disruptions

Yeast strains used in this study are listed in supplementary material Table S1. *GRX4* was disrupted by the one-step disruption method using the *kan*MX4 module (Wach et al., 1994), whereas the *GRX3* gene was disrupted using the *nat*MX4 module (Goldstein and McCusker, 1999).

### DNA manipulation and plasmids

We used the pHW4 vector (a gift from Pascual Sanz, Instituto de Biomedicine, Valencia, Spain), containing the ADH1 promoter and the GST sequence, to clone each of the sequences of interest and obtain the corresponding fusion proteins. The plasmid pMM470 was obtained by cloning Grx3 into the *Eco*RI and *Sma*I sites of

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pHW4 after amplifying Grx3 from genomic DNA using the oligos MMO455 and MMO456. The plasmid pMM483 contains the Grx4 ORF amplified with MMO521 and MMO522, and cloned into the *Eco*RI and *Sal*I sites of the pHW4 vector.

For two-hybrid analysis we constructed the following plasmids: pACTII-*GRX3*, *GRX3* was amplified by PCR with MMO453 and MMO454 to be cloned in the *Sma*I and *Eco*RI sites. pACTII-*GRX4* was cloned following the same strategy as with *GRX3* but the oligos designed were MMO523 and MMO524. For constructing pACTII-*AFT1*, AFT1 was amplified by means of MMO457 and MMO458, and subsequently cloned into the *Sma*I-*Xho*I sites. To obtain pGBT9-*GRX3* and pGBT9-*GRX4* plasmids, we amplified the two sequences from genomic DNA, respectively. Thus, MMO455 and MMO456 were used to amplify *GRX3*, which was then cloned into the *Eco*RI-*Sma*I sites. MMO170 and MMO171 were designed to amplify *GRX4* to be cloned into the *Pst*I-*Eco*RI sites.

To construct pTP17, containing the Trx domain of Grx3 in pGBT9 we used MMO455 and MMO492 oligos bearing the *Eco*RI-*Sam*I sites. The plasmid pTP20 contains the Grx domain of Grx3 cloned into pGBT9 in *Eco*RI-*Sma*I restrictions sites using MMO494 and MMO456. The plasmid pTP21 carries the Trx domain of Grx4 cloned into pGBT9 in *Eco*RI-*Sma*I sites by means of MMO170 and GSLOL51. The vector pTP19 bears the Grx domain of Grx4 constructed in pGBT9 in *Eco*RI-*Pst*I sites by using GSLOL52 and MMO171. As a control for positive nuclear interaction we used the pair of plasmids pACT2*SNF4* and pGBT9*SNF1* (see Vilella et al., 2004). And finally pACT2*YAK1* and pGBT9*GRX5* were used as a negative control for nuclear interaction (Vilella et al., 2004). The plasmid pMM351 is an integrative vector that contains three repetitions of the hemagglutinin (HA) epitope in the C-terminal position and also bears the regulatable *tet*$O_7$ promoter. *Eco*RV was used to linearise this vector to make it integrative at the *LEU3* locus. The following plasmids are derivatives of the former one upon cloning *AFT1*, *GRX3* and *GRX4*, ORFS, respectively as detailed: pMM358 carries the *AFT1* ORF cloned by using *Eco*RI and *Pst*I sites using GSLOL31 and GSLOL32 oligos. pMM367 bears *GRX3* ORF inserted at *Not*I and *Pst*I restriction sites using GSLOL33 and GSLOL34. pMM518 carries *GRX4* cloned at *Not*I and *Pst*1 sites by using MMO543 and MMO583. The plasmid pCM265 is a centromeric vector containing three repetitions of the HA epitope in C terminal position and the regulatable *tet*$O_7$ promoter (de la Torre-Ruiz et al., 2002). The following plasmids are derivatives of the former one upon cloning either the Grx or Trx domains of Grx3 and Grx4, respectively, into the *Pme*I-*Not*I restriction sites, as detailed: pMM491 bears the Grx domain of Grx3. pMM486 carries the Trx domain of Grx3. pMM506 bears the Grx domain of Grx4 and pMM502 carries the Trx domain of Grx4. All the oligonucleotides listed here are detailed in supplementary material Table S2.

### Total iron determination

The total iron concentration in the different strains was determined in exponentially growing cultures as described (Fish, 1988).

### Fluorescence-activated cell sorting (FACS)

This approach was used to characterise the DNA content in exponentially growing cells (de la Torre-Ruiz et al., 2002).

### Two-hybrid assays

Two-hybrid analyses were carried out basically as described in (Rodriguez-Navarro et al., 2002) using (1) the pACT2 plasmid, which contains the *ADH1* promoter and the *GAL4*-activating domain. To construct a hybrid protein, genes encoding the protein of interest were appropriately cloned, in frame, into the MCS sited located just after *GAL4*AD (for activating domain), and (2) the pGBT9 vector, which contains the *ADH1* promoter followed by the *GAL4*BD (binding domain) and a MCS in which each protein of interest was cloned. The targeting of each hybrid protein was achieved using specific nuclear localisation sequences contained in the *GAL4*-bindinng domain.

### In vivo cellular localisation of proteins

To study Aft1 subcellular localisation we cloned *AFT1* into the *Cla*I-*Eco*RI sites of the vector pUG35 (a gift from Dr J. Hegemann, Institute für Mikrobiologie, Düsseldorf, Germany) using both MMO420 and MMO421. pUG35 vector contains the green fluorescence protein yEGFP3 and the repressible Met25 promoter. We regulate protein expression levels by adding methionine to the culture medium to a final concentration of 25 μM.

### Pull-down assays and total protein extraction

Both methods were carried out as described by Garcia-Gimeno et al. (Garcia-Gimeno et al., 2003). Proteins retained by the affinity system were detected by immunoblot assays using anti-GST polyclonal antibody (Amersham) or anti-HA monoclonal antibody, and chemiluminescence reagents (Supersignal, Pierce)

### RNA preparation and northern blot analyses

RNA purification, northern blot and probe labelling with digoxygenin were carried out according to Gallego et al. (Gallego et al., 1997). Probes covering the entire open reading frame, without adjacent sequences, were generated by PCR from genomic DNA.

### Yeast extracts and immunoblot analyses

Both methods were performed as described in de la Torre-Ruiz et al. (de la Torre-Ruiz et al., 2002).

We would like to acknowledge Pascual Sanz for plasmid pHW4 and the protocol for pull-down assays. J. Hegemann for providing the pUG35 vector. We would like to mention Lidia Piedrafita for the excellent technical assistance. This work was supported by the project from the Instituto de Salud Carlos III (Spanish Ministry of Health and Consumption, PI030734). N.P.C. was granted a fellowship from the Generalitat de Catalunya (Spain).

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