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

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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., 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).

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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

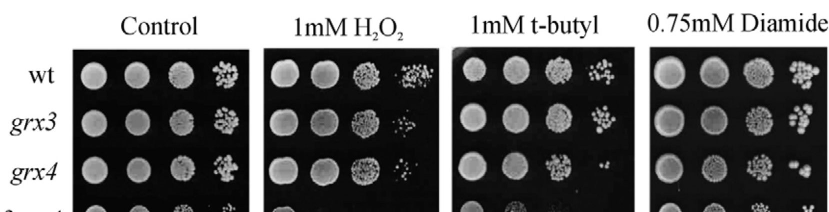

Fig. 1. Grx3 and Grx4 are required for survival upon treatment with hydrogen peroxide and t-butylhydroperoxide. Exponentially growing cells from wild-type, *grx3*, *grx4* and *grx3grx4* strains were harvested, serially diluted and spotted onto control SD plates or on SD plates containing 1 mM H_2O_2,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

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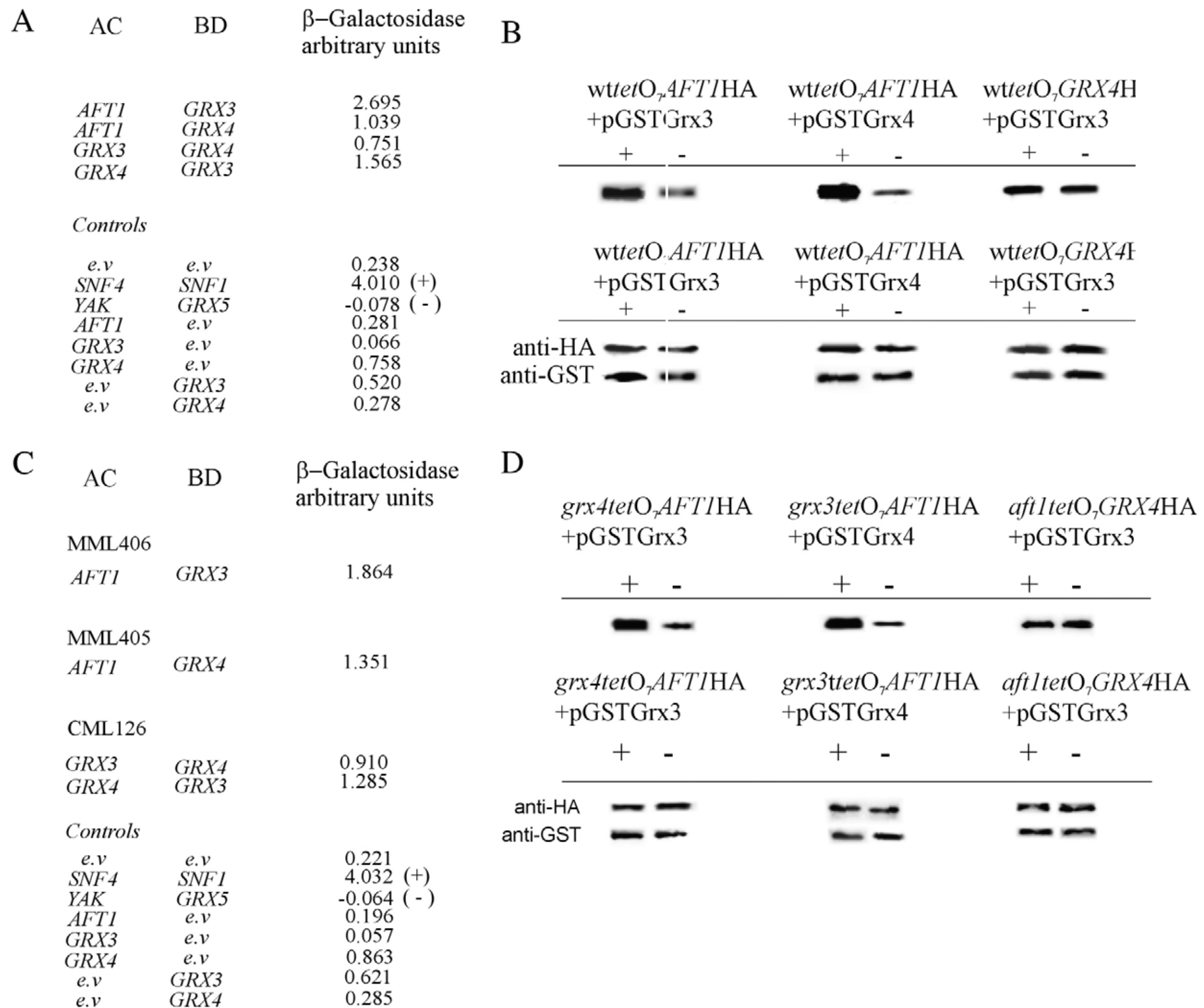

Fig. 2. In vivo and in vitro interaction of Grx3 and Aft1, Grx4 and Aft1 and Grx3 and Grx4. (A) Two-hybrid analyses for the following interactions: Grx3 with Aft1, Grx4 with Aft1 and Grx3 with Grx4. Values for interaction between *SNF4* and *SNF1* were used as a strong positive control for nuclear interaction (+). 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

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

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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_7Grx3HA, *grx3grx4tet*O_7Grx4HA, *grx3grx4tet*O_7Grx3HA+pGSTGrx4 and *tet*O_7Grx4HA+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

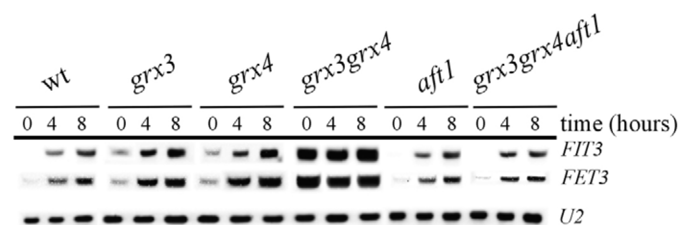

Fig. 3. Grx3 and Grx4 negatively regulate the expression of *FIT3* and *FET3* in a manner dependent on Aft1 activity. Cells from the following strains: wild type, *grx3*, *grx4*, *grx3grx4*, *aft1* and *grx3grx4aft1*, were exponentially grown in SD medium plus amino acids, at 30°C, then treated with 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,

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

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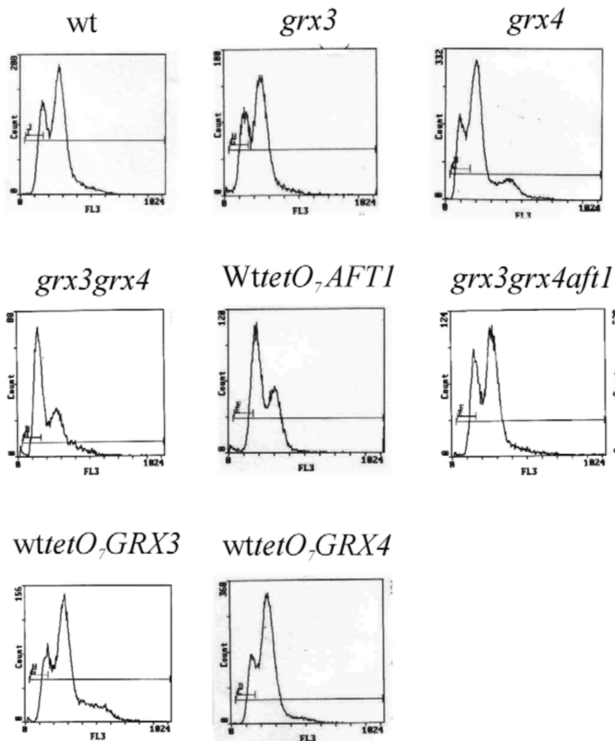

Fig. 5. The simultaneous absence of Grx3 and Grx4 induces accumulation of cells in G1. FACS profiles of different strains growing exponentially in SD medium plus amino acids. The strains are: wild type, *grx3*, *grx4*, *grx3grx4*, wild type overexpressing Aft1, *grx3grx4aft1*, wild type overexpressing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

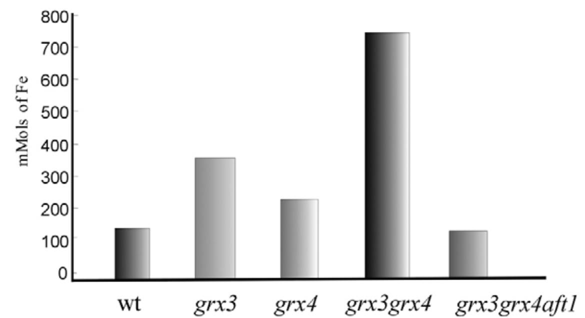

Fig. 6. Total iron accumulates in the cell in the absence of Grx3 and Grx4. Total iron concentration was spectrophotometrically determined as described in the Materials and Methods in exponentially growing cultures of wild-type, *grx3*, *grx4*, *grx3grx4* and *grx3grx4aft1* strains. Numerical values 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

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

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