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Yeast glutaredoxin, GRX4, functions as a glutathione S-transferase required for red ade pigment formation in *Saccharomyces cerevisiae*

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The adenine biosynthetic mutants ade1 and ade2 of *Saccharomyces cerevisiae* accumulate a characteristic red pigment in their vacuoles under adenine limiting conditions. This red pigmentation phenotype, widely used in a variety of genetic screens and assays, is the end product of a glutathione-mediated detoxification pathway, where the glutathione conjugates are transported into the vacuole. The glutathione conjugation step, however, has still remained unsolved. We show here, following a detailed analysis of all the members of the thioredoxin-fold superfamily, the involvement of the monothiol glutaredoxin GRX4 as essential for pigmentation. GRX4 plays multiple roles in the cell, and we show that the role in ade pigmentation does not derive from its regulatory role of the iron transcription factor, Aft1p, but a newly identified GST activity of the protein that we could demonstrate using purified Grx4p. Further, we demonstrate that the GRX domain of GRX4 and its active site cysteine C171 is critical for this activity. The findings thus solve a decades old enigma on a critical step in the formation of this red pigmentation.

Keywords. Glutathione; glutathione conjugation; glutathione S-transferase; GRX4; ade pigmentation; detoxification

1. Introduction

Mutations in the *ADE1* or *ADE2* genes of the yeast *Saccharomyces cerevisiae* accumulate a characteristic red pigment in the yeast vacuole under adenine limiting conditions. This red pigment is one of the earliest known phenotypes of yeast genetics (Roman 1956), and yet its formation is still only partially understood. The phenotype continues to be widely used in the field of yeast genetics in a variety of genetic screens or strategies (Weng and Nickoloff 1997; Barbour and Xiao 2006; Bharathi *et al.* 2016). The yeast *ADE1* and *ADE2* genes encode two enzymes in the multistep adenine biosynthetic pathway. *ADE1* codes for the enzyme *N*-succinyl-5-aminoimidazole-4-carboxamide riboside synthetase, while *ADE2* encodes the enzyme Phosphoribosylaminoimidazole carboxylase (supplementary figure 1) (Jones and Fink 1982).

During conditions of excess adenine in the medium, the adenine biosynthetic pathway is repressed. However during adenine deficiency in the medium, the pathway is derepressed, and the pathway proceeds to make adenine, an essential cellular nutrient. Under these conditions, when *ADE1* or *ADE2* are mutated, the precursors phosphoribosylaminoimidazole (AIR) and phosphoribosylaminoimidazole carboxylate (CAIR) accumulate. These metabolites are known to be toxic for the cells (Ishiguro 1989; Kalinina *et al.* 2014). Genetic studies have revealed that to remove these metabolic intermediates from the cytosol, the cells require glutathione and that the red pigment is an end product of a glutathione mediated detoxification

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pathway (Chaudhuri *et al.* 1997). In this detoxification pathway, toxic metabolites and xenobiotics are conjugated to glutathione and removed from the cytosol by glutathione conjugate pumps. Members of the ABC subfamily, the MRP family are glutathione conjugate transporters, and in yeast these are located at the vacuolar membrane (Li *et al.* 1996; Sharma *et al.* 2002). YCF1 and BPT1 are the glutathione conjugate transporters, along with some secondary efflux pumps, BAT1 (Sharma *et al.* 2003). Thus the efflux pumps and their contribution have been fairly well characterized in terms of the genes involved.

In the detoxification pathway, very little is known on the initial step of the conjugation of the toxic metabolites to glutathione in yeast. We have thus attempted to investigate this first step in the detoxification pathway, the conjugation to glutathione. As members of the thioredoxin superfamily were most likely to be involved in this conjugation process, we have taken an unbiased approach where deletions of all members of this family in yeast were evaluated on the consequences of pigmentation in *ade1Δ* and *ade2Δ* backgrounds. Our results reveal that the two-domain monothiol glutaredoxin, GRX4, is critical for the pigmentation. In addition to containing both thioredoxin and glutaredoxin domains, GRX4 also has an unusual regulatory role in the regulation of iron homeostasis. Studies describe hear reveal that the role of GRX4 in this process is not through its regulatory control on AFT1, but through the GST activity of the protein mediated by the GRX domain of GRX4

2. Materials and methods

2.1 *Chemicals and reagents*

All chemicals used were obtained from commercial sources and were of analytical grade. Media components, fine chemicals and reagents were purchased from Sigma Aldrich (St. Louis, USA), HiMedia (Mumbai, India), Merck. India Ltd. (Mumbai, India), USB Corporation (Ohio, USA) or Difco, USA. Oligonucleotides (primers) were designed using SnapGene (version 2.4.3) software and were purchased from Integrated DNA Technologies (IDT). Enzymes (Restriction enzymes, T4 DNA ligase, Calf Intestinal Phosphatase (CIP), Antarctic phosphatase, *Vent* DNA polymerase, *Phusion* High-fidelity DNA polymerase and other modifying enzymes), their buffers, dNTPs, DNA and protein molecular weight markers were purchased from New England Biolabs Inc, (Beverly, MA, USA) or Thermo Scientific. Gel-extraction kits and plasmid miniprep columns were obtained from BioNEER and Promega. Glutathione was obtained from Sigma-Aldrich, Western Blotting Detection Reagents were purchased from Amersham Biosciences GE Healthcare (UK). Anti-HA mouse Tag polyclonal antibody was bought from Cell Signaling (Danvers, MA, USA).

2.2 *Cell growth and spotting*

All *S. cerevisiae* strains were routinely maintained on YPD medium. Minimal media for *S. cerevisiae* was SD medium with supplements containing appropriate nutrients. Cells were grown at 30°C. For adenine-limited plates, the adenine concentration in the minimal medium was 8 mg/L (instead of 75 mg/L), and in YPD plates, yeast extract was reduced from 1 to 0.2%.

2.2.1 *Growth and maintenance of bacteria and yeast strains:* The *E. coli* DH5α strains were routinely grown in LB medium at 37°C. *E. coli* transformants were selected and maintained on LB medium supplemented with ampicillin.

The *S. cerevisiae* strains were regularly maintained on YPD medium and grown at 28–30°C. The yeast transformants were selected and maintained on SD medium with amino acid supplements as per requirements.

2.2.2 *Growth and pigmentation visualization by spotting:* For dilution spotting, yeast cells were grown in selective minimal medium. Primary cultures were grown for 12–16 h at 30°C, with shaking at 200 rpm. Primary cultures were used to inoculate a secondary culture in the selective minimal medium for 8 hours or till OD_{600} reaches between 0.6–0.8 at 30°C. Cells were centrifuged, washed with sterile water and resuspended into sterile water at OD_{600} = 0.2. Four serial dilutions were made in sterile water; OD_{600} = 0.2, 0.02, 0.002, 0.0002. 10 µL of each of these cell suspensions were spotted on the selective minimal medium plates. Plates were incubated at 30°C for 2–5 days and photographs were taken.

2.3 *Strains and plasmids*

Escherichia coli DH5α was used as the cloning host. BL21 (DE3) was used as expression host. The genotype for the *E. coli* and *S. cerevisiae* strains used in the study are given in supplementary table 1. The list of

various plasmids used in this study is given in supplementary table 2.

2.4 *Cloning of GRX3,GRX4 and the TRX and GRX domains of GRX4*

The *GRX3* and *GRX4* genes were PCR amplified from yeast genomic DNA using their respective primers and cloned into the yeast expression vector pRS315 TEF. The TRX domain of GRX4 (residues 8–106) and the GRX domain of GRX4 (residues 149–233) were also amplified by separate primers. A stop codon was included in the reverse primer of the TRX domain amplification, and an ATG codon was included in the forward primer of the GRX domain cloning. The amplified products were cloned and expressed downstream of the TEF promoters as well downstream of the CYC promoters. Site-directed mutagenesis was performed using the Splice Overlap Extension PCR (SOE PCR) method. The mutant created at position C171A in GRX domain of GRX4 was sequenced to confirm the presence of the desired nucleotide change.

GRX4 was also cloned with a His-tag into the *E. coli* expression vector pET23a. Primers used for cloning are listed in supplementary table 3.

2.5 *Construction of ADE1 and ADE2 deletions in different backgrounds*

The *ade1*::*URA3* and the *ade2*:: *HIS3* disruption cassette were created by PCR using primers designed to amplify *URA3* from pRS416 and *HIS3* from pRS313 with flanking regions on the primer corresponding to *ADE1* and ADE2 respectively. The PCR amplified products were purified by agarose gel electrophoresis followed by elution of the required DNA band. The different *S. cerevisiae* deletion strains were transformed with these disruption cassettes and selected on minimal media lacking histidine and uracil. Successful disruptants were confirmed by adenine auxotrophy.

2.6 *GRX4p purification*

2.6.1 *Recombinant expression and purification of His-tagged Grx4 protein in E. coli:* pET23a(+)-6X His-Grx4 was transformed in BL21 strain and transformants were plated on selection media The primary culture was grown overnight in LB media containing 100 μg/mL ampicillin for selection. The secondary culture was inoculated at 0.05 $O.D_{600}$ and allowed to grow until $O.D_{600}$ reached 0.6–0.8. The culture was induced using 1 mM IPTG and kept at 18°C for 18 h at 200 rpm.

The cells were harvested by centrifugation at 2500*g* for 10 min at 4°C. The cells were lysed by sonication using lysis buffer, pH 7.4 (50 mM Sodium phosphate monobasic, 300 mM NaCl, 5 mM Imidazole and 1 mM PMSF) with 10 s on and 15 s off cycle and 20% amplitude. The cell lysate was centrifuged at 10000 rpm for 30 min at 4°C. The supernatant obtained was incubated with washed Ni-NTA beads for 1 h at 4°C. The supernatant-bead slurry was centrifuged at 2700*g*, 4°C; the supernatant was aspirated and protein-bound beads were collected. Beads were subjected to three washes (centrifuged at 8000*g*, 4°C with wash buffer containing 50 mM Sodium phosphate monobasic, 300 mM NaCl, 20 mM Imidazole and 1 mM PMSF. The specific protein bound to beads was eluted in elution buffer containing 50 mM Sodium phosphate monobasic, 300 mM NaCl, 250 mM imidazole and 1 mM PMSF by centrifugation at 8000*g*, 4°C followed by collection of eluate which contains specific protein of interest. The protein fractions obtained were run on 10% SDS-PAGE to check the purity of the protein. The protein was flash-frozen in liquid nitrogen and stored at −80°C.

2.6.2 *Protein concentration and quantification:* The concentration of protein in the samples was estimated using Bradford reagent. Protein estimation was also carried out by scanning the protein at A_{280} and concentration was calculated using the extension coefficient of a protein.

2.7 *Measurement of GST activity of GRX4*

The glutathione S-transferase (GST) activity was assayed using the standard GST substrate 1-Chloro-2,4-dinitrobenzene (CDNB) (Gronwald and Plaisance 1998). The activity was assayed by the increase in the absorbance at A_{340} nm. Between 5–10 μg of purified Grx4p was used for the assay. The CDNB buffer contained 0.1 M potassium phosphate monobasic buffer, potassium hydroxide (1 M) to adjust the pH to 6.5, 1 mM EDTA. A total volume of the 1 mL reaction was set up and followed up for 10 min. The linearity of the reaction was determined by plotting the absorbance values against time.

3. Results

3.1 *Evaluation of the role of different thioredoxin superfamily members in ade pigment formation*

Glutathione S-transferases which are involved in the enzymatic conjugation of glutathione to metabolites and xenobiotics, are a large family of proteins that include a large number of classes and subclasses (Salinas and Wong 1999). In the yeast *Saccharomyces cerevisiae* there are a total of five GSTs that includes the three proteins GTO1, GTO2 and GTO3 that belong to the omega subfamily (Garcerá *et al.* 2006) and two uncanonical GSTs, GTT1 and GTT2 which are not classifiable into standard classes (Choi *et al.* 1998). The GSTs are a member of the larger thioredoxin-fold superfamily that includes the thioredoxins, glutaredoxins, sulfaredoxins, and the peroxiredoxins (Atkinson and Babbitt 2009). Interestingly many of these also play a role in glutathionylation and deglutathionylation (Greetham *et al.* 2010) and the glutaredoxins GRX1 and GRX2 also have GST activity (Collinson and Grant 2003). To examine these different proteins, we decided to take an unbiased approach and investigate the complete thioredoxin superfamily for their possible role in the ade pigmentation phenotype. This included the GTT1 and GTT2 proteins that were previously suggested not to play a role in the ade pigmentation process (Sharma *et al.* 2003). A total of 17 GSTs/GST-like proteins were identified including the glutaredoxin family (GRX1, GRX2, GRX3, GRX4, GRX5, GRX6 and GRX7), the thioredoxin family (TRX1, TRX2 and TRX3), GSTs (GTT1 and GTT2), sulfiredoxin (SRX1), peroxiredoxin (PRX1), the omega class GSTs (GTO1, GTO2 and GTO3), and the non-standard class GSTs (GTT1, GTT2). While the majority of these are cytosolically localized, some are mitochondrial, some in the ER, and some in the nucleus. As dual localization was also possible, we decided to subject all 17 genes to the analysis.

We procured knockouts of all these 17 genes from Euroscarf. In these deletion backgrounds, we separately deleted the *ADE1* and *ADE2* genes independently to obtain two different sets of deletions, one in the *ade1Δ* background, and one in the *ade2Δ* background. PCR-based ADE1::URA3 disruption cassette and ADE2::HIS3 disruption cassette were used to create the disruptions. The use of both ADE1 disruptions and ADE2 disruptions in the analysis was to ensure that the results were not specific to any one of the metabolites, AIR or CAIR. Further, the use of 2 different makers for the disruption, in one case URA3 and in the other case HIS3 was to enable us to conclude that the phenotype was not dependant on the nutritional state based on a particular marker, since the adenine pathway intersects with other pathways (Rébora *et al.* 2005), and ade-pigmentation is known to be otherwise affected by a large number of environmental and other factors (Dorfman 1969).

Following transformation with the disruption cassettes, the transformants were selected on medium containing adenine but lacking either URA (uracil) or HIS (histidine) (depending on the marker being used). Colonies were picked up and correct disruptants were selected based on their inability to grow on adenine (as these were expected to be adenine auxotroph). These disruptants were then analyzed under different conditions for growth and pigmentation.

With excess adenine in the media all the disruptions are white (figure 1A and B). In the absence of adenine in the media, no growth is seen confirming the adenine auxotrophy. Only under limiting adenine conditions (8 mg/L) could the typical ade pigmentation become apparent. The different mutants showed slight variations in color (figure 2A and B).

In the case of *grx4Δ,* there was no red pigmentation seen in both the *ade1Δ* and *ade2Δ* backgrounds. The *grx4Δ* colonies were almost completely white even in adenine limiting media (8 mg/L). These observations were common to both *ade1Δ* and *ade2Δ* backgrounds.

A few white colored micro-colonies emerged from some of the pink or red coloured colonies in the *grx5Δ* and *prx1Δ* backgrounds. These are most likely mitochondrial petites since both GRX5 and PRX1 have mitochondrial functions, and thus their deletions were possibly accentuating the spontaneous petite mutations formation in these backgrounds.

3.2 *Monothiol glutaredoxin GRX4 is required for the ade-pigment formation*

The *grx4Δ* strain consistently showed an absence of colour, in both *ade1Δ* and *ade2Δ* backgrounds even in the adenine limiting medium at 8 mg/L. To examine if lack of colour seen in the *grx4Δ* strains at 8 mg/L might be a consequence of the adenine threshold for pigmentation being different in the *grx4Δ*, we evaluated the pigmentation of all the mutants over a range of lower adenine concentrations. This was to examine whether at lower adenine concentrations some pigmentation might be visible in the *grx4Δ* background. We evaluated the pigmentation concentrations from 1 to 5 mg of added adenine in the medium. However at

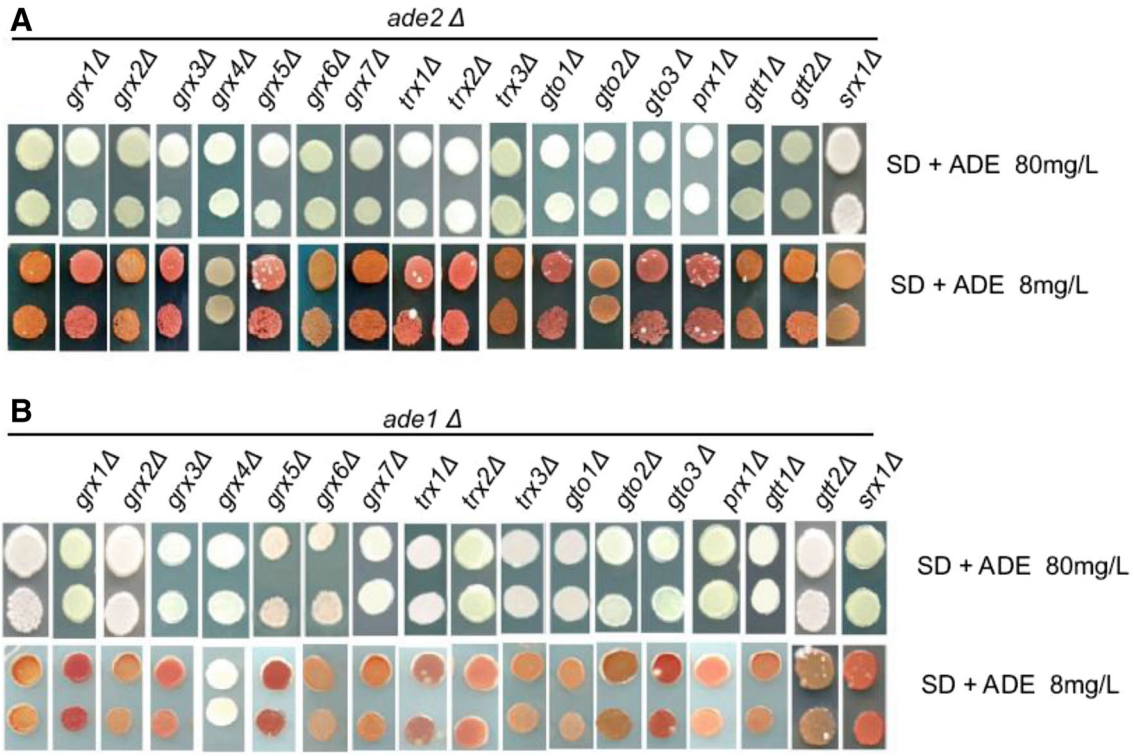

Figure 1. Red pigmentation phenotype after deletion of the member of the thioredoxin superfamily under limiting adenine condition: (**A**) *ade2Δ* background, (**B**) *ade1Δ* background. Two serial dilutions of these mutants were made in sterile water; OD_{600} = 0.2, and 0.02, 10 µl of each of these cell suspensions were spotted on limited adenine conditions.

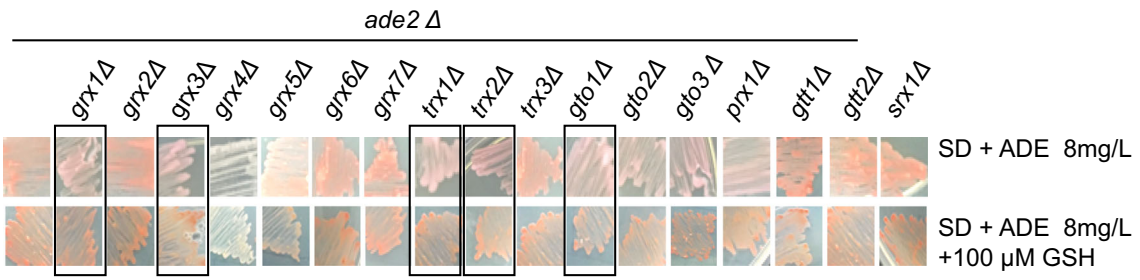

Figure 2. The effect of supplementing GSH on the red pigmentation in an *ade2Δ*background. The different deletions were grown in SD medium containing adenine at 8mg/l and supplemented with external glutathione (100 µM).

none of the concentrations could any pigmentation be observed in the *grx4Δ* strain and in fact at lower concentrations growth also became quite reduced in all the strains (data not shown).

Many of the members of the thioredoxin superfamily, especially the thioredoxins and glutaredoxins are part of the redox systems of the cell and have overlapping functions with glutathione (Grant 2001; Greetham *et al.* 2010). Glutathione has an essential function in mitochondrial iron-cluster formation, in addition to its bulk functions of redox and detoxification (Kumar *et al.* 2011). It was probable therefore, that the different deletions might divert some of the GSH towards more essential functions rather than in the detoxification function and in this way might have an impact on the levels of pigmentation. Increasing glutathione levels, would in such a case, increase the pigmentation. We were particularly interested to see if this might impart some pigmentation to the *grx4Δ* strain. We thus evaluated the deletion mutants under limiting ade conditions but which were supplemented with extra external glutathione. We observed that almost all the strains which were pink in colour became more red when supplemented with glutathione. However, in the case of

grx4Δ, even addition of glutathione did not impart any pigmentation (figure 2). The studies revealed that while the addition of glutathione to the medium enhances pigmentation levels, in the case of the *grx4Δ* strains, there was still a complete absence of pigmentation.

Temperature is one of the many environmental factors known to play a role in influencing pigmentation, so we also examined pigmentation levels of all the mutants at 30°C and 37°C to see if some differences in phenotype might be observed at the two temperatures. However, at 37°C, all the deletions behaved similarly in that they yielded very limited colour at the higher temperature (data not shown).

Together, these results clearly demonstrated a critical role for GRX4 in the ade pigment formation, and that it was not an indirect consequence of the intracellular levels of two important nutrient factors, adenine and glutathione, or other environmental factors.

To further confirm that the absence of pigmentation in both the *ade1Δgrx4Δ* and the *ade2Δgrx4Δ* strains was indeed due to the absence of *grx4Δ*, we complemented these mutants with the GRX4 gene expressed downstream of the TEF promoter in a single copy, centromeric plasmid. Transforming the *ade1Δgrx4Δ* or *ade2Δgrx4Δ* strains with this plasmid restored the red pigment phenotype to the mutants, confirming that the phenotype was indeed due to a disruption of GRX4 (figure 3A).

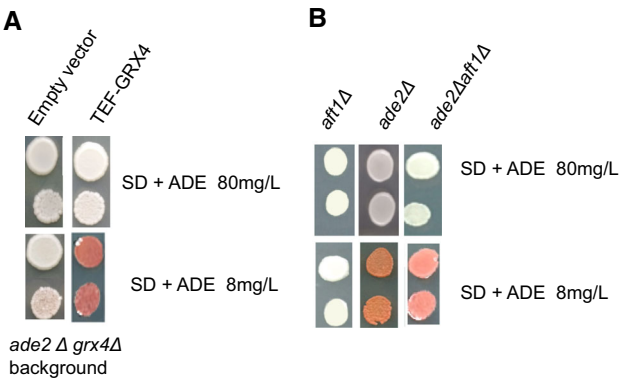

Figure 3. (**A**) Complementation of pigmentation defect in *grx4Δade2Δ* by WT GRX4. 0.2 OD_{600} cells of *grx4Δ ade2Δ* strain bearing TEF-GRX4 transformants were spotted in duplicate on limited adenine conditions. (**B**) Evaluation of the red pigmentation phenotype of *ade2Δ* stress in an *aft1Δ* background. 0.2 OD_{600} cells of *aft1Δ* in *ade2Δ* background strain was spotted in duplicate on limited adenine conditions.

3.3 *GRX4 function in the ade pigmentation does not depend on its Aft1p regulatory role*

Yeast GRX4 is a monothiol glutaredoxin (Herrero and de la Torre-Ruiz 2007). It is involved in radical responsive to hydroperoxide and superoxide and in protecting cells from oxidative damage. It also has a role in the nucleus as a transcriptional regulator of the iron-regulated transcription factor Aft1p (Pujol-Carrion *et al.* 2006). Along with Grx3p, Grx4p binds to Aft1p, the low-iron-sensing transcription factor in iron-replete conditions, promoting its dissociation from promoters (Ojeda *et al.* 2006; Pujol-Carrion *et al.* 2006; Jbel *et al.* 2011). GRX4 also contains a TRX domain and a GRX domain, regions that in other glutaredoxins/thioredoxins are involved in the glutathionylation and deglutathionylation processes (Pujol-Carrion *et al.* 2006; Pujol-Carrion and de la Torre-Ruiz 2010). Thus the precise role of GRX4 in adenine pigmentation remained unclear. One possibility was a direct involvement in glutathione conjugation. The second possibility was that it could also be involved through the regulation of the transcription activator Aft1p and the downstream effects of AFT1.

To examine if GRX4 might in fact be functioning in the ade pigmentation indirectly through transcriptional regulation, we decided to examine the consequences of disrupting the downstream gene, AFT1 to see if it might be responsible for the phenotype. AFT1 is known to be a transcriptional activator that functions under iron limiting conditions to upregulate many genes involved in iron homeostasis, including genes involved in iron uptake (Yamaguchi-Iwai *et al.* 1995), but also has iron-independent functions (Berthelet *et al.* 2010). To investigate the role of AFT1 in the ade pigmentation we examined how the *aft1Δ* affected the ade2 pigmentation. A disruption of the *ade2Δ* gene in an *aft1Δ* background was created and the pigmentation of the resultant strain was examined under ade limiting conditions. We observed a mildly decreased level of the pigment colour in the *ade2Δaft1Δ* background but did not observe the complete absence of pigmentation seen in the *grx4Δade2Δ* strains. Since iron is known to influence the ade pigmentation (Park *et al.* 2014), the altered pigmentation shades may be a consequence of the involvement of iron. However, more importantly, the almost complete lack of pigmentation seen in the *grx4Δ* strain was not observed in the *ade2Δaft1Δ* strain background suggesting that the mechanism of action of GRX4 was not primarily due to its regulatory role on the transcriptional activator Aft1p (figure 3b).

GRX4 has a paralog, GRX3, that arose from the whole genome duplication. Grx3p and Grx4p are highly homologous with 66% identity, and like Grx4p, Grx3p possesses both an N-terminal TRX-like domain and a C-terminal GRX-like domain (Pujol-Carrion and de la Torre-Ruiz 2010) (figure 4A). Furthermore Grx3p, like Grx4p binds to Aft1p regulating its transcriptional activation function processes (Ojeda *et al.* 2006; Pujol-Carrion *et al.* 2006). Since the GRX domains of GRX4 were very much similar to that of GRX3, we were interested to examine the role of GRX3 in the restoration of ade pigmentation. We therefore cloned and expressed GRX3 downstream of the strong TEF promoter. However, even though it was expressed from a strong promoter, GRX3 was not able to restore the ade pigmentation of the *grx4Δade2Δ* strain (figure 4B).

3.4 *GRX domain of Grx4p has a critical role in ade pigmentation*

Grx4p is an unusual member of the monothiol glutaredoxins in that it contains both a TRX domain and a GRX domain. The GRX domain plays a vital role in ROS detoxification. The thioredoxin-like domain is found to be essential for the nuclear location of Grx4, although there exist no reports regarding canonical nuclear localization sequences detectable in the Trx domains of Grx4p. The Trx and GRX domains of

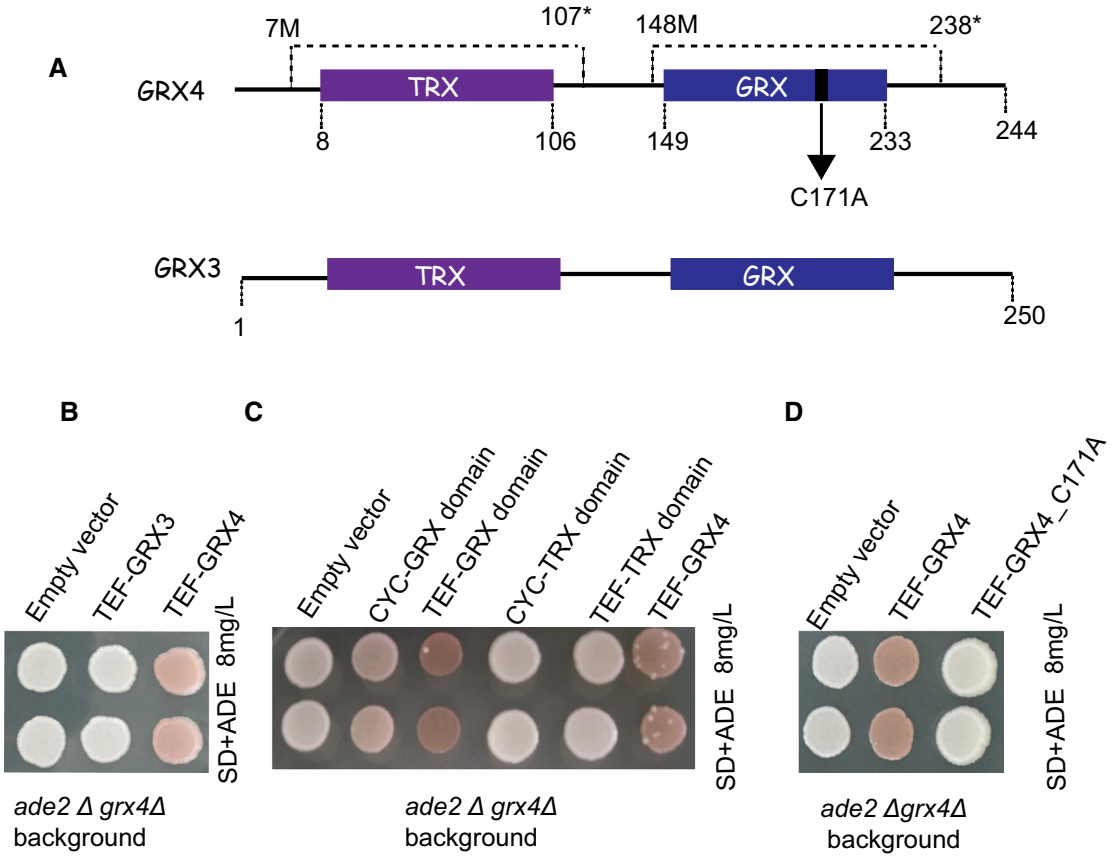

Figure 4. (**A**) Schematic representation of different domains in Grx3p and Grx4p. Grx4 and Grx4 with their respective are GRX and TRX domains. The truncated versions of the Grx4, GRX domain (8–106 amino acid residues) and TRX domain (148–238 amino acid residues) that were cloned under TEF and CYC promoter are indicated. The position of the active site mutant C171A in the GRX domain is indicated. (**B**) GRX3 overexpression fails to complement pigmentation of *grx4Δade2Δ* strain. 0.2 OD600 cells of *grx4Δ ade2Δ* strains bearing TEF-GRX3 was spotted in duplicate on SD plates with adenine at 8mg/l. (**C**) Comparison of the red pigmentation phenotype of TRX and GRX domains of Grx4 in ade pigmentation under promoters TEF and CYC. 0.2 OD600 cells of *grx4Δade2Δ* strain bearing plasmids with different TRX domains, TEF-GRX, TEF-TRX, CYC-GRX and CYC-TRX domains were spotted in duplicate on SD plates with adenine at 8mg/l. (**D**) Active site mutant C171A in GRX domain leads to failure to complement pigmentation defect. 0.2 OD600 cells of *grx4Δ ade2Δ* strain bearing TEF-C171A in GRX4 was spotted in duplicate on SD plates with adenine at 8mg/l.

Grx4p can act independently of each other (Pujol-Carrion and de la Torre-Ruiz 2010).

We attempted to decipher which of these domains might be important for the ade pigmentation. We therefore separately cloned and expressed the two domains downstream of the strong TEF promoter, and the much weaker CYC promoter. These were transformed into the *grx4Δade2Δ* strain and examined for the restoration of the pigmentation. We observed the restoration of pigmentation only by the GRX domain. The restoration was complete when the GRX was expressed downstream of the TEF promoter, and when expressed under the much weaker CYC promoter, the complementation was still visible though weak. In contrast, no complementation was observed with the TRX domain even when expressed downstream of the strong TEF promoter (figure 4C). To determine if the CXXS motif of GRX4 was required for the role in the GSH conjugation. The Cys 171 residue in the GRX domain of GRX4 was mutated to Ala. Complementation with the GRX4 clone bearing this mutation led to no restoration of the pigmentation, and suggested that the CGFS motif of the GRX domain of GRX4 has a crucial role in the ade pigmentation phenotype (figure 4D).

3.5 *Purification of GRX4 and evaluation of GST activity with a standard substrate*

Although evidence was pointing towards a GST activity of GRX4 in the ade pigmentation, in which the GRX domain and the cysteine 171 in the GRX motif was involved, there was no prior demonstration of a GST-activity for these monothiol glutaredoxins. The two classical dithiol glutaredoxins, GRX1 and GRX2, have been demonstrated to have GST activity (Collinson and Grant 2003), however, no such activity has been demonstrated for the monothiol glutaredoxins. These glutaredoxins have mostly been shown to play a role in protein glutathionylation and deglutathionylation (Greetham *et al.* 2010). We considered it necessary therefore, to purify the yeast GRX4p and examine activity towards its substrate *in vitro*. AIR and CAIR were the substrates for GRX4, however, as these were not available, we decided to examine if GST-activity for Grx4p could be demonstrated for a standard GST substrate such as CDNB. We purified recombinant his-tagged yeast Grx4p from *E. coli* using a Ni-NTA column. The purified Grx4p, a 25 kDa protein (supplementary figure 2a), was used to evaluate the enzymatic activity towards CDNB. We observed significant activity specially of 5.9 unit /mg proteins compared to 24.6 unit /mg proteins of the commercial equine GST towards CDNB indicating that Grx4p indeed has GST-activity (supplementary figure 2b). These experiments confirm the GST-activity of GRX4 and further demonstrates the direct involvement of Grx4 by its GST activity in AIR/CAIR conjugation with GSH, followed by the subsequent events of efflux into vacuole and the formation of the red pigmentation.

4. Discussion

In this study, we fill a critical missing link in the ade pigment formation of yeasts. This pigment phenotype is not only specific to *S. cerevisiae* but is found and exploited in other yeasts such as *S. pombe* (Chaudhuri *et al.* 1997), *K. lactis* (Zonneveld and Van der Zanden 1995) and *C. albicans* (Poulter and Rikkerink 1983). Although widely used in a variety of genetic screens, the full pathway leading to the pigment formation has surprisingly, still remained elusive. This is probably due to the complexity of genetic and environmental factors that affect the pigmentation. Although genetic studies carried out with the yeast *S. pombe* have revealed the involvement of glutathione and the glutathione conjugate pumps (Chaudhuri *et al.* 1997), concerted efforts have not yet been made to identify the enzymes involved in the conjugation process. Thus, this step has still remained unknown. In this study we took a comprehensive approach where instead of focusing on a few candidate genes as has been done in the past, we evaluated the role of all 17 members of the thioredoxin fold superfamily. The results yielded the somewhat surprising, but nonetheless convincing evidence of the involvement of GRX4. Since GRX4 has multiple roles in the cell and also carries multiple domains it was also necessary to pinpoint which of these roles might be important. As it all pointed to a GST activity of GRX4, an activity that has not been demonstrated for this class of proteins so far, it was necessary to establish through purification and *in vitro* activity measurements that GRX4 had indeed GST activity. We could demonstrate that it has significant activity towards the standard GST substrate, CDNB, and we predict that it is likely to show similar GST activity towards AIR and CAIR once these substrates become available and are tested. The ability of GRX4 to have not only roles in iron regulation, glutathionylation (Vall-llaura *et al.* 2016) and deglutathionylation,

oxidative stress and now glutathione S-transferase suggests that many of these glutaredoxins proteins have multiple functions.

Interestingly, neither of the known GSTs, GTT1, GTT2, GTO1, GTO2, GTO3 was found to be involved in the process. The glutaredoxins GRX1 and GRX2 which have also been shown to have GST activity also did not appear be involved. Thus there seems to be significant substrate specificities of the GST activity of these proteins even as they displayed activity towards standard substrates. Even the closely related GRX3, which is a paralog of GRX4 did not appear to play a role as seen from both through deletion analysis and overexpression studies. It is pertinent to note that when this study was initially initiated it was thought that there would be redundancy in the involvement of the members of the thioredoxin fold family, just as significant redundancy was observed in the glutathione conjugate pumps that transport the glutathione conjugates to the yeast vacuole (SHARMA *et al*. 2003). And yet surprisingly, no obvious redundancy was observed since the GRX4 deletions alone were almost completely white. Since iron also seems to have a role in ade pigment formation (Park *et al.* 2014), one immediate issue is whether the involvement of GRX4 has also some indirect links to iron metabolism. However, this will require studies on the post-vacuolar processing and interactions that could be important for the pigmentation, and awaits more detailed investigations.

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