INTRODUCTION

Reactive oxygen species (ROS) are generated continuously through the process of respiration in all aerobic organisms, and may cause the oxidative deterioration of lipid, DNA and protein function in cells (Temple et al., 2005). ROS damage is often exacerbated during stress. To counter this, cells have evolved a range of enzymic and non-enzymic antioxidant defence mechanisms. The majority of these serve a preventative role in scavenging ROS before they exert damage. Others repair incurred oxidative damage, including phospholipid hydroperoxide glutathione peroxidases (lipid peroxidation) (Avery et al., 2004) and 8-oxoG DNA glycosylases (DNA oxidation) (Boiteux & Radicella, 1999). The principal enzymic mechanism for reversing protein oxidation acts on the oxidation product of just one amino acid residue, methionine. This specificity for Met reflects the fact that Met is particularly susceptible to oxidation compared with other amino acids. Furthermore, Met oxidation has been linked to Alzheimer’s (Schoneich, 1999) and Parkinson’s (Wassef et al., 2007) diseases. Methionine sulphoxide reductases (MSRs) are conserved across nearly all organisms from bacteria to humans (Kryukov et al., 2002), and have been the focus of considerable attention in recent years. Two MSR activities have been characterized in the yeast Saccharomyces cerevisiae: MsrA (encoded by MXR1) reduces the S stereoisomer of methionine sulphoxide (MetO), while MsrB (encoded by the YCL033c ORF, which we term here MXR2) reduces the R stereoisomer of MetO (Koc et al., 2004; Kryukov et al., 2002). Consistent with defence against oxidative damage, mutants deficient in MSR activity are hypersensitive to pro-oxidants such as H₂O₂, paraquat and Cr, while MSR overexpression enhances resistance (Kryukov et al., 2002; Moskovitz et al., 1997, 1998; Sumner et al., 2005). In addition to countering protein...
damage caused by MetO, it has been proposed that MSR activity acts to support an ROS-scavenging function of reduced and surface-exposed Met residues (Levine et al., 1996; Melkani et al., 2006). Furthermore, reversible oxidation of Met residues may serve as a mechanism for regulating protein activity and in cellular signalling processes (for reviews see Bigelow & Squire, 2005; Moskovitz, 2005).

Besides methionine residues, iron–sulphur (FeS) clusters are exquisitely ROS-sensitive components of many (>120) cellular proteins. FeS clusters are highly abundant and diversely employed enzymic cofactors that have ancient origins (Imlay, 2006). FeS cluster biogenesis is the only known process for which mitochondria are essential to yeast cells (Lill & Kispal, 2000). FeS clusters participate in electron transfer (featuring commonly in redox enzymes), substrate binding and activation, iron–sulphur–storage and regulation of gene expression (Johnson et al., 2005). Haemoglobin formation in differentiating red blood cells is regulated through FeS cluster assembly (Wingert et al., 2005), and decreased activity of FeS enzymes is a characteristic feature of the neurodegenerative disease Friedrich’s ataxia (Shan et al., 2007). The vulnerability of FeS clusters to oxygen and oxidative stress greatly complicates their roles in biology. Oxidation of FeS causes loss of protein function, and at the same time releases free Fe which may participate in Fenton catalysis to exacerbate oxidative stress (Kryukov et al., 2002) to the members themselves and Cu resistance. The LEU2 marker, amplified from pRS315 as template, was used for further gene deletions in the mexα background. SFH-PCR was used to disrupt the following genes in the mexα background and, where specified in the Results, also in the BY4741 wild-type strain: CTR1, FET3, FET4, SOD1, SUP5, SUP1-1, AFT2 (all with the His3MX6 marker), AFT1 (with the hphNT1 marker). It was confirmed that the markers themselves did not influence Cu resistance. The LEU2 marker, amplified from pRS315 as template, was used for disruption of SUP1-2 in the mexα-1Δ mexα-1Δ background. CTR3 was disrupted in the mexα-1Δ, mexα-1Δ and sod2Δ strains with hphNT1. A disruption construct described elsewhere (Portnoy et al., 2001) was used to delete CTR2, following linearization of plasmid pS411 with BamHI and transformation into S. cerevisiae BY4741 or mexα cells.

**Plasmids.** Standard cloning procedures (Ausubel et al., 2007), reagents from New England Biolabs and the cloning host Escherichia coli DH5α were used throughout. For complementation, a fragment comprising the MXR1 ORF and native promoter was cloned between the NotI and BamHI sites of the single-copy vector pRS315-hphNT1. pRS315-hphNT1 was constructed previously by ligating the hphNT1 marker (see below), 150 µg hygromycin ml⁻¹ (Invitrogen). Diagnostic PCR (Longtine et al., 1998) was used to confirm appropriate gene disruption in transformants. Where used, colony PCR for diagnosis of gene disruption was according to Amberg et al. (2005). An mexα double mutant (mexα::KAN, mexα-1::hphNT1) isogenic with BY4741 was constructed using pFA6a-hphNT1 (Janke et al., 2004) as the template for SFH-PCR-based disruption of Mxr2 in the S. cerevisiae mexα background. A previously constructed mexα-1Δ double-mutant strain (mexα-1::URA3, mexα-2::KanMX4, isogenic with BY4741) (Kryukov et al., 2002) was used for further gene deletions in the mexα background. SFH-PCR was used to disrupt the following genes in the mexα background and, where specified in the Results, also in the BY4741 wild-type strain: CTR1, FET3, FET4, SOD1, SUP5, SUP1-1, AFT2 (all with the His3MX6 marker), AFT1 (with the hphNT1 marker). It was confirmed that the markers themselves did not influence Cu resistance. The LEU2 marker, amplified from pRS315 as template, was used for disruption of SUP1-2 in the mexα-1Δ mexα-1Δ background. CTR3 was disrupted in the mexα-1Δ, mexα-1Δ and sod2Δ strains with hphNT1. A disruption construct described elsewhere (Portnoy et al., 2001) was used to delete CTR2, following linearization of plasmid pS411 with BamHI and transformation into S. cerevisiae BY4741 or mexα cells.

**Growth conditions and toxicity assays.** Organisms were maintained either in liquid YEPD medium or, where specified, in YNB medium supplemented with the appropriate amino acids or nucleic acid bases (Ausubel et al., 2007). The same media were used for preparation of experimental cultures, by subculture from stationary-phase culture and growth to mid-/late-exponential phase (OD₆₀₀ ~2.0) at 30 °C, 120 r.p.m. (Bishop et al., 2007). Growth in the broth media after addition of Cu(NO₃)₂ or paraquat (from filter-sterilized stock solutions) was followed at OD₆₀₀ in 300 μl volumes in 48-well plates (Greiner Bio-One) incubated with shaking in a BioTek Powerwave microplate spectrophotometer (Smith et al., 2007). For qualitative viability assays on solid medium, dilution series from experimental cultures adjusted to OD₆₀₀ ~0.2, 0.02, 0.002 and 0.0002 and
0.0002 were spotted (8 µl) onto medium solidified with agar (1.6 %, w/v) and supplemented as specified. An l-MetO supplement containing a mixture of R and S MetO isomers was obtained from Sigma-Aldrich. Quantitative viability tests on solid medium were based on colony forming ability, as described previously (Smith et al., 2007).

**Determination of Cu uptake.** Cu(NO$_3$)$_2$ was added to experimental cultures (OD$_{600}$ > 2.0) to a final concentration of 3 mM and, after 20 min incubation, cells (25 ml samples) were harvested by centrifugation (740 g, 5 min). Control incubations at 4 °C were set up in parallel to enable discrimination of Cu that was passively bound to cells (Avery et al., 1996). For analysis by atomic absorption spectrophotometry (AAS), cell digests were prepared with HNO$_3$ as described previously (Avery et al., 1996). Samples were filtered, diluted with 3 M HNO$_3$, and analysed for Cu content using a SpectraAA 220FS atomic absorption spectrophotometer (Varian) that was calibrated with 0–0.5 mg ml$^{-1}$ standard solutions of Cu(NO$_3$)$_2$. To support AAS measurements of Cu uptake, a colorimetric assay employing bathocuproine disulphonic acid (BCS) (Ramirez et al., 2005) was used to determine Cu concentrations in supernatants after removal of cells by centrifugation (see above). Cellular Cu uptake was determined by difference versus Cu determinations in parallel control flasks that lacked cells.

**Microarray analysis.** RNA was extracted from cells of experimental cultures (OD$_{600}$ > 1.0) using the RNeasy Mini kit (Qiagen), after cell breakage with a mini-Beadbeater (Biospec Products) three times for 30 s interspersed with incubations on ice. Residual DNA was removed by treatment with RNase-free DNase (Promega, 0.05 U µg$^{-1}$). For each yeast strain, RNA extracts from each of six replicate cultures were pooled into two samples (each comprising RNA from three replicate cultures), snap-frozen in liquid nitrogen and stored at −20 °C until use. The integrity of RNA and cRNA was confirmed in all samples before analysis using an Agilent Bioanalyzer. These analyses, RNA preparation, hybridizations to GeneChip Yeast Genome S98 Arrays (Affymetrix) and analysis with an Affymetrix GeneChip Reader, were performed as a service by the NASC Affymetrix Service, University of Nottingham. Raw signal intensity data from hybridizations were normalized after removal of the highest and lowest 2 % of signal values, and gene signals scoring ‘marginal’ or ‘absent’ were removed from the datasets (Payne et al., 2008). Genes showing > 25 % difference in signal intensities between replicate samples were also removed from the analysis. Fold up/down-regulation values for individual transcripts were subsequently determined for mean signal values derived from the test (RNA from mutant) samples with reference to the corresponding control (wild-type) analyses.

**Determination of individual transcript abundances.** Cellular RNA was prepared as described above. The absence of protein or DNA contamination in RNA samples was confirmed according to A$_{260}$/A$_{230}$ ratios and standard PCR tests, respectively. For reverse-transcription reactions, 1 µl dNTP mix (10 mM each of dATP, dCTP, dGTP and dTTP), 1 µl 25 µg ml$^{-1}$ oligo (dT)$_{20}$ primer (Invitrogen) and 1 µg RNA template were combined and made up to 13 µl with nuclease-free water. The mixture was heated to 65 °C for 5 min, followed by incubation on ice for ≥ 1 min. The contents of the tube were collected by brief centrifugation and 1 µl 0.1 M DTT, 4 µl 5 x first strand buffer and 1 µl SuperScript III reverse transcriptase (200 U µl$^{-1}$, Invitrogen) were added. After incubation at 50 °C for 60 min, the reaction was inactivated by heating to 70 °C for 15 min. Residual RNA was removed by addition of 1 µl (2 U) E. coli RNase H (Invitrogen) and incubation at 37 °C for 20 min.

The relative abundance method was used to determine resultant cDNA levels by quantitative real-time (qRT)-PCR. For reactions, 0.4 µM each of gene-specific primers (HPLC purification scale, Sigma–Genosys), 2 µl cDNA template from first-strand cDNA synthesis (10$^{-1}$ dilution), and 2 x QuantiTect SYBR Green PCR Master Mix were combined and made up to 25 µl with RNase-free water in 0.2 ml tubes (Stratagene). PCRs (95 °C for 10 min, followed by (95 °C for 30 s, 55 °C for 1 min, 72 °C for 30 s) for 40 cycles, followed by 95 °C for 1 min, and 55 °C for 20.5 min) were monitored using an MX4000 real-time PCR thermocycler (Stratagene). The results were normalized and analysed with the MX4000 software.

For semiquantitative RT-PCR, 10$^{-1}$, 10$^{-2}$ and 10$^{-3}$ dilutions of the cDNA were used as the template in PCRs (95 °C for 1 min, followed by (95 °C for 30 s, 55 °C for 1 min, 72 °C for 1 min), 30 cycles, followed by 72 °C for 10 min and then 4 °C). The PCR products were examined by agarose gel electrophoresis and the intensities of the bands were compared between wild-type and mutant strains as specified, using template dilutions at which the reaction had not progressed to saturation. ACT1 was used as the reference mRNA.

**Protein extraction and enzyme assays.** Protein extracts were prepared as described elsewhere (Cashikar et al., 2005). Incubations were under nitrogen to protect FeS clusters. Protein extracts in supernatants were transferred to clean tubes and protein concentrations determined (Bradford, 1976). The assay of aconitase activity in protein extracts (from 2 x 10$^8$ cells) under nitrogen was performed as described elsewhere (Wallace et al., 2005). The change in absorbance was recorded at 1 min intervals for 8 min in UV cuvettes and the specific activity (per mg protein) calculated from the linear portion of the resultant plot. Isopropylmalate dehydratase (Leu1) activity was assayed as described by Kohlhaw (1988). The change in absorbance was recorded over 2 min following substrate addition, and the specific activity (per mg protein) calculated from the linear portion of the resultant plot.

**In vivo $^{55}$Fe-labelling studies.** Cultures (25 ml) were grown overnight in YEPD to mid-exponential phase (OD$_{600}$ > 0.5) in 125 ml flasks, and 12.8 Ci (473.6 kBq) $^{55}$FeCl$_3$ (Perkin-Elmer) was added. For measurement of FeS cluster biosynthesis, cells were harvested after incubation for 1 or 2 h with $^{55}$Fe. For measurement of FeS cluster turnover, the $^{55}$Fe pre-loaded cells were washed and resuspended in YEPD medium lacking $^{55}$Fe, and incubated for a further 1 or 2 h before harvesting. Protein extracts were prepared and immunoprecipitations with antibodies specific for Bio2p or Leu1p (gifts from Roland Lill, University of Marburg) were performed as described elsewhere (Molik et al., 2007). Incorporation isotope was quantified in 1 ml scintillation fluid (Emulsifier Safe, Perkin-Elmer) using a Packard Tri-Carb 2100TR liquid scintillation analyser.

**RESULTS**

**MSR activity sensitizes S. cerevisiae to copper**

During a study on the contribution of oxidative damage repair proteins to metal resistance in S. cerevisiae, we observed an unexpected Cu-resistance phenotype in a mxr1A/mxr2A double mutant (hereafter referred to as mxrΔ), which lacks MetO-reducing activity. Growth of this mxrΔ mutant (Kryukov et al., 2002) was only mildly affected by Cu(NO$_3$)$_2$ at a concentration which strongly inhibited growth of the wild-type (Fig. 1a). [Note that high (mM) Cu concentrations are needed to observe growth-inhibitory effects in rich medium (YEPD) such that used here, as much of the added Cu is biologically unavailable in complexes with medium components (Avery et al., 1996;
Hughes & Poole, 1991). The mxrΔ double mutant exhibited stronger Cu resistance than the mxr1Δ and mxr2Δ single mutants, which are defective for reduction of different MetO stereoisomers. The Cu resistance of the mxrΔ mutant was evident also from growth on Cu-supplemented agar (Fig. 1b), was suppressed by complementation with the MXR1 gene (Fig. 1b) and was confirmed in an independently constructed mxrΔ double mutant from our laboratory. The Cu-resistance phenotype could be mimicked by growing wild-type cells in MetO-supplemented medium, whereas Met supplementation had no effect. MetO also abolished the relative resistance of the mxrΔ mutant (Fig. 1c). These data suggested that it is specifically the accumulation of MetO (rather than Met depletion or similar) in mxrΔ cells that gives rise to Cu resistance. Yeast mutants lacking both MSRs have been reported previously to be sensitive to other pro-oxidants (Kryukov et al., 2002; Sumner et al., 2005). Those sensitivity phenotypes were confirmed during this study, indicating that the resistance phenotype was specific to Cu.

**Decreased Cu uptake is not responsible for the Cu resistance of mxrΔ cells**

To explain the Cu resistance of mxrΔ cells it was first suggested that, in the absence of MSR activity, oxidation of Met residues in Cu-uptake proteins causes inactivation and a decrease in Cu accumulation. The high-affinity Cu transporter Ctr1p is enriched in Met residues [proteins that interact with MSR are usually Met-rich (Alamuri & Maier, 2006; Le et al., 2008)], some of which are required for Cu uptake (Puig et al., 2002). However, epistasis tests showed that MXR gene deletion conferred Cu resistance also in a ctr1Δ background (data not shown), indicating that loss of Ctr1p activity is not the cause of Cu resistance in mxrΔ cells. Loss of activity of Ctr2p (the putative low-affinity vacuolar Cu transporter) was also ruled out as, unlike MXR gene deletion, CTR2 deletion did not improve growth on Cu-supplemented agar. We also demonstrated with PCR that CTR3, which encodes a high-affinity Cu transporter, was disrupted by a Ty2 transposon insertion in the strains used here, as in most S. cerevisiae laboratory strains (Knight et al., 1996). Therefore, Ctr3p activity was not involved here. Epistasis experiments with mutants deficient in Fet4p, a low-affinity copper transporter, suggested that Fet4p inactivation partly contributes to Cu resistance in the mxrΔ mutant (see Supplementary Fig. S1). Moreover, Cu-accumulation tests indicated that an effect on Cu uptake was not the cause of Cu resistance in mxrΔ cells. According to two independent methods, calculated to exclude (Fig. 2a) or include (Fig. 2b) surface-associated Cu, Cu accumulation was up to twofold higher in mxrΔ than in wild-type
cells. It was inferred that for both Cu accumulation and Cu resistance to be elevated in mxrΔ cells, intracellular Cu must be less available to exert toxicity.

Transcript levels of gene products mediating Fe/Cu homeostasis and resistance are elevated in mxrΔ versus wild-type cells

In light of the above results, it was hypothesized that gene products which modulate the availability of free Cu in the cell are upregulated in mxrΔ versus wild-type cells. To test this, the transcriptomes of the two strains were compared. A preliminary microarray experiment in our laboratory indicated that ~30 genes were upregulated at least twofold in mxrΔ cells versus the wild-type (Supplementary Table S1). These included CCC2, which encodes a Cu transporter and, most strikingly, a set of genes of the iron regulon (Puig et al., 2005; Rutherford et al., 2003): FIT2, FIT3, ARN2, ARN3(SIT1), ARN4(ENB1) and TIS11. To explore this further, we analysed the more comprehensive microarray datasets produced elsewhere (Koc et al., 2004) for the same strain comparison. Those analyses distinguished 540 genes that were at least twofold upregulated in the mxrΔ mutant versus the wild-type. Again, Fe-regulon genes were among the most strongly upregulated, including FET3 (~3.8-fold), FIT2 (3.0-fold), and ARN4 (2.7-fold) (Fig. 3a). Over half of the 10 most highly regulated Fe-regulon genes (Puig et al., 2005; Rutherford et al., 2003) were induced ≥1.5-fold in the mxrΔ mutant. In addition, several gene functions that specifically modulate intracellular Cu availability were upregulated in mxrΔ cells, including CUP1 (~2.0-fold), SOD1 (1.5-fold), FET3 (3.8-fold) and CUP5 (2.6-fold) (Fig. 3b, c). Key transcriptomics data were validated with either qRT-PCR or semiquantitative RT-PCR. There were some quantitative differences in upregulation between the PCR-based and the microarray assays. Nonetheless, according to both, each of the four genes tested (CUP1, CUP5, FET3 and YHB1) was markedly upregulated in mxrΔ versus wild-type cells (Fig. 3b, c).

It was reasoned that the upregulation of one or more of the identified Cu-resistance determinants in the mxrΔ mutant could explain the elevated Cu resistance of these cells. Consistent with this, multicopy expression of SOD1 and FET3 was confirmed here to elevate the Cu resistance of wild-type S. cerevisiae (Supplementary Fig. S2). Conversely, CUP5 did not influence Cu resistance in our hands. SOD1, CUP1 and FET3 were selected initially for further study.

The Cu-binding Fet3 protein is required for the Cu-resistance phenotype of the mxrΔ mutant

To test whether SOD1, CUP1 or FET3 was required for Cu resistance in the mxrΔ double mutant, in which they were each upregulated, these genes were deleted in the mxrΔ and wild-type backgrounds. Deletion of SOD1 did not alter the relative Cu resistances of the mxrΔ and wild-type strains (data not shown), indicating that Sod1p is not required for the mxrΔ phenotype. Although the MSR-deficient cells retained some Cu resistance following deletion of CUP1, the loss of resistance resulting from Cup1p deficiency was more marked for mxrΔ cells than for the wild-type. Thus, no colony formation by the wild-type was detectable at a Cu concentration yielding ~40 % viability of mxrΔ cells (Fig. 4a), whereas cup1Δ cells retained >15 % viability at a Cu concentration that yielded <40 % viability in mxrΔ/ cup1Δ cells (Fig. 4b). Therefore, CUP1 upregulation in mxrΔ cells (above) may partially contribute to their Cu resistance. In contrast, FET3 was required for the phenotype. As expected (Stoj et al., 2007), FET3 deletion resulted in a sensitization to Cu in both the wild-type and the mxrΔ background (note the different Cu concentrations in Fig. 4c versus Fig. 4a). However, this sensitization was much greater in the mxrΔ mutant than in the wild-type. Put another way, an absence of MSR activity had the opposite effect on Cu resistance in the fet3Δ background to that in the wild-type (Fig. 4a, c). The Cu sensitivity of mxrΔ/fet3Δ cells relative to fet3Δ cells was similar to that seen for other pro-oxidants in comparisons of mxr mutant and wild-type cells (Kryukov et al., 2002; Moskovitz et al., 1997; Sumner et al., 2005). The results indicated that the unexpected Cu resistance of mxrΔ cells could be accounted for by the FET3 upregulation observed in this mutant. A partial role for CUP1 in the phenotype (above) was further supported by observations that removal of MSR activity in a cup1Δ/fet3Δ background caused a greater relative sensitization to Cu than occurred in a fet3Δ background (Supplementary Fig. S3), but the more important role evident for FET3 provided the focus for subsequent experiments.
FeS cluster-dependent functions are deficient in the mxrΔ mutant

FET3 transcription is known to be under the regulation of the Aft2 and, in particular, the Aft1 transcription factors. These have overlapping roles in the regulation of iron utilization and homeostasis (Rutherford et al., 2003, 2005). The possible activation of Aft1 and/or Aft2 in mxrΔ cells was suggested by the upregulation not only of FET3 (and associated Cu resistance) but also of other Aft-dependent genes (Rutherford et al., 2003) such as ARN1, ARN4, FIT2 and FRE6 (Fig. 3a). We expressed an AFT1-1up allele, which produces constitutively active Aft1p (Yamaguchi-Iwai et al., 1995). AFT1-1up expression in wild-type cells incubated in YEPD mimicked the phenotype of Cu resistance seen in mxrΔ cells (Fig. 5). In contrast, AFT1-1up expression in the mxrΔ mutant had no further effect on Cu resistance. The results were consistent with Aft activation determining Cu resistance in mxrΔ cells (see Fig. 6 for scheme).

Aft1p (and consequently FET3) is usually activated under Fe-limited conditions. This activation has been traced largely to defective FeS cluster biogenesis in mitochondria (Chen et al., 2004; Rutherford et al., 2005). Accordingly, the transcriptomic responses to FeS biogenesis defects and Aft1 activation are strikingly similar (Hausmann et al., 2008). To test for FeS cluster defects in mxrΔ cells, the activities of the [4Fe–4S]-containing enzymes aconitase (Aco1p) and isopropylmalate isomerase (Leu1p) were assayed. Aconitase specific activity was reproducibly ~30 % lower in protein extracts from mxrΔ cells than in extracts from wild-type cells (Fig. 7a). This activity defect was despite an approximately sixfold higher level of ACO1 mRNA in mxrΔ cells (Koc et al., 2004). In conjunction with a ~50 % decrease in Leu1p activity in the mxrΔ mutant (Fig. 7a), these data supported the hypothesis that mxrΔ cells have a FeS cluster defect.

If, as inferred, FeS cluster defects in mxrΔ cells are the cause of FET3 upregulation and Cu resistance (Fig. 6), then it should be possible to mimic these phenotypes by manipulating the integrity of FeS clusters. This was tested by creating conditions conducive to FeS cluster degradation, achieved using a sod2Δ mutant defective for Mn-
superoxide dismutase activity; like the process of FeS biosynthesis, Sod2p localizes to mitochondria, where it scavenges superoxide radicals, which are major antagonists of FeS cluster activity. Thus, Sod2p activity protects the integrity of mitochondrial FeS clusters (Irazusta et al., 2006; Strain et al., 1998). Consistent with this, the \( \text{FET3} \) gene appeared to be upregulated in \( \text{sod2}^{-} \) cells relative to the wild-type (Fig. 7b). Furthermore, the \( \text{sod2}^{-} \) mutant reproducibly showed a slightly higher Cu resistance than the wild-type in YEPD medium (Fig. 7c). This phenotype was similar, albeit less marked, to the phenotype of \( \text{mxr}^{-} \) cells described above. To test whether \( \text{FET3} \) was required for Cu resistance of the \( \text{sod2}^{-} \) mutant, as in the \( \text{mxr}^{-} \) mutant (Fig. 4a, c), the effect of \( \text{SOD2} \) deletion was examined also in a \( \text{fet3}^{-} \) background. Fet3p deficiency abolished the slight Cu resistance otherwise associated with \( \text{SOD2} \) deletion (Fig. 7c, d). Thus, the \( \text{FET3} \)-dependent Cu resistance seen in \( \text{mxr}^{-} \) cells could be reproduced, albeit less strikingly, in a mutant known to be limited for FeS cluster integrity.

**Turnover of \([4\text{Fe}–4\text{S}]\) clusters is increased in the \( \text{mxr}^{-} \) mutant**

It was speculated that the FeS cluster defect in \( \text{mxr}^{-} \) cells was due either to an increased rate of FeS cluster turnover or to decreased FeS cluster biosynthesis. To resolve these possibilities, the association of radiolabelled \( ^{55}\text{Fe} \) with FeS proteins was measured. This was accomplished by...
immunoprecipitation of FeS proteins of interest from cells after incubation with $^{55}$FeCl$_3$, and quantification of isotope in the immunoprecipitates. To distinguish FeS biosynthesis from FeS turnover, FeS proteins were immunoprecipitated from cells both during $^{55}$Fe incorporation (biosynthesis) and during a period after transferring $^{55}$Fe-loaded cells to $^{55}$Fe-free medium (turnover). First, we performed immunoprecipitations with the mitochondrial FeS protein biotin synthase (Bio2p). Analysis of immunoprecipitated protein with SDS-PAGE confirmed that the Bio2p protein levels in $\text{mxr}^D$ and wild-type strains were similar (Supplementary Fig. S4). The incorporation of $^{55}$Fe into Bio2p after 1 h was decreased by about one-third in the $\text{mxr}^D$ mutant (Fig. 8a), possibly suggesting a defect in FeS biosynthesis. A comparable difference was also observed after 2 h of $^{55}$Fe incorporation (Supplementary Fig. S5). However, analyses of $^{55}$Fe turnover indicated that a similar proportion (~30%) of the Bio2p-associated $^{55}$Fe of wild-type cells was lost within 1 h in $^{55}$Fe-free medium, whereas the labelled Bio2p was stable in $\text{mxr}^D$ cells (Fig. 8a). These data may be rationalized by the fact that biotin synthases contain a [2Fe–2S] cluster in addition to the oxygen-labile [4Fe–4S] cluster (Jarrett, 2005). Previous data indicate that the additional ~30% of $^{55}$Fe incorporated into (and lost from) Bio2p in wild-type cells, observed here, could correspond to the labile [4Fe–4S] cluster (Mühlenhoff et al., 2007). If so, then our data suggest that there is no detectable incorporation of $^{55}$Fe into the [4Fe–4S] cluster of Bio2p in $\text{mxr}^D$ cells, consistent with a [4Fe–4S]-specific biosynthesis defect in the $\text{mxr}^D$ mutant. However, considering the rapid turnover of $^{55}$Fe in Bio2p measured in the wild-type, our data also leave open the possibility that the inability to detect $^{55}$Fe incorporation to Bio2p of $\text{mxr}^D$ cells results from a significantly elevated [4Fe–4S] turnover rate (i.e. clusters are turned over at least as fast as they are synthesized).

To help resolve these possibilities, we also performed $^{55}$Fe labelling and immunoprecipitation with Leu1p. Unlike Bio2p, Leu1p contains only a [4Fe–4S] cluster. Moreover, we considered that the cytosolic localization of Leu1p should help to give slower FeS turnover rates than those suggested above for mitochondrial Bio2p, mitochondria being the major source of ROS in respiring cells. This was
bore out by the data as, unlike with Bio2p (Fig. 8a), no turnover of $^{55}$Fe-labelled Leu1p was detected within 1 h of transferring labelled wild-type cells to $^{55}$Fe-free medium (Fig. 8b). In contrast, $\text{mxr}^{-}$ cells treated in the same way exhibited a $>50\%$ decrease in $^{55}$Fe-labelled Leu1p. There was no significant difference in the incorporation of label into Leu1p of $\text{mxr}^{-}$ and wild-type cells. The data collectively indicate a [4Fe–4S] cluster defect in $\text{mxr}^{-}$ cells, which, at least in the case of Leu1p, can be attributed to a higher rate of [4Fe–4S] turnover than in wild-type cells.

**DISCUSSION**

This study reveals a novel role for the MSR proteins of *S. cerevisiae* in helping to preserve the function of cellular FeS clusters. Previous work has established an antioxidant function for the MSR enzymes, methionines being especially susceptible to oxidation compared with other amino acids. Such MSR activity has been proposed to protect against the damaging effects of MetO on protein function, to support ROS scavenging via reduced Met, and to act as a mechanism for regulating protein activity (Moskovitz, 2005; Oien & Moskovitz, 2008). However, a link to FeS protein function has not previously been reported. This is an important discovery, as the requirement to maintain the integrity of essential FeS proteins is a key challenge to organisms with an aerobic lifestyle (Imlay, 2006). Therefore, the ubiquitous MSR proteins could be an important factor facilitating aerobicity.

The finding that the deletion of both $\text{MXR1}$ and, to a lesser extent, of $\text{MXR2}$ contributed to the Cu-resistance phenotype of the $\text{mxr}^{-}$ double mutant was consistent with the relative contributions of these proteins to other phenotypes in yeast (Koc et al., 2004; Kryukov et al., 2002; Sumner et al., 2005). This indicates that both the S and R stereoisomers of MetO are a factor in the FeS cluster defects that led to Cu resistance. The Cu-resistance phenotype of $\text{mxr}^{-}$ cells occurred despite an increased level of Cu accumulation. Therefore, Cu must be less available to exert toxicity in the $\text{mxr}^{-}$ mutant, as borne out by the upregulation of a number of Cu-binding proteins in this strain, in particular Fet3p. $\text{FET3}$ transcription is principally under Aft1p control (Courel et al., 2005; Rutherford et al., 2003; Yamaguchi-Iwai et al., 1995), and the microarray and AFT1-1 expression evidence was consistent with Aft1p dependency here (Fig. 6). The low-affinity Cu transporter Fet4p has also been reported to be under Aft1p control (Waters & Eide, 2002), and is upregulated approximately twofold in the $\text{mxr}^{-}$ mutant (Koc et al., 2004). Therefore, Fet4p could account for the elevated Cu uptake of this strain.

The protection of both mitochondrial (Aco1p, Bio2p) and cytosolic (Leu1p) FeS proteins by MSR activity indicated here suggests that the MSRs localize to both of these subcellular compartments. Mammalian MsrA and MsrB proteins have been localized variously to mitochondria, the cytosol and nucleus (Kim & Gladyshev, 2004). To our knowledge, MSR localization has not been examined directly in yeast. Global protein localization studies (Hu et al., 2003; Kumar et al., 2002) indicate that MsrA (encoded by $\text{MXRI}$) occurs in the cytosol and nucleus (there are no data for yeast MsrB). Moreover, the Predator tool for identification of N-terminal targeting sequences (Small et al., 2004) gives an 81% probability that the yeast MsrB (encoded by $\text{MXR2}$) localizes to mitochondria (no localization sequence is predicted for MsrA). A mammalian mitochondrion-targeted MsrB protein is sufficient to rescue a yeast $\text{mxr}^{-}$ double mutant, indicating the importance of mitochondrial MetO reduction (Kim & Gladyshev, 2004). These predictions imply that MsrA protects FeS cluster integrity primarily in cytosolic and nuclear proteins, and MsrB in mitochondrial FeS proteins [including the FeS-related signal that is exported to Aft1p (Rutherford et al., 2005)]. The question has been posed elsewhere (Moskovitz, 2005), what is the dominant role of mitochondrial MSR activity? The present study reveals that preservation of FeS cluster function, at the site of FeS biosynthesis, could be the answer.
One question arising from our study is how does MSR activity protect FeS-cluster function? FeS-cluster deficiencies can result from depletion of mitochondrial Fe (Li & Kaplan, 2004). However, the possibility that a mxrΔ defect in mitochondrial Fe accumulation is responsible is unlikely, as Fe supplementation in the medium up to 3 mM does not alter the relative Cu sensitivities of mxrΔ and wild-type strains (T. Sideri and S. V. Avery, unpublished data). Furthermore, our 55Fe-labelling studies suggested that the FeS defect in mxrΔ cells is primarily attributable to an enhanced FeS-turnover rate rather than decreased FeS biosynthesis, substantiating that Fe availability is not the primary cause.

It is unlikely that the FeS defect is due to some generic antioxidant activity of MSRs, as other antioxidant genes in yeast do not yield a Cu-resistance phenotype upon deletion (Avery, 2001; Jo et al., 2008). One exception is SOD2, as corroborated here. Sod2p has a specific role in preserving mitochondrial FeS-cluster integrity by scavenging mitochondrial superoxide radicals that are primary antagonists of FeS function (Irazusta et al., 2006). It has been proposed that MSRs indirectly enhance scavenging of free radicals such as superoxide by regenerating reduced Met residues that act as antioxidants (Levine et al., 1996; Melkani et al., 2006). However, Met depletion was not responsible for the mxrΔ phenotype, as Met supplementation of the medium did not suppress Cu resistance. In contrast, the addition of MetO to wild-type cultures mimicked the phenotype, indicating that Met oxidation (rather than depletion) was causative. A number of proteins are reported to be inactivated by Met oxidation (Alamuri & Maier, 2006; Ezraty et al., 2004; Sun et al., 1999). However, FeS proteins are not among those characterized. Furthermore, none of the MSR-interacting proteins that have been identified in bacteria (Alamuri & Maier, 2006) or in protein-interaction studies with yeast (http://www.yeastgenome.org/) are FeS proteins. Bacterial MSR is reported to bind proteins with Met contents approximately twofold higher than average (Alamuri & Maier, 2006), whereas the average Met content of yeast FeS proteins is only slightly higher (at 2.6%) than that of all yeast proteins (2.3%).

If the yeast MSRs do not interact directly with FeS proteins, they may modulate the activities of proteins that themselves influence FeS function. Met oxidation can cause conformational changes in proteins, as MetO is more hydrophilic than Met (Moskovitz, 2005). The helix-breaking character of MetO is thought to be exploited in regulation and signalling (Bigelow & Squier, 2005; Ciobra et al., 1997). It should also be noted that MetO may initiate other oxidative reactions that themselves exert damage. For example, oxidation of the Met-35 residue of β-amyloid peptides is thought to be linked to catalysis of free radical production (Pogocki, 2003). Yeasts lacking MsrA exhibit increased levels of protein carbonylation (Oien & Moskovitz, 2007), a marker of broader oxidative protein damage than solely Met oxidation (Sumner et al., 2005). Furthermore, a general decrease in mitochondrial ROS production and oxidative damage in calorie-restricted rats has been linked specifically to methionine (and therefore presumably MetO) restriction (Sanz et al., 2006). Although these studies did not examine FeS-cluster function, they have highlighted how Met oxidation may trigger a cascade of oxidative events leading to the phenotype. Considering that FeS clusters are major targets of superoxide action (Imlay, 2006), as underscored here with Sod2p manipulations, any catalysis of (mitochondrial) superoxide generation that is associated with MetO formation (Pogocki, 2003) could explain the FeS defects in mxrΔ cells.

A relationship between cellular MSR activity and FeS-cluster function, revealed here, could be widely conserved. Both types of function are found among almost all organisms, and both have ancient origins (Delaye et al., 2007; Imlay, 2006). Furthermore, defects in both are associated with degeneration and disease. For example, aberrant Fe homeostasis (e.g., due to FeS-cluster defects) contributes to the ageing process (Atamna et al., 2002), while a role for MSR activity in extending lifespan is conserved across different organisms (Koc et al., 2004). In addition, a major hallmark of Friedreich’s ataxia is decreased activity of FeS enzymes (Shan et al., 2007), and ataxia symptoms have been reported in MsrA-deficient mice (Moskovitz et al., 2001). While such disease states were not a focus of this study, our results have revealed a relationship that contributes to our understanding of ROS-related degeneration and the sustenance of FeS-cluster activity in aerobic settings.

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