Methionine sulfoxide reductases protect iron–sulphur clusters from oxidative inactivation in yeast

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Methionine residues and iron–sulphur (FeS) clusters are primary targets of reactive oxygen species in the proteins of micro-organisms. Here, we show that methionine redox modifications help to preserve essential FeS cluster activities in yeast. Mutants defective for the highly conserved methionine sulfoxide reductases (MSRs; which re-reduce oxidized methionines) are sensitive to many pro-oxidants, but here exhibited an unexpected copper resistance. This phenotype was mimicked by methionine sulfoxide supplementation. Microarray analyses highlighted several Cu and Fe homeostasis genes that were upregulated in the *mxrΔ* double mutant, which lacks both of the yeast MSRs. Of the upregulated genes, the Cu-binding Fe transporter Fet3p proved to be required for the Cu-resistance phenotype. *FET3* is known to be regulated by the Aft1 transcription factor, which responds to low mitochondrial FeS-cluster status. Here, constitutive Aft1p expression in the wild-type reproduced the Cu-resistance phenotype, and FeS-cluster functions were found to be defective in the *mxrΔ* mutant. Genetic perturbation of FeS activity also mimicked *FET3*-dependent Cu resistance. ⁵⁵Fe-labelling studies showed that FeS clusters are turned over more rapidly in the *mxrΔ* mutant than the wild-type, consistent with elevated oxidative targeting of the clusters in MSR-deficient cells. The potential underlying molecular mechanisms of this targeting are discussed. Moreover, the results indicate an important new role for cellular MSR enzymes in helping to protect the essential function of FeS clusters in aerobic settings.

Received 25 July 2008

Revised 15 October 2008

Accepted 22 October 2008

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, 2005) and Parkinson's (Wassef *et al.*, 2007) diseases. Methionine sulfoxide 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 sulfoxide (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

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Abbreviations: AAS, atomic absorption spectrophotometry; BCS, bathocuproine disulphonic acid; FeS cluster, iron–sulphur cluster; MetO, methionine sulfoxide; MSR, methionine sulfoxide reductase; qRT-PCR, quantitative real-time PCR; ROS, reactive oxygen species.

Five supplementary figures, showing Cu resistance in the *fet4Δ*, *mxrΔ* and *mxrΔ/fet4Δ* mutants, that overexpression of *FET3* or *SOD1* confers Cu resistance, that *CUP1* may partially contribute to the Cu resistance of the *mxrΔ* mutant, Bio2 protein in immunoprecipitates from wild-type and *mxrΔ* cells, and determination of ⁵⁵Fe incorporation into FeS proteins, and a supplementary table listing genes upregulated at least twofold in *msr* versus wild-type cells, are available with the online version of this paper.

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 & Squier, 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 Friedreich'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 (Keyser & Imlay, 1996; Srinivasan *et al.*, 2000). Denaturation of FeS clusters is not the only way in which these co-factors may influence cellular Fe availability. A signal which represses the Fe regulon (and so the uptake of Fe) at high cellular Fe levels in yeast is transmitted through a product of the mitochondrial FeS biogenesis machinery (Chen *et al.*, 2004; Kumanovics *et al.*, 2008; Rutherford *et al.*, 2005). Thus, Fe levels influence FeS biogenesis, which in turn regulates Fe homeostasis. Iron homeostasis in yeast also interplays closely with homeostasis of another essential but potentially toxic Fenton catalyst, copper (Askwith & Kaplan, 1998). The high-affinity Fe transporter Fet3p requires bound copper for activity, and this Cu binding contributes to cellular Cu resistance (Stoj *et al.*, 2007). However, direct links between cellular FeS cluster status and Cu transport or toxicity have not been established to date. The same is true of FeS clusters and Met residues, despite their shared notoriety as highly oxidation-sensitive components of cellular proteins.

In this report, an unexpected Cu-resistance phenotype of MSR-deficient *S. cerevisiae* cells was traced to activation of the Fe regulon, specifically *FET3*. Moreover, it was established that MSR activity helps to preserve the function of cellular FeS clusters. The finding suggests that the ubiquity of MSR enzymes could be an adaptation that enables the operation of FeS clusters in aerobic settings.

METHODS

Yeast strains. *S. cerevisiae* BY4741 (*MATa*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*) and the isogenic deletion strains *aft1Δ*, *aft2Δ*, *ctr1Δ*, *cup5Δ*,

fet3Δ, *fet4Δ*, *mxr1Δ*, *mxr2Δ* (*YCL033cΔ*), *sod1Δ*, and *sod2Δ*, in which the specified ORFs are replaced with the *KanMX4* marker, were obtained from Euroscarf. Short flanking homology (SFH)-PCR (Longtine *et al.*, 1998) was used to disrupt additional genes (see below) in this study; all primer sequences are available on request. SFH-PCR products were transformed into *S. cerevisiae* by the lithium acetate method (Gietz & Woods, 2002), and transformants were selected on yeast nitrogen base (YNB) without amino acids (Formedium), supplemented as required to give appropriate selection (Ausubel *et al.*, 2007). To select for the *hphNT1* marker (see below), transformants were plated on YEPD medium (see below) supplemented with 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 *mxrΔ* double mutant (*mxr1::KAN*, *mxr2::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* *mxr1Δ* background. A previously constructed *mxr* double-mutant strain (*mxr1::URA3*, *mxr2::KanMX4*, isogenic with BY4741) (Kryukov *et al.*, 2002) was used for further gene deletions in the *mxrΔ* background. SFH-PCR was used to disrupt the following genes in the *mxrΔ* background and, where specified in the Results, also in the BY4741 wild-type strain: *CTR1*, *FET3*, *FET4*, *SOD1*, *CUP5*, *CUP1-1*, *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 *CUP1-2* in the *cup1-1Δ* and *mxrΔ/cup1-1Δ* backgrounds. *FET3* was disrupted in the *cup1Δ*, *mxrΔ/cup1Δ* and *sod2Δ* strains with *hphNT1*. A disruption construct described elsewhere (Portnoy *et al.*, 2001) was used to delete *CTR2*, following linearization of plasmid pJS411 with *Bam*HI and transformation into *S. cerevisiae* BY4741 or *mxrΔ* 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 *Not*I and *Bam*HI sites of the single-copy vector pRS315-hphNT1. pRS315-hphNT1 was constructed previously by ligating the hphNT1 marker excised from pFA6a-hphNT1 between the *Bbs*I and *Nar*I sites of pRS315. For overexpression of *SOD1* or *FET3*, fragments encompassing these ORFs plus their native promoters were amplified from yeast genomic DNA and ligated between the *Kpn*I and *Sal*I (*SOD1*) or *Eco*RI and *Xba*I (*FET3*) sites of the multicopy vector YEp352. Yeast transformants were selected on YNB-minus-uracil medium. An *AFT1-1^{up}* fragment was released from pRS313-*AFT1-1^{up}* (Chen & Kaplan, 2000) by digestion with *Sac*I and *Spe*I. This 3.6 kb fragment was ligated into pRS315-hphNT1. Yeast transformants were selected in YEPD agar supplemented with 150 µg hygromycin ml⁻¹. Plasmid-transformed yeast cells were grown throughout, for maintenance or for experimental purposes, in the relevant medium selective for the plasmid.

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 ~2.0, 0.2, 0.02, 0.002 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(NO₃)₂ was added to experimental cultures (OD₆₀₀ ~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; Lin & Kosman, 1990). For analysis by atomic absorption spectrophotometry (AAS), cell digests were prepared with HNO₃ as described previously (Avery *et al.*, 1996). Samples were filtered, diluted with 3 M HNO₃, and analysed for Cu content using a SpectrAA 220FS atomic absorption spectrophotometer (Varian) that was calibrated with 0–0.5 mg ml⁻¹ standard solutions of Cu(NO₃)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 (OD₆₀₀ ~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 Bioanalyser. 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 A₂₆₀/A₂₈₀ ratios and standard PCR tests, respectively. For reverse-transcription reactions, 1 µl dNTP mix (10 mM each of dATP, dCTP, dTTP and dGTP), 1 µl 2.5 µg µl⁻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 × 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 × 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 × 10⁸ 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 ⁵⁵Fe-labelling studies. Cultures (25 ml) were grown overnight in YEPD to mid-exponential phase (OD₆₀₀ ~0.5) in 125 ml flasks, and 12.8 µCi (473.6 kBq) ⁵⁵FeCl₃ (Perkin-Elmer) was added. For measurement of FeS cluster biosynthesis, cells were harvested after incubation for 1 or 2 h with ⁵⁵Fe. For measurement of FeS cluster turnover, the ⁵⁵Fe pre-loaded cells were washed and resuspended in YEPD medium lacking ⁵⁵Fe, 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). Incorporated 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 *mxr1Δ/mxr2Δ* 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(NO₃)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 as that used here, as much of the added Cu is biologically unavailable in complexes with medium components (Avery *et al.*, 1996;

Fig. 1. MSR activity sensitizes YEPD-grown *S. cerevisiae* to copper. (a) Exponential-phase cells of the wild-type (BY4741) (○) or isogenic deletion strains $mxr1\Delta$ (●), $mxr2\Delta$ (□) or $mxr1\Delta, mxr2\Delta$ (' $mxr\Delta$ ') (■) were inoculated into either unsupplemented YEPD broth (upper panel) or YEPD supplemented with 12 mM $\text{Cu}(\text{NO}_3)_2$ (lower panel) and incubated with shaking in a microplate spectrophotometer. (b, c) Exponential-phase cells were adjusted to $\text{OD}_{600} \sim 2.0$. Tenfold dilution series were spotted (8 μl) from left to right on YEPD agar supplemented or not supplemented with 14 mM $\text{Cu}(\text{NO}_3)_2$ and L-MetO, as indicated. '+ev', cells transformed with the single-copy 'empty' vector pRS315-hphN; '+MXR1', cells transformed with pRS315-hphN-MXR1. Media used for these transformed cells were additionally supplemented with 150 μg hygromycin ml^{-1} . Plates were observed after incubation at 30 °C for 4 days. Typical results from one of several independent experiments are shown.

Hughes & Poole, 1991)]. The $mxr\Delta$ double mutant exhibited stronger Cu resistance than the $mxr1\Delta$ and $mxr2\Delta$ single mutants, which are defective for reduction of different MetO stereoisomers. The Cu resistance of the $mxr\Delta$ 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 $mxr\Delta$ 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 $mxr\Delta$ mutant (Fig. 1c). These data suggested that it is specifically the accumulation of MetO (rather than Met depletion or similar) in $mxr\Delta$ 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 $mxr\Delta$ cells

To explain the Cu resistance of $mxr\Delta$ 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 *ctr1* Δ background (data not shown), indicating that loss of Ctr1p activity is not the cause of Cu resistance in $mxr\Delta$ 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 $mxr\Delta$ mutant (see Supplementary Fig. S1). Moreover, Cu-accumulation tests indicated that an effect on Cu uptake was not the cause of Cu resistance in $mxr\Delta$ 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 $mxr\Delta$ than in wild-type

Fig. 2. Cellular Cu accumulation is increased in the *mxrΔ* mutant. Exponential-phase cells of the wild-type (white bars) or *mxrΔ* (black bars) strains were harvested after 20 min incubation in YEPD broth in the presence of 3 mM $\text{Cu(NO}_3)_2$. (a) Cellular Cu levels were determined by AAS, after washing of cells and digestion with HNO_3 . Surface-bound Cu was determined in parallel incubations at 4 °C, and these values were subtracted from total cellular Cu to give intracellular Cu levels. (b) Total cellular Cu accumulation was determined according to BCS-assisted measurement of Cu in medium supernatants. Mean $\pm$ SEM data from three independent determinations are shown.

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.

(a)

Fe regulon genes highly upregulated by Fe depletion (Puig <i>et al.</i> , 2005)	Fold induction in <i>msrΔ</i> versus wild-type cells (Koc <i>et al.</i> , 2004)	Fe regulon genes highly upregulated by constitutive <i>AFT1</i> activation (Rutherford <i>et al.</i> , 2003)	Fold induction in <i>msrΔ</i> versus wild-type cells (Koc <i>et al.</i> , 2004)
<i>FIT3</i>	1.6	<i>FIT3</i>	1.6
<i>FIT2</i>	3.0	<i>FIT2</i>	3.0
<i>ARN2 (TAF1)</i>	1.5	<i>FET3</i>	3.8
<i>ARN1</i>	2.0	<i>FIT1</i>	1.0
<i>FIT1</i>	1.0	<i>YOR387C</i>	0.9
<i>FET3</i>	3.8	<i>ARN4 (ENB1)</i>	2.7
<i>ARN3 (SIT1)</i>	1.7	<i>ARN3 (SIT1)</i>	1.7
<i>TIS11 (CTH2)</i>	0.9	<i>TIS11 (CTH2)</i>	0.9
<i>FTR1</i>	1.3	<i>FTR1</i>	1.3
<i>ARN4 (ENB1)</i>	2.7	<i>FRE2</i>	0.6

(b)

(c)

Fig. 3. Upregulation of Fe-regulon and Cu-resistance genes in the *msrΔ* mutant. (a) The table lists the 10 yeast Fe-regulon genes identified previously as the most highly upregulated in response to Fe depletion (Puig *et al.*, 2005), or during constitutive Fe-regulon activation via *AFT1-1^{up}* expression (Rutherford *et al.*, 2003). The genes are listed in order with the most highly upregulated first. The data in columns 2 and 4 indicate the *msrΔ*:wild-type expression ratio for the listed genes, derived from other published microarray datasets (Koc *et al.*, 2004). (b) Levels of *CUP1*, *CUP5* and *YHB1* upregulation in *msrΔ* versus wild-type cells were derived from microarray data (Koc *et al.*, 2004) (white bars) and from transcript-specific qRT-PCR analyses (black bars). The latter data were normalized against *ACT1* mRNA determinations in the same samples, and then against the corresponding determinations for wild-type samples to yield values for upregulation. Mean data $\pm$ SEM from two independent biological replicates are shown. (c) *FET3* transcript levels were examined with semiquantitative RT-PCR [this was deemed adequate for *FET3* expression as these analyses were performed later in the study, when microarray and phenotypic (below) studies had already substantiated a role for *FET3*]. Lanes 1 and 2, replicate wild-type samples; lanes 3 and 4, replicate *msrΔ* samples. The microarray data for *FET3* (not shown) indicated a 3.8-fold upregulation in *msrΔ* versus wild-type cells.

FeS cluster-dependent functions are deficient in the *msrΔ* 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 *msrΔ* 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 *AFT1-1^{up}* allele, which produces constitutively active Aft1p (Yamaguchi-Iwai *et al.*, 1995). *AFT1-1^{up}* expression in wild-type cells incubated in YEPD mimicked the phenotype of Cu resistance seen in *msrΔ* cells (Fig. 5). In contrast, *AFT1-1^{up}* expression in the *msrΔ* mutant had no further effect on Cu resistance. The results were consistent with Aft activation determining Cu resistance in *msrΔ* 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 *msrΔ* 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 *msrΔ* cells than in extracts from wild-type cells (Fig. 7a). This activity defect was despite an approximately sixfold higher level of *ACO1* mRNA in *msrΔ* cells (Koc *et al.*, 2004). In conjunction with a >50 % decrease in Leu1p activity in the *msrΔ* mutant (Fig. 7a), these data supported the hypothesis that *msrΔ* cells have a FeS cluster defect.

If, as inferred, FeS cluster defects in *msrΔ* 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-

Fig. 4. *FET3* is required for the Cu resistance of *mxrΔ* cells. Exponential-phase cells ($OD_{600} \sim 2.0$) that were either wild-type (white bars) or deleted for the *MXR* genes (black bars) were washed, diluted and spread-plated onto YEPD agar supplemented or not supplemented with the specified concentrations of $Cu(NO_3)_2$. Colonies were enumerated after incubation for 4 days at 30 °C and percentage viability was calculated with reference to growth on control plates lacking Cu. The strains tested were: (a) *S. cerevisiae* BY4741 (wild-type) (white bars) and *mxrΔ* (black bars); (b) *cup1Δ* (white bars) and *mxrΔ/cup1Δ* (black bars); and (c) *fet3Δ* (white bars) and *mxrΔ/fet3Δ* (black bars). Mean $\pm$ SEM data from three independent determinations are shown.

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 *FET3* gene appeared to be upregulated in *sod2Δ* cells relative to the wild-type (Fig. 7b). Furthermore, the *sod2Δ* 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 *mxrΔ* cells described above. To test whether *FET3* was required for Cu resistance of the *sod2Δ* mutant, as in the *mxrΔ* mutant (Fig. 4a, c), the effect of *SOD2* deletion was examined also in a *fet3Δ* background. Fet3p deficiency abolished the slight Cu resistance otherwise associated with *SOD2* deletion (Fig. 7c, d). Thus, the *FET3*-dependent Cu resistance seen in *mxrΔ* cells could be reproduced, albeit less strikingly, in a mutant known to be limited for FeS cluster integrity.

Fig. 5. Constitutive activation of Aft1p mimics the Cu resistance of *mxrΔ* cells in YEPD medium. Exponential-phase wild-type and *mxrΔ* cells transformed with either pRS315-hphNT1-*AFT1-1^{up}* (black bars) or the empty vector pRS315-hphNT1 (white bars) were spread-plated onto YEPD agar supplemented with 13 or 14 mM $Cu(NO_3)_2$, as indicated. Viability (colony formation) was determined after incubation for 4 days at 30 °C, and percentage viability was calculated with reference to growth on control plates lacking Cu. Mean $\pm$ SEM data from three independent determinations are shown.

Turnover of [4Fe-4S] clusters is increased in the *mxrΔ* mutant

It was speculated that the FeS cluster defect in *mxrΔ* 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}Fe with FeS proteins was measured. This was accomplished by

Fig. 6. Proposed scheme explaining the Cu resistance of *mxrΔ* cells. The relative Cu resistance of the *mxrΔ* mutant was dependent on *FET3*, a component of the Fe regulon of yeast that is activated by Aft1p. Aft1p is responsive to low FeS-cluster status in cells. It is proposed in this paper that high MetO levels (like high superoxide levels) stimulate the turnover of FeS clusters, so activating Aft1p and inducing *FET3*-dependent Cu resistance.

Fig. 7. FeS-protein defects in the *mxrΔ* mutant, and mimicry of the *mxrΔ* phenotype by *SOD2* deletion. (a) Aconitase and isopropylmalate isomerase activities were determined in protein extracts from exponential-phase wild-type and *mxrΔ* cells. Mean $\pm$ SEM data from three independent determinations are shown. (b) Levels of *FET3* and *ACT1* mRNA were determined in RNA extracts from exponential-phase wild-type or *sod2Δ* cells, using semiquantitative RT-PCR. Lanes 1 and 2, replicate wild-type samples; lanes 3 and 4, replicate *sod2Δ* samples. [Note that the controls (lanes 1 and 2) are the same as in Fig. 3(c), as these analyses were part of the same experiment.] (c) Growth of wild-type ($\circ$) and *sod2Δ* ($\bullet$) cells was monitored in YEPD medium supplemented with 10 mM $\text{Cu}(\text{NO}_3)_2$, with shaking in a microplate spectrophotometer. (d) Growth of *fet3Δ* ($\square$) or *fet3Δ/sod2Δ* ($\blacksquare$) mutants in YEPD medium supplemented with 10 mM $\text{Cu}(\text{NO}_3)_2$. Note that there were no growth differences between strains in control YEPD medium lacking $\text{Cu}(\text{NO}_3)_2$ (results not shown). Typical data from one of at least three independent experiments are shown in each case. Absolute measurements varied by $>10\%$ between certain replicate experiments, but the effects of the gene deletions relative to wild-type varied by $<10\%$. The growth effects described in (c) and (d) were relatively small, but reproducible in every experiment.

immunoprecipitation of FeS proteins of interest from cells after incubation with $^{55}\text{FeCl}_3$, 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 Bio2 protein levels in *mxrΔ* 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 *mxrΔ* 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 ($\sim 30\%$) 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 *mxrΔ* cells (Fig. 8a). These data may be rationalized by the fact that biotin synthases contain a $[2\text{Fe}-2\text{S}]$ cluster in addition to

the oxygen-labile $[4\text{Fe}-4\text{S}]$ cluster (Jarrett, 2005). Previous data indicate that the additional $\sim 30\%$ of ^{55}Fe incorporated into (and lost from) Bio2p in wild-type cells, observed here, could correspond to the labile $[4\text{Fe}-4\text{S}]$ cluster (Mühlenhoff *et al.*, 2007). If so, then our data suggest that there is no detectable incorporation of ^{55}Fe into the $[4\text{Fe}-4\text{S}]$ cluster of Bio2p in *mxrΔ* cells, consistent with a $[4\text{Fe}-4\text{S}]$ -specific biosynthesis defect in the *mxrΔ* 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 *mxrΔ* cells results from a significantly elevated $[4\text{Fe}-4\text{S}]$ 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 $[4\text{Fe}-4\text{S}]$ 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

Fig. 8. Determination of ^{55}Fe incorporation into FeS proteins. Exponential-phase wild-type or *mxrΔ* cells were incubated for up to 2 h in YEPD supplemented with $12.8 \mu\text{Ci ml}^{-1}$ ($473.6 \text{ kBq ml}^{-1}$) $^{55}\text{FeCl}_3$, before protein extracts were prepared and immunoprecipitations performed with anti-Bio2p (a) or anti-Leu1p (b) antibodies. ^{55}Fe levels in immunoprecipitates prepared from 0.5 mg protein extract were determined by scintillation counting. The data shown are for immunoprecipitates obtained after 1 h labelling in ^{55}Fe -supplemented medium (white bars), or after 1 h labelling followed by 1 h incubation in ^{55}Fe -free medium (black bars). (Note that similar data were obtained with samples obtained after 2 h incubations; Supplementary Fig. S5.) The data are means $\pm$ SEM from three replicate determinations, and typical results from one of at least two independent experiments are shown. WT, wild-type.

borne 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, *mxrΔ* 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 *mxrΔ* and wild-type cells. The data collectively indicate a [4Fe–4S] cluster defect in *mxrΔ* 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 aerobically.

The finding that the deletion of both *MXR1* and, to a lesser extent, of *MXR2* contributed to the Cu-resistance phenotype of the *mxrΔ* 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 *mxrΔ* cells occurred despite an increased level of Cu accumulation. Therefore, Cu must be less available to exert toxicity in the *mxrΔ* mutant, as borne out by the upregulation of a number of Cu-binding proteins in this strain, in particular Fet3p. *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^{up}* 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 *mxrΔ* 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 (Huh *et al.*, 2003; Kumar *et al.*, 2002) indicate that MsrA (encoded by *MXR1*) occurs in the cytosol and nucleus (there are no data for yeast MsrB). Moreover, the Predotar tool for identification of N-terminal targeting sequences (Small *et al.*, 2004) gives an 81% probability that the yeast MsrB (encoded by *MXR2*) localizes to mitochondria (no localization sequence is predicted for MsrA). A mammalian mitochondrion-targeted MsrB protein is sufficient to rescue a yeast *mxrΔ* 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; Ciorba *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.

ACKNOWLEDGEMENTS

This work was supported by a grant from the National Institutes of Health (R01 GM57945). We thank Roland Lill (University of Marburg) for his kind gifts of anti-Bio2p and anti-Leu1p antibodies. We also thank Victor Gladyshev (University of Nebraska) for sharing his *mxrΔ* double mutant, Jerry Kaplan (University of Utah) for plasmid pRS313-*AFT1*-*I^{HP}*, and Valeria Culotta (Johns Hopkins University) for plasmid pJS411. We are grateful for expert technical assistance from Lee Shunburne.

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Edited by: D. J. Jamieson