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3 **Methionine sulfoxide reductases revisited: Free**

4 **methionine as a primary target of H₂O₂ stress in**

5 **auxotrophic fission yeast**

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20 Running title: Met, a first defence line against H₂O₂

21 Keywords: methionine, methionine oxidation, H₂O₂ reactivity, methionine sulfoxide reductases,

22 antioxidant defenses

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SUMMARY

Amino acid methionine can suffer reversible oxidation to sulfoxide and further irreversible over-oxidation to methionine sulfone. As part of the cellular antioxidant scavenging activities are the methionine sulfoxide reductases (Msrs), with a reported role in methionine sulfoxide reduction, both free and in proteins. Three families of Msrs have been described, but the fission yeast genome only includes one representative for two of these families: MsrA/Mxr1 and MsrB/Mxr2. We have investigated their role in methionine reduction and H₂O₂ sensitivity. We show here that MsrA/Mxr1 is able to reduce free oxidized methionine. Cells lacking each one of the genes are not significantly sensitive to different types of oxidative stresses, neither display altered life span. However, only when deletion of *msrA/mxr1* is combined with deletion of *met6*, which confers methionine auxotrophy, the survival upon H₂O₂ stress decreases by 100-fold. In fact, cells lacking only Met6, and which therefore require addition of methionine to the growth media, are extremely sensitive to H₂O₂ stress. These and other evidences suggest that oxidation of free methionine is a primary target of peroxide toxicity in cells devoid of methionine biosynthetic capacity, and that an important role of Msrs is to recycle this oxidized free amino acid.

1 INTRODUCTION

2
3 Oxidative stress causes damage to all biomolecules and in particular to proteins (for reviews, see
4 Madian and Regnier, 2010; Nystrom, 2005; Souza *et al.*, 2008). Thus, hydrogen peroxide (H_2O_2)
5 has been widely reported to react and irreversibly damage several amino acid residues, to induce
6 peptidyl breaks and to trigger protein loss-of-function and aggregation. Only the sulfur-containing
7 amino acids methionine (Met) and cysteine (Cys) have been reported to suffer reversible
8 modifications by H_2O_2 . In the case of Cys, its oxidation to sulfenic acid or disulfide can be
9 reduced back to the thiol form by the action of classical electron donors such as thioredoxin and
10 glutaredoxin (Meyer *et al.*, 2009).

11 Met is one of the amino acids more readily oxidized by H_2O_2 , either free in the cell or
12 bound to proteins. When exposed to mild oxidizing conditions, a mixture of two enantiomers of
13 Met sulfoxide are formed, L-Met-S-sulfoxide [Met-(S)-O] and L-Met-R-sulfoxide [Met-(R)-O],
14 which can be further oxidized to Met sulfone under stronger oxidizing conditions (Weissbach *et*
15 *al.*, 2005). The reversible nature of sulfenic acid and disulfide bonds has been used by peroxide
16 sensors of antioxidant pathways as a sensing mechanism (for a review, see D'Autreaux and
17 Toledano, 2007). Similarly, reversible oxidation of Met to Met sulfoxides was soon suggested to
18 be a mechanism for modulating protein activity (Ciorba *et al.*, 1997; Kanayama *et al.*, 2002), and
19 even to have a protective antioxidant role as a quencher of oxidants (Levine *et al.*, 1996; Luo and
20 Levine, 2009). Therefore, catalyzed reduction of this modification could be an antioxidant
21 mechanism and/or a protein regulatory mechanism (see below).

22 Met sulfoxide reductases (Msrs) have been described to be both repair enzymes of
23 oxidized free and protein-bound Met, and indirect scavengers of reactive oxygen species (for
24 reviews, see Cabreiro *et al.*, 2006; Lee *et al.*, 2009; Moskovitz, 2005; Oien and Moskovitz, 2008;
25 Stadtman *et al.*, 2005; Tarrago and Gladyshev, 2012). These enzymes belong to three
26 sequence-unrelated protein families: MsrA, MsrB and free Msrs (fMsrs). While representatives of
27 the MsrA and MsrB families exist in almost all cell types, fMsrs were initially isolated in
28 *Escherichia coli* (Lowther *et al.*, 2002) and their presence has been made extensive to about half
29 of prokaryotes and eukaryotic microbes, but they are missing in higher eukaryotes (Le *et al.*,
30 2009). At least in bacteria and yeast, members of each of these families are specialized in

1 catalyzing substrate reduction with great specificity. Thus, while MsrAs reduce the enantiomer
2 Met-(S)-O both free and in proteins, MsrBs seem to be specialized in the reduction of protein-
3 bound Met-(R)-O, and fMsrs recycle free Met-(R)-O (Le *et al.*, 2009; Lowther *et al.*, 2002; Tarrago
4 and Gladyshev, 2012). As a general trait, the catalytic mechanism of Met reduction by Msrs
5 involves formation of an intramolecular disulfide bond in the Msr which needs to be reduced by
6 thioredoxin (Black *et al.*, 1960; Lowther *et al.*, 2000) or other electron donors such as glutaredoxin
7 (Tarrago *et al.*, 2009) or thionein (Sagher *et al.*, 2006). Cells devoid of MsrA enzymes display
8 several phenotypes, such as inability to use oxidized Met as a Met source (Le *et al.*, 2009;
9 Moskowitz *et al.*, 1998), sensitivity to grow in the presence of peroxides (Minniti *et al.*, 2009;
10 Moskowitz *et al.*, 1997; Romero *et al.*, 2004; St John *et al.*, 2001), or impaired chronological aging
11 (Koc *et al.*, 2004; Koc and Gladyshev, 2007). Over-expression of the gene can cause the
12 opposite effects (Moskovitz *et al.*, 1998). Similar, albeit weaker, phenotypes have been
13 described for cells lacking MsrB enzymes (for a review, see Tarrago and Gladyshev, 2012).

14 In a recent proteomic screen searching for substrates of thioredoxin in fission yeast, we
15 isolated peptides of two putative Met sulfoxide reductases, MsrA/Mxr1 and MsrB/Mxr2 (Garcia-
16 Santamarina *et al.*, 2013). Due to the reported participation of the Msr families in the reduction of
17 oxidized Met and in H₂O₂ tolerance in other model organisms, we decided to characterize their
18 role in protection against extracellular peroxides in *S. pombe*, and to confirm the participation of
19 different electron donors in their recycling.

RESULTS

MsrA/Mxr1, but not MsrB/Mxr2, is able to reduce free oxidized Met and utilize it as a Met source

We recently analyzed the oxidized thiol proteome of wild-type cells, and compared it to that of cells lacking the main cytosolic thioredoxin, Trx1 (Garcia-Santamarina *et al.*, 2013). Peptides from most known thioredoxin substrates were reversibly oxidized in $\Delta trx1$ cells, and among them we identified peptides from two putative Msrs (Garcia-Santamarina *et al.*, 2013). In the *S. pombe* genome, we only found one representative for the MsrA and MsrB families, named Mxr1 and Mxr2, respectively (Fig. 1A), while no members of the fMsrs or biotin sulfoxide reductase (Pollock and Barber, 1997) families are present. Even though they have been recently reported by the groups of Veal and Lim (Day *et al.*, 2012; Jo *et al.*, 2013), their role in Met reduction is still unknown.

MsrA and MsrB family members have been described to recycle the Met sulfoxide enantiomers S and R, respectively. *S. pombe* is able to synthesize Met, but cells lacking homoserine O-acetyltransferase, Met6, are auxotrophic for this amino acid (Fig. 5A) (Ma *et al.*, 2007). We tested the capacity of Mxr1 and Mxr2 to reduce free oxidized Met by deleting the *mxr1* or *mxr2* genes in cells auxotrophic for Met, and providing L-Met sulfoxide as the only source of Met. Cells lacking Mxr1 and Met6 cannot use Met sulfoxide as a Met source (Fig. 1B), which suggests that Mxr1 reduces oxidized Met. It is worth mentioning that the commercial Met sulfoxide added to the growth media comes as a mixture of R- and S-diastereomers. That partially explains the high concentrations of Met sulfoxide compared to reduced Met required to allow full growth of $\Delta met6$ cells (0.14 mM *versus* 5 mM; Fig. 1B). In any case, $\Delta met6 \Delta mxr2$ cells do not display any growth defect in the presence of Met sulfoxide compared to strain $\Delta met6$ (Fig. 1B), which suggests that Mxr2 is not required to reduce free oxidized Met.

Fission yeast is only able to reduce the Met-(S)-O enantiomer in an MsrA/Mxr1-dependent manner

To specifically define the participation of Mxr1 in the reduction of the R or S enantiomers, we purified both forms from the commercial mixture. According to analytical characterization (see

Experimental Procedures), our Met-(S)-O was 92% pure and Met-(R)-O was only enriched to 82% (and therefore still contained 18% of the S form). We recorded the growth of $\Delta met6$, $\Delta met6 \Delta mxr1$, $\Delta met6 \Delta mxr2$ and $\Delta met6 \Delta mxr1 \Delta mxr2$ strains in minimal medium supplemented with different Met sources. As shown in Figure 2A, the Met auxotrophic strain $\Delta met6$ is able to grow in minimal medium supplemented with 0.14 mM Met, 0.42 mM Met-(R,S)-O and 0.42 mM Met-(S)-O. However, addition of Met-(R)-O as the only Met source was not able to sustain growth of $\Delta met6$ cells. Importantly, the use of Met-(S)-O from either the racemic mixture or from the enriched enantiomer preparation was fully dependent on the presence of MsrA/Mxr1, but not on MsrB/Mxr2 (Fig. 2A). Similarly, the Met auxotrophic strains were only able to grow on plates when the Met-(S)-O enantiomer was supplied as a Met source (Fig. 2B). Only when the enriched preparation of Met-(R)-O was added to minimal media plates at high concentrations, sufficient to provide around 0.25 mM Met-(S)-O, growth of $\Delta met6$ cells was sustained (Fig. S1).

Trx1 and Grx1, the main cytosolic thioredoxin and glutaredoxin in fission yeast, are the electron donors for Mxr1

The catalytic action of Msrs seems to involve the formation of unstable sulfurane-to-sulfonium cation intermediates between the reductase and its substrate, Met sulfoxide (Dokainish and Gauld, 2013), which are then resolved through the release of Met and formation of a sulfenic acid in the Msr, which then reacts with another Cys to form an internal disulfide (for a review, see (Tarrago and Gladyshev, 2012). Recycling of this oxidized Msr requires the presence of an electron donor, usually thioredoxin (for reviews, see (Boschi-Muller *et al.*, 2005; Vieira Dos Santos *et al.*, 2007) (Fig. 3A). To test the role of the two *S. pombe* cytosolic thioredoxins, Trx1 and Trx3, and the main cytosolic glutaredoxin, Grx1, in Mxr1 turnover, we constructed double mutants with each gene deletion and that of *met6*. The single deletion strains were not auxotrophic for methionine (Fig. S2); only cells lacking Trx1 are auxotrophic for cysteine, as previously reported, since Trx1 is the only electron donor for phosphoadenosine phosphosulfate reductase (Met16 in *S. pombe*; Fig. 5A) (Garcia-Santamarina *et al.*, 2013). However, cells lacking either *trx1* or *grx1* in combination with *met6* deletion were partially unable to use Met sulfoxide as a Met source (Fig. 3B). A strain lacking both Grx1 and Trx1 in a $\Delta met6$ background

1 displayed a more severe growth defect in the presence of Met sulfoxide. We conclude that Trx1
2 and Grx1 are alternative electron donors for Mxr1, as demonstrated *in vivo*.

3 The recycling of oxidized Msr by an electron donor occurs through the formation of a
4 transient mixed disulfide intermediate, which can be trapped by mutating the Cys residue required
5 for resolution. To confirm that Trx1 can recycle Mxr1, we expressed an HA-tagged version of the
6 Msr in a *trx1.C33S* background. Since Cys33 in Trx1 resolves the mixed disulfides with its
7 substrates, the Trx1.C33S mutant should allow *in vivo* trapping of an intermolecular disulfide
8 between Trx1 with Mxr1. As shown in Figure 3C, a DTT sensitive, slow migrating band was
9 detected by anti-HA immuno-blotting of extracts from a strain co-expressing Mxr1-HA and
10 Trx1.C33S. Mxr2 seems to be also a substrate of Trx1, since a mixed-disulfide intermediate
11 between Mxr2-HA and Trx1.C33S could be detected in extracts from a strain co-expressing both
12 tagged proteins (Fig. 3C). Even though the function of Mxr2 in free oxidized Met reduction is still
13 to be demonstrated, a minor protective role to oxidative stress of Mxr2 over-expression has
14 recently been reported (Jo *et al.*, 2013).

15 16 **Absence of Mxr1 or Mxr2 *per se* does not trigger sensitivity to oxidative stress, but only** 17 **when combined with Met auxotrophy**

18 In general, induction of an enzymatic activity upon specific environmental conditions can shed
19 light on the physiological role of the protein. Thus, transcriptional activation of the *mxr1* or *mxr2*
20 genes upon H₂O₂ stress could connote a role in adaptation to peroxides. Indeed, both transcripts
21 accumulate 2-3-fold upon mild peroxide concentrations, those known to activate the transcription
22 factor Pap1 (Chen *et al.*, 2008; Vivancos *et al.*, 2004; Vivancos *et al.*, 2006). The peroxide
23 induction of *mxr1* and *mxr2* transcription was abolished in cells lacking the transcription factor
24 Pap1 or the MAP kinase Sty1, both required for the activation of antioxidant responses in fission
25 yeast (Fig. 4A and Fig. S3A) (for a review, see (Vivancos *et al.*, 2006).

26 This prompted us to investigate whether strains lacking the Msrs display growth defects
27 in H₂O₂-containing plates. As shown in Figures 4B and S3BC, strains $\Delta mxr1$, $\Delta mxr2$, and the
28 double deletion mutant $\Delta mxr1 \Delta mxr2$ grew as wild-type when plated on solid media containing
29 different concentrations of H₂O₂. The mutant strains were neither affected by the presence on
30 plates of cadmium sulfate (Fig. S4A), diamide (Fig. S4B), menadione (Fig. S4C), camptothecin

(Fig. S4D), sodium hypochlorite (Fig. S4E), nitroso glutathione (Fig. S4F) or acidified nitrite (Fig. S4F); cells lacking the MAP kinase Sty1 or the transcription factor Pap1 were used as oxidant-sensitive control strains. Furthermore, over-expression of Mxr1 did not increase the tolerance to H₂O₂ on plates (Fig. S5A). Similarly, very high doses of peroxides did not affect the survival of $\Delta mxr1$ or $\Delta mxr2$ liquid cultures (Fig. 4C and S5BC).

We also tested whether deletion of *mxr1* or *mxr2* genes modulated life span in fission yeast, as proposed for other model organisms (Koc *et al.*, 2004; Koc and Gladyshev, 2007). Again, cells lacking the MAP kinase Sty1 were used as a positive control, since this mutant strain displays impaired survival at stationary phase under low glucose conditions (Zuin *et al.*, 2010). As observed in Figure 4D, lack of Msrs did not affect chronological aging in fission yeast under calorie restriction (MM plates).

A clear oxidative stress phenotype, however, was observed when deletion of the Mxr1-coding gene was combined with *met6* deletion (Fig. 4E), with an approximately 100-fold increase in the killing effect of H₂O₂ compared to $\Delta met6$ strain. Interestingly enough, deletion of *met6* alone was sufficient to render cells very sensitive to H₂O₂, to a similar extent as cells lacking Pap1, our positive control (Fig. 4E).

Cells depending on Met for growth are extremely sensitive to oxidative stress

We investigated the role of Met6 in oxidative stress survival. Met6 participates in the synthesis of Met, as shown in Fig. 5A (adapted from Baudouin-Cornu and Labarre, 2006). To test whether the inability to synthesize either amino acid was the cause of H₂O₂ sensitivity, we decided to analyze whether other deletion strains of the Cys and Met pathways were or not sensitive to peroxides. We chose to compare cells deleted in *cys1a*, *met16*, *met6* or *gcs1*, coding for Cys synthase, phosphoadenosine phosphosulfate reductase, homoserine O-acetyltransferase and glutamate-cysteine ligase, respectively (Fig. 5A, in blue) (for a review on the budding and fission yeast sulfur metabolic pathways, see (Baudouin-Cornu and Labarre, 2006). Since the synthesis of Cys, Met and glutathione (GSH) are intimately connected, we first tested which nutritional supplements could sustain the growth of each one of the mutants in minimal media conditions. As expected, cells deleted in *Gcs1* can only grow in the presence of GSH, but not with Cys or Met alone (Fig. 5B). Cells lacking Met16 are able to grow in the presence of either GSH, Cys or Met (although

1 growth of $\Delta met16$ strain does not reach wild-type levels in Met; Fig. 5B, right panel). Growth of
2 cells lacking Cys1a in minimal media can only be restored in the presence of Cys or GSH, but not
3 Met. Lastly, the synthesis of Met from Cys requires O-acetylhomoserine, since cells lacking Met6
4 can only grow in the presence of Met or Met sulfoxide, but not of Cys (Fig. 5B and 5C). As
5 summarized in Figure 5D, $\Delta met6$ is the only strain which completely relies on extracellular Met for
6 growth.

7 We then tested which one of these four strains displayed sensitivity to peroxides. On
8 plates containing all three supplements, only $\Delta met6$ cells display a severe H_2O_2 phenotype
9 almost comparable to cells lacking Pap1 (Fig. 6A). $\Delta met6$ cells also displayed sensitivity to other
10 oxidative stress insults such as menadione, diethylmaleate, cadmium or sodium hypochlorite (Fig.
11 S6). The sensitivity to oxidative stress of $\Delta met6$ cells could be fully suppressed by increasing the
12 concentration of extracellular Met (compare the toxicity over strain $\Delta met6$ of 0.5 mM H_2O_2 in
13 plates with 0.08 mM Met *versus* 0.28 mM Met; Fig. 4E). In fact, higher concentrations of Met
14 could even suppress the defects of cells lacking Pap1 (0.84 mM; data not shown), suggesting
15 that Met can block H_2O_2 toxicity by directly reacting with it in the *S. pombe* acidic media,
16 something that has previously been reported (Brot and Weissbach, 1983; Caldwell and Tappel,
17 1964; Savige and Fontana, 1977; Vogt, 1995). This protective effect can also be observed by
18 adding Cys to the growth media, even though the concentrations have to be higher to accomplish
19 full suppression (Fig. 6B). Importantly enough, $\Delta met6$ cells do not display intrinsic sensitivity to
20 H_2O_2 , as shown by measuring survival to acute peroxide stress in liquid cultures (Fig. 6C).

21 Cells lacking Met6 were also sensitive to heat shock, that is, when plates were placed at
22 37°C (Fig. 6D, left panels). We again used cells lacking Sty1 as a heat sensitive strain. Again,
23 the sensitivity of strain $\Delta met6$ (but not that of strain $\Delta sty1$) to high temperature could be
24 suppressed by addition of high amounts of free Met (Fig. 6D, 0.84 mM Met). We suspect that
25 Met is sensitive to both peroxide oxidation and probably temperature inactivation, and cells
26 without the capacity to synthesize Met will display sensitivity to grow on solid plates in which Met
27 has been exhausted. Indeed, $\Delta met6$ cells do not display intrinsic sensitivity to heat shock, as
28 cells lacking Sty1 do: after 2 hours of treatment at 48°C to liquid cultures, the survival of strain
29 $\Delta sty1$ was reduced by at least five orders of magnitude, while cells lacking *met6* behave as wild-

1 type cells (Fig. 6E). These experiments suggest that oxidants and temperature can deplete Met
2 from the media, and impair the growth of cells auxotrophic for Met.

3

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DISCUSSION

Msrs are part of the antioxidant battery of enzymes expressed by aerobic cells, since Met can be reversibly oxidized by H_2O_2 to Met sulfoxides. MsrA family members are supposed to reduce the (S)-sulfoxide enantiomer, while MsrB members have been described to recycle the (R)-enantiomer. We have analyzed the role of the two Msrs in fission yeast, MsrA/Mxr1 and MsrB/Mxr2, and shown here that both proteins are substrates of thioredoxin. Mxr1 is required to utilize Met-(S)-sulfoxide as the only Met source in cells auxotrophic for this amino acid, which agrees with the predicted role of other protein members of the MsrA family of enzymes in free Met sulfoxide recycling; Mxr2 has, if any, a very secondary role in the reduction of oxidized Met (compare the growth of strains Δmet6 and $\Delta\text{met6} \Delta\text{mxr2}$ in Fig. 2B). Another conclusion from our study is that fission yeast can utilize only one enantiomer, S, as the only Met source. Interestingly enough, mammalian cells have been also described to be unable to reduce the (R)-enantiomer, even though they express three MsrB isozymes (Lee *et al.*, 2008). Met is an essential amino acid in animal cells, and has to be provided with the diet, and therefore the inability to use the oxidized Met-(R)-O as a Met source seems to be a metabolic disadvantage. Why bacteria and budding yeast have free Met-(R)-O reducing capacity and *S. pombe* and higher eukaryotes do not is, at the very least, puzzling.

As noted in the Introduction, Met in proteins has been proposed to be a target of oxidation by peroxides, with this post-translational modification altering the protein structure and potentially inducing loss-of-function, protein carbonylation (Moskovitz *et al.*, 2001; Moskovitz and Oien, 2010) and protein aggregation/degradation. Therefore, Msrs have been suggested to act as antioxidant enzymes able to repair damaged proteins. Indeed, it has also been proposed that Met in proteins, and their recycling by Msrs, can serve as an antioxidant strategy since they can be a primary target of H_2O_2 -driven damage. We tried to confirm these hypotheses in fission yeast by immuno-blotting protein extracts with commercial anti-Met sulfoxide antibodies, but the pattern of oxidation did not change upon H_2O_2 addition or in extracts from Δmxr1 cells (data not shown). It is worth pointing out that the usefulness of these commercial antibodies is currently a matter of debate (Moskovitz, 2013; Wehr and Levine, 2012, , 2013). We have only been able to demonstrate that the participation of Mxr1 in the antioxidant stress defence is somehow

secondary to its role in recycling the available free Met pool, and can only be highlighted in cells unable to synthesize Met. Thus, while $\Delta met6$ cells are very sensitive to H_2O_2 , the double deletion strain $\Delta met6 \Delta mxr1$ is 100-fold more sensitive to peroxides on plates.

The idea of free Met from the culture media being a first line of defence against peroxides comes from the analysis of the $\Delta met6$ phenotype. While analyzing the role of Met6 in the sulfur metabolic pathways (Fig. 5A), we first tested whether intracellular Cys accumulation in $\Delta met6$ cells could contribute to H_2O_2 sensitivity. We discarded this hypothesis since the double deletion strains $\Delta cys1a \Delta met6$ and $\Delta met16 \Delta met6$ are still sensitive to peroxides (Fig. S7A). Secondly, homoserine accumulation does not seem to be the cause of H_2O_2 sensitivity of $\Delta met6$ cells, since over-expression of enzymes from the methyl cycle, Thr1 or Sam1, did not suppress the oxidative stress phenotype of strain $\Delta met6$ (Fig. S7B). We were left with the idea that Met depletion from the culture media had to be the cause of growth cessation of $\Delta met6$ strain upon H_2O_2 stress. Indeed, gradual increments of Met could suppress the deleterious effects of peroxides, through a double effect: providing the Met required for protein synthesis first, and blocking H_2O_2 toxicity by establishing a first target barrier.

Our experiments therefore indicate that addition of Met in the media may block H_2O_2 toxicity, preventing cell damage. But they also suggest that in cells unable to synthesize Met, such as $\Delta met6$ strain, and which therefore rely on extracellular Met for protein synthesis, the role of Msrs would be essential to permit reduction of free Met sulfoxide and therefore allow protein synthesis. It is worth to point out that the *mxr1* and *mxr2* mRNAs are triggered around 2-fold only in cells lacking Met6 when they grow in the presence of oxidized Met (Fig. S8), what seems to confirm that Msrs are important in cells auxotrophic for Met. This may not seem essential in all microbes, but some model systems which have been used for testing the role of Msrs in oxidative stress survival happen to be auxotrophic for Met [that is the case of *Helicobacter pylori* (Alamuri and Maier, 2004; Nedenskov, 1994), or some strains used for studies in the budding yeast (Kryukov *et al.*, 2002)]. Furthermore, Met has to be provided to mammals with food (Fukagawa, 2006), and therefore we propose that in animal cells recycling free oxidized Met has to be the main role of Msrs.

EXPERIMENTAL PROCEDURES

Materials

H₂O₂, L-methionine, L-methionine-(R,S)-sulfoxide, glutathione (GSH), sodium hypochlorite (NaOCl), sodium nitrite, S-nitrosoglutathione (SGNO), *tert*-butyl hydroperoxide (*t*-BOOH), cadmium sulphate (CdSO₄), diamide, and menadione are from Sigma-Aldrich. Cys is from Merck.

Strains and growth conditions

The genotypes of strains used in this study are outlined in Table 1. Strains were grown in standard *S. pombe* media [minimal media (MM) or rich media containing 0.25 g/l of lysine, adenine, uracil, leucine, and histidine (YE5S)] as described (Alfa *et al.*, 1993).

Plasmids

The *mxr1*, *sam1*, and *thr1* coding sequences were PCR amplified from an *S. pombe* cDNA library using primers specific for the open reading frames. All PCR-fragments were cloned into the *nmt* (*no message in thiamine*)-driven expression vector pRep.3x (Maundrell, 1993), which additionally included a DNA fragment coding for the HA epitope, inserted between the *nmt* promoter and the three different ORFs, to yield p474.3x (*pHA-mxr1.3x*), p438.3x (*pHA-sam1.3x*), and p439.3x (*pHA-thr1.3x*), respectively.

Survival and auxotrophy assays by sequential spots

Strains were grown at 30°C in YE5S media until they reached an OD₆₀₀ ~ 0.5. Cells were then diluted from 5x10⁷ to 5x10² in serial 1/10 dilutions in a 96-well microplate. 2 µl of each concentration, containing from 10⁵ to 10 cells, were then spotted with a replicator on solid agar plates of YE5S or MM containing the indicated concentrations of stressors or Cys, Met, Met-(R,S)-O, and GSH. For the auxotrophy assays, cells were washed twice in MM prior to dilution. For the stationary phase experiment, an OD₆₀₀ ~ 0.5 was considered only for the first-day plate.

Preparation of the two diastereoisomers of Met-O

1 L-Met-(S)-sulfoxide and L-Met-(R)-sulfoxide were prepared according to previously described
2 (Sharov *et al.*, 1999) with some minor modifications. Briefly, 1.2 g of L-Met-(R,S)-sulfoxide, with
3 an approximate composition of 50% R / 50% S according to the manufacturer (Sigma), were
4 dissolved in 8 ml of H₂O in a boiling water bath. After slight cooling, a methanolic solution of
5 picric acid (2 g wet weight/16 ml) was slowly added to give a yellow precipitate. After cooling to
6 room temperature, the filtrate was separated from the supernatant by centrifugation (this
7 supernatant was kept to liberate L-Met-(R)-O, see below), washed with ice-cold methanol and re-
8 dissolved in 20 ml of H₂O. To liberate the sulfoxide, the required amount of amylamine (Sigma)
9 was added to reach a pH to around 8.0. L-Met-(S)-O was re-precipitated by addition of 200 ml of
10 acetone, and collected by filtration in cellulose paper. The filtrate was washed three times with
11 acetone and dried under vacuum yielding 263 mg of dried product. The product had a specific
12 rotation of $[\alpha]_D^{25} = +81.9^\circ$ in H₂O, indicating a mixture of 90.1 % L-Met-(S)-O and 9.9 % L-Met-
13 (R)-O. The original supernatant was evaporated under vacuum to remove methanol, re-dissolved
14 in 20 ml H₂O and the required amount of amylamine to reach pH 8.0. 200 ml of acetone were
15 added to re-precipitate the free sulfoxide. The pellet was filtrated through cellulose paper,
16 washed three times with acetone and dried yielding 255 mg of dried product. To obtain a higher
17 enrichment in L-Met-(R)-O, the whole procedure was repeated again with the obtained product,
18 yielding at the end 130 mg of powder. This had a specific rotation of $[\alpha]_D^{25} = -64.1^\circ$ in H₂O,
19 measured on a Perkin-Elmer model 241 polarimeter, indicating a mixture of 82 % L-Met-(R)-O
20 and 18 % L-Met-(S)-O.

22 **Preparation of *S. pombe* trichloroacetic acid (TCA) extracts and immunoblot analysis**

23 To analyze the *in vivo* redox status of Msrs, TCA extracts *S. pombe* cultures (5 ml) at an OD₆₀₀ of
24 0.5 were pelleted just after the addition of 10% TCA (from a 100% stock) and washed in 20%
25 TCA. The pellets were resuspended in 100 µl of 12.5% TCA and lysed with glass beads by
26 vortexing for 5 min. Cell lysates were pelleted, washed in ice-cold acetone, and dried at 55°C for
27 8 min. Pellets were resuspended and incubated for 15 min in 50 µl of a solution containing 1%
28 SDS, 100 mM Tris-HCl (pH 8.0), 1 mM EDTA and 75 mM iodoacetamide, allowing in this way
29 alkylation of free thiols in proteins. The alkylated samples were electrophoretically separated by
30 non-reducing [loading buffer without dithiothreitol (DTT)] or reducing (loading buffer with DTT)

SDS-PAGE, and proteins were immunodetected with house-made monoclonal anti-HA antiserum (12CA5).

RNA analysis

Total RNA from exponentially growing *S. pombe* cells was extracted, processed, and transferred to a membrane as previously reported (Castillo *et al.*, 2002). Membranes were hybridized with [α - 32 P] dCTP-labeled *mxr1*, *mxr2*, and *act1*. Ribosomal RNA (rRNA) and *act1* were used as loading controls.

Growth curves in liquid

To measure cellular growth, we used an assay based on automatic measurements of optical densities for small (100 μ l) cell cultures. Cells were grown to an OD₆₀₀ of 0.5 under continuous shaking in Erlenmeyer flasks and then diluted to an OD₆₀₀ of 0.1. 100 μ l of the diluted cultures (treated or not with the indicated amounts of stressors or Met sources) were placed into a 96-well non-coated polystyrene microplate covered with an adhesive plate seal. A Power Wave microplate scanning spectrophotometer (Bio-Tek) was used to obtain the growth curves. The OD₆₀₀ was automatically recorded using Gen5 software. The software was set as follows: OD was measured at 600 nm, incubation temperature was kept at 30°C, the microplates were subjected to continuous shaking and the readings were done every 10 min during 48 hours.

ACKNOWLEDGEMENTS

We thank David Andreu and members of his laboratory for advice regarding Met sulfoxide analysis. We also acknowledge Rosa Giera and Cristina Minguillón for providing a polarimeter for enantiomer characterization. This work was supported by the Spanish Ministry of Science and Innovation (BFU2009-06933, BFU2012-32045), PLAN E and FEDER, by the Spanish program Consolider-Ingenio 2010 Grant CSD 2007-0020, and by SGR2009-196 from Generalitat de Catalunya (Spain) to E.H. E. H. and J.A. are recipients of ICREA Academia Awards (Generalitat de Catalunya). The authors declare no conflict of interests.

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1 Table 1. Genotypes of strains used in this study

Strain	Genotype	Origin
972	<i>h⁻</i>	(Leupold, 1970)
PF5	<i>h⁻ mxr1::kanMX6</i>	This work
PF4	<i>h⁻ mxr2::kanMX6</i>	This work
PF10	<i>h⁻ mxr1::kanMX6 mxr2::natMX6</i>	This work
SG138	<i>h⁻ met6::hph</i>	This work
SG136	<i>h⁻ met6::hph mxr1::kanMX6</i>	This work
SG143	<i>h⁻ met6::hph mxr2::kanMX6</i>	This work
SG137	<i>h⁻ met6::hph mxr1::kanMX6 mxr2::natMX6</i>	This work
SG240	<i>h⁻ met6::hph trx3::kanMX6</i>	This work
SG231	<i>h⁻ met6::hph grx1::kanMX6</i>	This work
SG191	<i>h⁻ met6::hph trx1::ura4+ ura4-D18 grx1::kanMX6</i>	This work
SG52	<i>h⁻ mxr1-HA::natMX6</i>	This work
SG65	<i>h⁻ mxr1-HA::natMX6 trx1::kanMX6</i>	This work
SG183	<i>h⁻ mxr1-HA::kanMX6 trx1.C33S</i>	This work
SB43	<i>h⁻ mxr2-HA::natMX6</i>	This work
SB51	<i>h⁺ mxr2-HA::natMX6 trx1::kanMX6</i>	This work
SG182	<i>h⁻ mxr2-HA::kanMX6 trx1.C33S</i>	This work
AV25	<i>h⁻ pap1::kanMX6</i>	(Jara <i>et al.</i> , 2008)
AV18	<i>h⁻ sty1::kanMX6</i>	(Zuin <i>et al.</i> , 2005)
SG178	<i>h⁻ cys1a::ura4+ ura4-D18</i>	(Garcia-Santamarina <i>et al.</i> , 2013)
NG77	<i>h⁻ gcs1::kanMX6</i>	This work
SG171	<i>h⁺ met16::kanMX6</i>	(Garcia-Santamarina <i>et al.</i> , 2013)
666	<i>h⁺ ade6-M216 ura4-D18 leu1-32</i>	(Kim <i>et al.</i> , 2010)
Δ <i>mxr1</i>	<i>h⁺ mxr1::kanMX6 ade6-M216 ura4-D18 leu1-32</i>	(Kim <i>et al.</i> , 2010)
SG257	<i>h⁻ trx3::nat</i>	This work
SB36	<i>h⁺ grx1::kanMX6 ura4-D18</i>	This work
MJ15	<i>h⁺ trx1::kanMX6</i>	(Garcia-Santamarina <i>et al.</i> , 2013)
SG239	<i>h⁻ met6::hph cys1a::ura4+ ura4-D18</i>	This work
SG246	<i>h⁻ met6::hph met16::kanMX6</i>	This work
SG119	<i>h⁺ met6::hph ade6-M216 ura4-D18 leu1-32</i>	This work

2

FIGURE AND TABLE LEGENDS

Fig. 1. *S. pombe* MsrA/Mxr1, but not MsrB/Mxr2, is able to utilize free oxidized Met as a Met source.

A. Schematic representation of *S. pombe* Msrs. The relative positions of all cysteine residues (C) and conserved regions (striped boxes) are indicated. In both proteins, all Cys are conserved when comparing the sequence with other organisms (i.e. *E. coli*). By sequence homology, C10 and C122 are the catalytic Cys of Mxr1 and Mxr2, respectively, whereas C159 and C67 are the recycling Cys of Mxr1 and Mxr2, respectively (Boschi-Muller *et al.*, 2005; Lowther *et al.*, 2002).

B. *S. pombe* cells lacking Met6 and Mxr1 do not grow in Met sulfoxide as a sole source of Met. Exponentially growing cultures of strains 972 (WT), PF5 ($\Delta m x r 1$), PF4 ($\Delta m x r 2$), PF10 ($\Delta m x r 1 \Delta m x r 2$), SG138 ($\Delta m e t 6$), SG136 ($\Delta m x r 1 \Delta m e t 6$), SG143 ($\Delta m x r 2 \Delta m e t 6$), and SG137 ($\Delta m x r 1 \Delta m x r 2 \Delta m e t 6$) were serial diluted and spotted on YE5S, MM, MM containing 0.14 mM Met, and in MM containing 5 mM Met sulfoxide (Met-O). This experiment is representative of four biological replicates.

Fig. 2. *S. pombe* is only able to reduce the Met-(S)-O enantiomer (but not the R) in an MsrA/Mxr1-dependent manner.

A. Exponentially growing cultures of strains SG138 ($\Delta m e t 6$), SG136 ($\Delta m x r 1 \Delta m e t 6$), SG143 ($\Delta m x r 2 \Delta m e t 6$), and SG137 ($\Delta m x r 1 \Delta m x r 2 \Delta m e t 6$) in rich media (YE5S) were washed twice with MM, diluted to an OD₆₀₀ of 0.1 and grown into micro-culture wells in MM with the indicated amounts of Met, Met-(S,R)-O, Met-(S)-O, and Met-(R)-O. Growth was monitored by measuring OD₆₀₀ every 10 min at 30 °C for 25 hours. This experiment is representative of three biological replicates.

B. Exponentially growing cultures of strains 972 (WT), SG138 ($\Delta m e t 6$), SG136 ($\Delta m x r 1 \Delta m e t 6$), SG143 ($\Delta m x r 2 \Delta m e t 6$), and SG137 ($\Delta m x r 1 \Delta m x r 2 \Delta m e t 6$) in rich media (YE5S) were washed twice with MM, serial diluted and spotted on MM plates containing the indicated concentrations of Met, Met-(S,R)-O, Met-(S)-O and Met-(R)-O. This experiment was done once.

Fig. 3. Trx1 and Grx1 are alternative electron donors for Mxr1 in *S. pombe*.

A. Scheme representing the enzymatic steps of Msr catalysis, and recycling by an electron donor. A catalytically active Cys in the Msr nucleophilically attacks on the sulphur of the Met-O substrate resulting in sulfurane-to-sulfonium cation intermediates, to then release reduced Met and a sulfenic acid (SOH) in the Msr. The SOH readily reacts with a different Cys on the Msr to yield an intramolecular disulfide. The catalytically active Cys of a reduced electron donor (E) breaks the Msr disulfide to form a transient Msr-E intermediate. Oxidized E is then released and the Msr is reduced and ready again to enter in a new catalytic cycle. Adapted from Dokainish and Gauld, 2013).

B. *In vivo* electron donors for *S. pombe* Mxr1. Cultures of exponentially growing strains 972 (WT), SG138 ($\Delta met6$), SG128 ($\Delta trx1 \Delta met6$), SG231 ($\Delta grx1 \Delta met6$), SG240 ($\Delta trx3 \Delta met6$), and SG191 ($\Delta grx1 \Delta trx1 \Delta met6$) were serially diluted and spotted on MM, MM containing 0.14 mM Met, and in MM containing 5 mM Met sulfoxide (Met-O). This experiment is representative of five biological replicates.

C. Trapping the transient Mxr1- and Mxr2-Trx1 mixed disulfide using the mutant Trx1.C33S. Upper panel, immuno-detection of Mxr1-HA from TCA extracts of strains SG52 (*mxr1-HA* WT), SG65 (*mxr1-HA* $\Delta trx1$), SG183 (*mxr1-HA* *trx1.C33S*), under non-reducing (-DTT) and reducing (+DTT) electrophoresis. Lower panel, immunodetection of Mxr2-HA from TCA extracts of strains SB43 (*mxr2-HA* WT), SB51 (*mxr2-HA* $\Delta trx1$), SG182 (*mxr2-HA* *trx1.C33S*), under non-reducing (-DTT) and reducing (+DTT) electrophoresis. Since Cys33 in Trx1 resolves mixed disulfides with its substrates, the Trx1.C33S mutant allows *in vivo* trapping of an intermolecular disulfide of Trx1 either with Mxr1 or with Mxr2.

Fig. 4. *S. pombe* cells lacking Msrs are not sensitive to oxidative stress, unless they are auxotrophic for Met.

A. Transcriptional analysis of *mxr1* and *mxr2* in oxidative stress. Cultures of strains 972 (WT), AV25 ($\Delta pap1$), PF5 ($\Delta mxr1$), PF4 ($\Delta mxr2$), and PF10 ($\Delta mxr1 \Delta mxr2$) were treated (+) or not (-) with 0.2 mM H₂O₂ during 15 min. RNA was obtained and analyzed by Northern blot with probes for *mxr1* and *mxr2*. *act1* and ribosomal RNA (*rRNA*) were used as loading controls. This experiment is representative of two biological replicates.

B. Absence of Mxr1 and/or Mxr2 does not trigger sensitivity to H₂O₂. Exponentially growing cultures of strains 972 (WT), AV18 ($\Delta sty1$), PF5 ($\Delta mxr1$), PF4 ($\Delta mxr2$), and PF10 ($\Delta mxr1 \Delta mxr2$)

1 were serial diluted and spotted on plates without (untreated) or with 1 mM H₂O₂. This experiment
2 is representative of two biological replicates.

3 C. Lack of Msrs does not protect from acute oxidative stress in fission yeast. Exponentially
4 growing cultures of strains as in A were left untreated or were treated with 25 mM H₂O₂ (arrow)
5 for the indicated times. Upon treatment cells were serial diluted and spotted on YE5S plates.
6 This experiment is representative of two biological replicates.

7 D. Lack of Msrs does not affect chronological aging in *S. pombe*. Strains as in B were grown in
8 MM. Serial dilutions of the logarithmic (day 1) and stationary phase (day 8 and day 22) cultures
9 were spotted onto MM plates. This experiment is representative of two biological replicates.

10 E. Cells lacking Mxr1 and auxotrophic for Met are very sensitive to H₂O₂. Exponentially growing
11 cultures of strains 972 (WT), AV25 ($\Delta pap1$), AV18 ($\Delta sty1$), SG138 ($\Delta met6$), SG143 ($\Delta mxr2$
12 $\Delta met6$), SG136 ($\Delta mxr1 \Delta met6$), and SG137 ($\Delta mxr1 \Delta mxr2 \Delta met6$) were serial diluted and spotted
13 on MM plates containing either 0.08 mM Met (untreated) and either 0.08 mM Met or 0.28 mM Met
14 and 0.5 mM H₂O₂. This experiment is representative of five biological replicates.

15
16 **Fig. 5.** Only mutant *S. pombe* cells which require Met for growth are sensitive to H₂O₂ stress.

17 A. Schematic representation of the Met, Cys and GSH biosynthetic pathways. Enzymatic roles
18 for Met16, Met6, Cys1a, and Gcs1 are indicated.

19 B. Nutritional requirements of cells lacking Cys1a, Gcs1, Met6 and Met16. Cultures of
20 exponentially growing strains 972 (WT), SG178 ($\Delta cys1a$), NG77 ($\Delta gcs1$), SG138 ($\Delta met6$), and
21 SG171 ($\Delta met16$) were serial diluted and spotted on YE5S, or on MM plates containing the
22 indicated concentrations of Met, Cys and GSH. This experiment is representative of three
23 biological replicates.

24 C. Oxidized Met is also a Met source for cells with Met auxotrophy. Cultures of exponentially
25 growing strains 972 (WT), and SG138 ($\Delta met6$) were serial diluted and spotted on MM plates, or
26 on MM plates containing the indicated concentrations of Met and Met sulfoxide (Met-O). This
27 experiment is representative of two biological replicates.

28 D. Table summarizing the nutritional requirements of cells lacking Cys1a, Gcs1, Met6 or Met16.

29
30 **Fig. 6.** Increasing extracellular concentrations of Met ameliorate the sensitivity to H₂O₂ of $\Delta met6$.

1 A. $\Delta met6$ strain is as sensitive as $\Delta pap1$ to peroxides. Cultures of exponentially growing strains
2 972 (WT), AV25 ($\Delta pap1$), SG138 ($\Delta met6$), SG171 ($\Delta met16$), SG178 ($\Delta cys1a$), and NG77 ($\Delta gcs1$),
3 were serial diluted and spotted on MM plates containing the indicated concentrations of Met, Cys
4 and GSH and H_2O_2 . This experiment is representative of two biological replicates.

5 B. Extracellular Cys is only able to partially restore the growth of a $\Delta met6$ strain upon oxidative
6 stress. Cultures of exponentially growing strains 972 (WT), AV25 ($\Delta pap1$), AV18 ($\Delta sty1$), and
7 SG138 ($\Delta met6$) were serial diluted and spotted on MM plates containing the indicated
8 concentrations of Met, Cys and H_2O_2 . This experiment is representative of two biological
9 replicates.

10 (C to E) $\Delta met6$ is not intrinsically sensitive to heat shock or oxidative stress, but rather to Met
11 depletion in the growing media.

12 C. Exponentially growing cultures of strains 972 (WT), AV18 ($\Delta sty1$), PF5 ($\Delta mxr1$), and SG138
13 ($\Delta met6$) were left untreated or were treated with 25 mM H_2O_2 (arrow) for the indicated times.
14 Upon treatment cells were serial diluted and spotted on MM plates containing 0.14 mM Met. This
15 experiment is representative of two biological replicates.

16 D. Exponentially growing cultures of strains 972 (WT), AV18 ($\Delta sty1$), AV25 ($\Delta pap1$), and SG138
17 ($\Delta met6$) were serial diluted and spotted on MM plates containing the indicated concentrations of
18 Met and incubated either at 30°C or at 37°C as indicated. This experiment is representative of
19 four biological replicates.

20 E. Cultures of exponentially growing strains 972 (WT), AV18 ($\Delta sty1$), and SG138 ($\Delta met6$) were
21 incubated either at 30°C (untreated) or at 48°C for the indicated times (arrow) and then serial
22 diluted and spotted on MM plates containing 0.08 mM Met; plates were incubated at 30°C. This
23 experiment is representative of two biological replicates.

24
25 **Table 1. Genotypes of strains used in this study**

García-Santamarina et al. Fig. 1

B

García-Santamarina et al. Fig. 2

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