



Mitochondrial localization of fission yeast manganese superoxide dismutase is required for its lysine acetylation and for cellular stress resistance and respiratory growth

Hidekazu Takahashi^{a,*}, Takehiro Suzuki^{b,c}, Atsuko Shirai^{a,1}, Akihisa Matsuyama^a, Naoshi Dohmae^{b,c}, Minoru Yoshida^{a,c,*}

^a Chemical Genetics Laboratory/Chemical Genomics Research Group, RIKEN Advanced Science Institute, Wako, Saitama 351-0198, Japan

^b Biomolecular Characterization Team, RIKEN Advanced Science Institute, Wako, Saitama 351-0198, Japan

^c CREST Research Project, Japan Science and Technology Corporation, Kawaguchi, Saitama 332-0012, Japan

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ABSTRACT

Manganese-dependent superoxide dismutase (MnSOD) is localized in the mitochondria and is important for oxidative stress resistance. Although transcriptional regulation of MnSOD has been relatively well studied, much less is known about the protein's posttranslational regulation. In budding yeast, MnSOD is activated after mitochondrial import by manganese ion incorporation. Here we characterize posttranslational modification of MnSOD in the fission yeast *Schizosaccharomyces pombe*. Fission yeast MnSOD is acetylated at the 25th lysine residue. This acetylation was diminished by deletion of N-terminal mitochondrial targeting sequence, suggesting that MnSOD is acetylated after import into mitochondria. Mitochondrial localization of MnSOD is not essential for the enzyme activity, but is crucial for oxidative stress resistance and growth under respiratory conditions of fission yeast. These results suggest that, unlike the situation in budding yeast, *S. pombe* MnSOD is already active even before mitochondrial localization; nonetheless, mitochondrial localization is critical to allow the cell to cope with reactive oxygen species generated inside or outside of mitochondria.

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1. Introduction

Living organisms utilize a variety of antioxidant mechanisms. Cells are constantly exposed to reactive oxygen species (ROS), such as superoxide radicals. ROS are generated either internally in the mitochondria as a byproduct of metabolic reactions such as oxidative phosphorylation, or externally in the environment. Superoxide dismutase (SOD) is a key enzyme that protects the cell from ROS. SOD catalyzes dismutation of superoxide anions into oxygen and hydrogen peroxide. Most eukaryotes contain two or more families of genes that encode SODs. These SOD families differ in metal cofactor and subcellular localization. Copper/zinc-dependent SOD (CuZnSOD) is primarily localized in the cytosol, and requires copper and zinc as cofactors. On the other hand, manganese-dependent SOD (MnSOD) is mainly localized in the mitochondria, due

to the presence of a mitochondrial targeting sequence (MTS). Fission yeast *Schizosaccharomyces pombe* contains both CuZnSOD and mitochondrial MnSOD [1–3]. Deletion of either one of two SOD genes results in hypersensitivity to oxidative stress-generating agents, such as menadione [1,2].

The mechanism of regulation of MnSOD expression has been well studied. In *Saccharomyces cerevisiae*, MnSOD is induced upon stress or the diauxic shift. Induction of budding yeast MnSOD expression depends on the Msn2/4p stress-responsive transcription factors and/or the Hap heme activator complex [4,5]. MnSOD is required for normal lifespan in *S. cerevisiae* [6,7]. In *S. pombe*, MnSOD is transcriptionally up-regulated via the Wis1-Sty1 MAPK pathway when cells are treated with heat, KCl or menadione [1].

Unlike transcriptional regulation, the posttranslational regulation of MnSOD remains relatively unexplored. In *S. cerevisiae*, MnSOD localized in the cytosol after translation is inactive due to the absence of the manganese cofactor that associates with MnSOD after mitochondrial import [8]. It is unknown whether such regulation by metal binding and/or other forms of posttranslational regulation also occurs in *S. pombe* MnSOD. Recently, MnSOD was identified by a high-throughput immunoblot screening as one of the lysine-acetylated proteins of fission yeast [9]. In

* Corresponding authors at: Chemical Genetics Laboratory/Chemical Genomics Research Group, RIKEN Advanced Science Institute, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan. Fax: +81 48 462 4676.

E-mail addresses: hidetakashi@riken.jp (H. Takahashi), yoshidam@riken.jp (M. Yoshida).

¹ Present address: Laboratory for Chromatin Dynamics, RIKEN Center for Developmental Biology, Kobe 650-0047, Japan.

this study, we analyzed the role played by the mitochondrial localization signal and lysine acetylation of the *S. pombe* MnSOD in determining the protein's catalytic activity and cellular oxidative stress resistance.

2. Materials and methods

2.1. Fission yeast strains

The fission yeast strains used in this study are listed in [Supplementary Table 1](#). We generated plasmids containing the native MnSOD gene promoter, followed by each MnSOD wild-type/mutant construct with a C-terminal FLAG₂-His₆ (MnSOD–FFH) tag, as detailed in [Supplementary methods](#). The DNA primers used in this study are listed in [Supplementary Table 2](#). These plasmids were digested with NotI and then integrated into the *leu1-32* locus [10] of the MnSODΔ mutant named SpHT84, in which the entire SPAC1486.01 ORF was replaced by the *hphMX* cassette amplified from pAG32 [11].

2.2. Culture media

Rich YE medium contains 5 g/L yeast extract and 20 g/L glucose. Agar was added to a final concentration of 20 g/L. Minimal MB medium contains 0.5 g/L KH₂PO₄, 0.36 g/L KOAc, 0.5 g/L MgSO₄, 0.1 g/L NaCl, 0.1 g/L CaCl₂, 5 g/L (NH₄)₂SO₄, 5 g/L glucose, 500 μg/L H₃BO₃, 40 μg/L CuSO₄, 100 μg/L KI, 200 μg/L FeCl₃, 400 μg/L MnSO₄, 200 μg/L Na₂MoO₄, 400 μg/L ZnSO₄, 10 μg/L biotin, 1 mg/L calcium pantothenate, 10 mg/L nicotinic acid and 10 mg/L myo-inositol [12].

2.3. Purification of MnSOD

Each MnSOD–FFH protein was expressed under the MnSOD promoter integrated into the *leu1* locus on chromosome 2. To purify MnSOD–FFHs, fission yeast strains were cultured in liquid MB and harvested at OD₆₀₀ = ~3. MnSOD–FFHs were purified using the His₆-tag. To determine acetylation sites, purification was carried out under denaturing conditions, whereas a non-denaturing purification was utilized for the *in vitro* MnSOD assay. These procedures are detailed in [Supplementary methods](#).

2.4. Immunoblotting

Similar amounts of purified MnSODs were separated on a 12% SDS–PAGE gel and transferred onto an ImmobilonFL PVDF membrane (Millipore). The membrane was blocked for 90 min in PBSTSM (phosphate-buffered saline containing 0.1% Tween-20 and 3% Difco skim milk). To detect lysine acetylation, the membrane was incubated overnight at 4 °C in PBSTSM containing an AKL5C1 anti-acetyl-lysine mouse antibody (1:100, Santa Cruz Biotechnology) and an anti-His₆-tag rabbit antibody (1:5000, MBL International). The membrane was washed four times with PBST and incubated in PBSTSM containing an Alexa Fluor 680-conjugated goat anti-mouse IgG (1:5000, Invitrogen) and an IRDye 800CW-conjugated goat anti-rabbit IgG (1:10,000, Rockland). The membrane was washed four times in PBST. The fluorescent signals were detected with an Odyssey Imaging System (Li-COR).

2.5. Mass spectrometry

MnSOD–FFH was purified from a wild-type strain, SpHT122 under denaturing conditions as described above. An aliquot of the protein was run on a 5–20% SuperSep SDS–PAGE gel (Wako) and stained with Quick-CBB PLUS (Wako). The MnSOD band was

reduced with dithiothreitol, carboxymethylated by iodoacetic acid, and digested with trypsin. An aliquot of the digest was analyzed by nano-liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) as previously described [13].

2.6. Multiple alignment of MnSOD amino acid sequences

Multiple alignment of MnSOD orthologs in eight model organisms was generated by the ClustalW program [14]. The GenBank accession numbers used are as follows: *Homo sapiens*, NP_001019636; *Mus musculus*, NP_038699; *Caenorhabditis elegans*, AAB53822; *Drosophila melanogaster*, NP_476925; *Arabidopsis thaliana*, NP_187703; *S. cerevisiae*, AAS56147; *S. pombe*, NP_594089; *Escherichia coli*, NP_418344.

2.7. In vitro SOD assay

MnSOD–FFHs were purified in parallel from SpHT121, 122, 142, 172, 198 and 109 under non-denaturing conditions as described above. The amount of MnSOD–FFH in each sample was determined by immunoblotting followed by densitometry. Each protein was twofold serially diluted, and only signals in the linear range were quantified. The SOD activity was assayed using an SOD Assay Kit–WST (Dojin). Each sample was diluted such that the activity is never saturated. To calculate specific activity, the average value of three measurements was divided by the amount of protein. Specific activities are reported relative to that of wild type, which is set to 1. Averages and standard deviations shown in [Fig. 4](#) were calculated from the results of three independent culturing of the strains.

3. Results and discussion

3.1. MnSOD is acetylated probably after mitochondrial import in fission yeast

Global analysis of protein acetylation in *S. pombe* suggested that MnSOD is an acetylated protein [9]. To confirm the acetylation of lysine residue(s), we expressed C-terminally tagged MnSOD under the native promoter in fission yeast. The purified protein was immunoblotted with an anti-acetyl lysine antibody ([Fig. 1A](#)). The wild-type (WT) MnSOD protein reacted with this antibody (second lane in each panel), confirming the previous immunoblot screening [9].

The N-terminal 21 amino acid residues of MnSOD correspond to a mitochondrial targeting sequence (MTS), which is cleaved upon the import of MnSOD into the mitochondrion ([Fig. 2A](#)) [1]. To see whether MnSOD is acetylated before or after mitochondrial import, we expressed a version of MnSOD that lacked the 2nd–21st amino acid residues. If MnSOD is acetylated in the cytosol before transport into the mitochondrion, then keeping MnSOD in the cytosol by deletion of the MTS should not decrease the acetylation level. However, the MnSOD protein that lacked an MTS exhibited diminished acetylation (first lane in each panel of [Fig. 1A](#)), suggesting that MnSOD is acetylated not in the cytosol but in the mitochondria after import.

3.2. Identification of acetylated residues in fission yeast MnSOD

To locate the acetylated lysine residues of MnSOD, we systematically replaced each of the protein's 18 lysine (K) residues with arginine (R). These mutant MnSOD proteins were expressed and purified from fission yeast cells. Immunoblotting with the anti-acetyl lysine antibody demonstrated that only the 25th lysine residue (K25) was crucial for acetylation of MnSOD ([Fig. 1A](#)). This residue is therefore likely to be the major acetylation site *in vivo*.

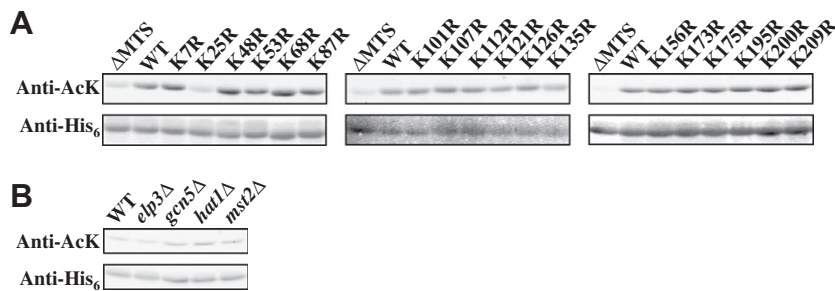


Fig. 1. MnSOD is acetylated at the 25th lysine residue. (A) Immunoblotting, using an anti-acetyl-lysine (anti-AcK) antibody, of MnSOD mutants in which individual lysine residues (K) have been substituted with arginine (R). Each protein was expressed under the native MnSOD promoter integrated at the *leu1* locus, and purified from fission yeast utilizing a C-terminal His₆-tag. Each membrane was re-blotted with anti-His₆ antibody. ΔMTS lacks the mitochondrial targeting sequence (the 2nd–21st residues). Strains used: SpHT121–140. (B) Immunoblotting, using an anti-acetyl-lysine antibody, of MnSODs purified from several known histone acetyltransferase gene deletion mutants. Strains used: SpHT105, 169, 170, 109 and 110.

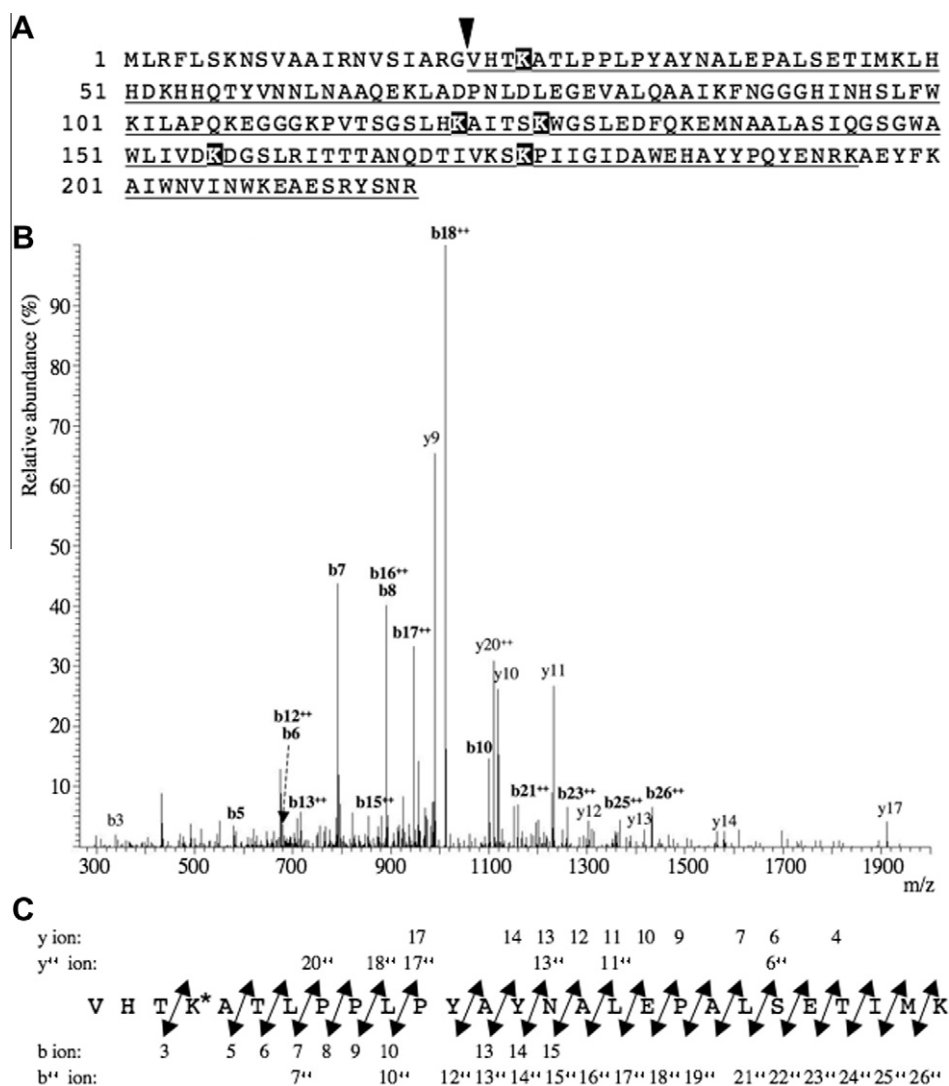


Fig. 2. Identification of acetylated lysine residues in MnSOD by tandem mass spectrometry (MS). (A) Amino acid sequence of MnSOD, with a summary of MS. The peptides covered by MS were underlined. Five candidate acetylated lysine (K) residues (K25, K121, K126, K156 and K175) detected were highlighted in black. The arrowhead indicates the mitochondrial targeting sequence cleavage site [1]. (B) The MS fragmentation spectra of the acetylated K25-containing peptide. The bold letters represent the peptides containing acetylated K25. Only some of the peaks are indicated. (C) The amino acid sequence of the acetylated K25 (indicated with “*”)–containing peptide. All the b, b⁺, y and y⁺ ions detected are denoted above or below the sequence.

None of 18 lysine-residue mutant proteins appeared larger in molecular weight than the ΔMTS mutant, suggesting that lysine acetylation is not required for cleavage of MTS from premature

MnSOD. We tested whether any of the four known histone acetyltransferase genes are involved in acetylation of MnSOD in vivo (Fig. 1B). None of the deletion mutants decreased the acetylation,

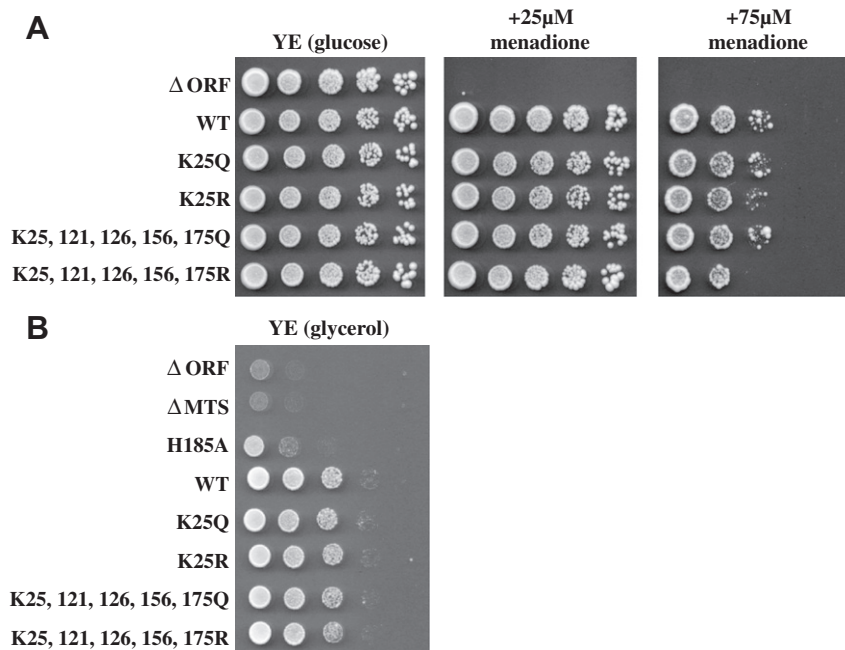


Fig. 3. Oxidative stress resistance of MnSOD lysine residue mutants. Each MnSOD mutant gene was expressed under the native MnSOD promoter integrated at the *leu1* locus in the MnSODΔ strain. (A) Serial dilution assay of the MnSOD acetylated lysine mutants, grown on rich media containing menadione. Strains used: SpHT142, 122, 154, 124, 172 and 209. The ΔORF strain contains the pDUAL-FFH vector only at the *leu1* locus (SpHT142). (B) Serial dilution assay of the MnSOD acetylated lysine mutants, grown on rich medium containing glycerol. Strains used: SpHT142, 121, 198, 122, 154, 124, 172 and 209.

raising the possibility that a unique acetylating mechanism/enzyme may exist in the mitochondrion.

To directly detect the acetylation at K25 of MnSOD, we conducted tandem mass spectrometry (LC–MS/MS) of purified wild-type MnSOD. We have identified five acetylated sites, K25, K121, K126, K156 and K175 (Fig. 2A), among which only the acetylation at K25 could be detected by immunoblotting (Fig. 1A). It is not clear why we did not detect acetylation at the other four sites by immunoblotting. It is possible that the acetylation levels at the other four residues are lower than the detection threshold level, or that the anti-acetyl lysine antibody has lower affinity toward these four sites.

To see whether the acetylated residues are conserved throughout evolution, we aligned *S. pombe* MnSOD amino acid sequence with orthologs in other model organisms (Supplementary Fig. 1). None of the acetylated lysine residues was invariantly conserved in all eight species, although K25 and K156 were retained at the equivalent positions in five out of the other seven species. Mass spectrometry-based proteome-wide determination of human acetylated proteins identified two acetylated lysine residues in human MnSOD [15]. Very recently, mouse MnSOD activity was shown to be regulated by the acetylation at K122 [16]. However, none of the three lysine residues identified in mammals were in identical positions to the acetylation site in fission yeast, nor were they conserved in the fission yeast ortholog (Supplementary Fig. 1).

3.3. None of the lysine residues of MnSOD are required for oxidative stress resistance in fission yeast

Fission yeast MnSODΔ mutant is hypersensitive to an oxidative stress reagent, menadione [1]. We analyzed the effects of arginine substitution of each lysine residue of MnSOD on menadione resistance. Arginine mimics non-acetylated lysine. Unexpectedly, none of the lysine residues, irrespective of acetylation, was important for the menadione resistance (Supplementary Fig. 2A). We next mutated each lysine residue to glutamine (Q), which mimics

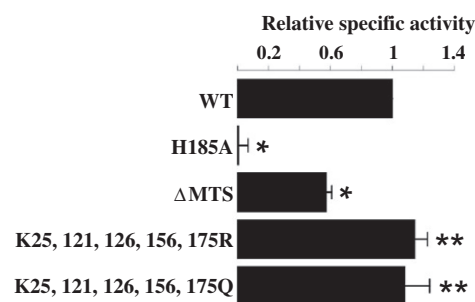


Fig. 4. *In vitro* SOD enzyme assay of the MnSOD mutants. The MnSODs with a C-terminal His₆-tag, which were expressed and purified from SpHT122, 198, 121, 172 and 209, were assayed as described in Section 2. The black bar represents the average value of specific activities of three independently purified samples. The values are reported relative to that of the wild-type (WT) sample. Standard deviations are shown. The single star represents the samples that were significantly different from WT (*p*-value <0.025). The double star denotes that the difference from WT was not significant.

acetyl-lysine. None of these glutamine-substituted mutants showed altered sensitivity to menadione compared to wild type (WT) (Supplementary Fig. 2B). To test the possibility that the five potentially acetylated residues are functionally redundant, we replaced all five lysine residues simultaneously with either arginine or glutamine residues. However, none of the resulting mutants exhibited obvious changes in menadione sensitivity (Fig. 3A). We also tested menadione resistance on minimal media in addition to rich medium; we obtained basically the same results on either media (data not shown). Thus, the physiological significance of MnSOD acetylation is unclear. We next examined whether MnSOD is important for fission yeast growth under respiratory conditions. We tested growth on rich medium containing 3% glycerol as the carbon source. Under these conditions, deletion of the entire ORF resulted in growth defect, suggesting that the level of endoge-

nously generated ROS becomes too high in the absence of MnSOD (Fig. 3B). Again, the acetylated residues were dispensable for growth under these conditions.

3.4. Mitochondrial localization of MnSOD is required for oxidative stress resistance in fission yeast

On the other hand, deletion of the MTS caused menadione hypersensitivity (Supplementary Fig. 2B, 2nd lane from the left). The Δ MTS mutant was as sensitive as the strain lacking the entire MnSOD ORF (Δ ORF). The Δ MTS mutant also exhibited a growth defect in YE containing glycerol as the sole carbon source (Fig. 3B, lane 2). In budding yeast, MnSOD that is present in the cytosol before mitochondrial import is inactive due to the lack of a manganese ion, which binds and activates MnSOD after mitochondrial import [8]. Therefore, it seemed likely that oxidative stress hypersensitivity caused by the deletion of the MTS from MnSOD is due to the lack of enzymatic activity of MnSOD when it cannot be imported into mitochondria. To test this possibility, we purified MnSOD from WT and the Δ MTS mutant, and compared their SOD activities. Unexpectedly, the MTS-deleted MnSOD retained ~60% of SOD activity relative to WT (Fig. 4, lane 3). This result implies that deletion of the MnSOD MTS caused oxidative stress hypersensitivity because ROS inside mitochondria can no longer be scavenged. The mutation of histidine residues critical for binding to manganese ion (H185) exhibited only a background level of activity (Fig. 4, lane 2), indicating that our purified samples are not contaminated by other SOD activities such as CuZnSOD. On the other hand, the simultaneous substitution of the five potentially acetylated lysine residues did not significantly alter enzymatic activity (Fig. 4, lanes 4 and 5). Again, acetylation at the five lysine residues may not be important, or may play functional roles in aspects other than catalytic activity.

In summary, we found that posttranslational regulation of MnSOD in fission yeast is different from that in budding yeast. The MnSOD present in the cytosol before mitochondrial import was fairly active, suggesting that a substantial amount of manganese ion is present in the cytosol and can be incorporated into the apoenzyme. In mammals, a large number of mitochondrial proteins are acetylated by unknown mechanisms [15,17,18]. Histone acetylation level reflects nucleocytoplasmic acetyl-coenzyme A metabolism [19], suggesting that the level of acetyl-coenzyme A is one of the determinants of protein acetylation. Acetylation of MnSOD at K25 depended on the protein's mitochondrial localization. Therefore, the fission yeast MnSOD will be a good model to study mitochondrial protein acetylation and mitochondrial acetyl-coenzyme A metabolism.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.01.103.

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