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NOTE

A Protective Role of Methionine-*R*-Sulfoxide Reductase against Cadmium in *Schizosaccharomyces pombe*

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The *Schizosaccharomyces pombe* cells harboring the methionine-*R*-sulfoxide reductase (MsrB)-overexpressing recombinant plasmid pFMetSO exhibited better growth than vector control cells, when shifted into fresh medium containing cadmium chloride (abbreviated as Cd). Although both groups of cells contained enhanced reactive oxygen species (ROS) and nitric oxide (NO) levels in the presence of Cd, ROS and NO levels were significantly lower in the *S. pombe* cells harboring pFMetSO than in vector control cells. Conversely, the *S. pombe* cells harboring pFMetSO possessed higher total glutathione (GSH) levels and a greater reduced/oxidized GSH ratio than vector control cells under the same conditions.

Keywords: cadmium, fission yeast, methionine-*R*-sulfoxide reductase, *msrB*$^+$, *Schizosaccharomyces pombe*

Cadmium (Cd^{2+}) is a redox-inactive heavy metal that cannot undergo simple oxidation reactions. Cadmium is an environmental toxin that can be liberated by natural phenomena, such as erosion of sedimentary rocks and volcanic eruptions, or by anthropogenic activities, including nickel-cadmium battery production, application of alloys and paints, and cigarette smoking (Hallenbeck, 1986; WHO, 2003). Its biochemical toxicity is generally attributed to four major processes: (1) induction of oxidative stress; (2) interference with intracellular signaling; (3) interference with DNA repair; and (4) competition with essential elements such as calcium, iron, zinc and manganese (Mielniczki-Pereira *et al.*, 2011). An alternative hypothesis for cadmium toxicity is the depletion of cytosolic glutathione (GSH) pools, which reduces the activities of glutathione-dependent enzymes that are involved in oxidative stress defense and other essential functions, namely glutathione peroxidases, glutathione S-transferases and glutaredoxins (Baudouin-Cornu and Labarre, 2006). Although the molecular mechanism by which cadmium leads to reactive oxygen species (ROS) production and oxidative stress is still largely unknown, it is expected to be an indirect mechanism, since cadmium cannot perform simple oxidation reactions (Gardarin *et al.*, 2010).

Cells are equipped with several mechanisms to counter cadmium toxicity. Bacteria use efflux pumps to expel cadmium ions (Silver and Phung, 1996). In *Saccharomyces cerevisiae* cells, cadmium ions are sequestered by metallothioneins due to their high cysteine content (Li *et al.*, 1997). In other eukaryotic cells, including the yeast *Schizosaccharomyces pombe*, phytochelatin synthase mediates a crucial function in cadmium detoxification through conversion of glutathione into phytochelatin, an oligomer of glutathione that binds cadmium, resulting in the formation of low-molecular-weight complexes being transferred to the vacuole by the ABC-type transporter HMT1 (Ortiz *et al.*, 1995; Clemens *et al.*, 1999). In eukaryotic cells, cadmium can be detoxified by conjugation with thiol-containing compounds, including GSH and metallothioneins (Cobbett and Goldsbrough, 2002). In *S. cerevisiae*, glutathione peroxidase 3 has been shown to be induced in the presence of Cd and to protect phospholipids during cadmium-induced oxidative stress (Muthukumar *et al.*, 2011).

Methionine sulfoxide reductases catalyze thiol-dependent reduction of free and protein-bound methionine sulfoxides to corresponding methionines. Methionine-*S*-sulfoxide (Met-*S*-SO) and methionine-*R*-sulfoxide (Met-*R*-SO), oxidatively generated from L-methionine by ROS, can be reduced by methionine-*S*-sulfoxide reductase (MsrA) and methionine-*R*-sulfoxide reductase (MsrB), respectively. In a variety of prokaryotic and eukaryotic organisms, both MsrA and MsrB act as antioxidants and protein repair enzymes and participate in various physiological and pathological processes, such as aging, cancer, rheumatoid arthritis, schizophrenia, and neurodegenerative disorders such as Alzheimer's and Parkinson's diseases (Kim and Gladyshev, 2007; Walss-Bass *et al.*, 2009; De Luca *et al.*, 2010; Martin *et al.*, 2010).

In a previous work (Jo *et al.*, 2013), the *S. pombe msrB*$^+$ gene was cloned into the shuttle vector pRS316 to generate the recombinant plasmid pFMetSO. *S. pombe* MsrB was demonstrated to play a protective role against oxidative, nitrosative and thermal stresses probably through diminishing intracellular ROS level (Jo *et al.*, 2013). In this work, we examined whether MsrB plays a protective role against cadmium toxicity which has numerous undesirable effects on human health.

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Bovine serum albumin (BSA), Bradford reagent, cadmium chloride (abbreviated as Cd), NADPH, 5,5′-dithio-(2-nitrobenzoic acid) (DTNB), Griess reagent, 2′,7′-dichlorodihydrofluorescein-diacetate (DCFH-DA), 2-vinylpyridine, GSH and glutathione reductase (GR) were purchased from Sigma Chemical Co. (USA). Yeast extract, peptone and agar were obtained from Amersham Life Science (USA). All other chemicals used were of the highest grade commercially available.

S. pombe KP1 (h^+ *leu1-32 ura4-294*), a derivative of *S. pombe* heterothallic haploid strain 975h^+, was used in this work. Previously constructed (Jo *et al.*, 2013) *S. pombe* cells harboring pFMetSO or pRS316 (Myers *et al.*, 1986) were used. The yeast cells were grown in yeast extract peptone dextrose medium (pH 6.5) which contains 1% yeast extract, 2% peptone, and 1% glucose. The yeast cells were incubated with shaking at 30°C, and their growth was monitored by measuring absorbance at a wavelength of 600 nm. Yeast cells used in the experiments were obtained preferentially from the early exponential growth phase.

The desired number of the yeast cells was harvested by centrifugation. They were re-suspended in 20 mM Tris buffer (pH 8.0) with 2 mM EDTA and disrupted using a glass bead beater. Cellular extracts were obtained after centrifugation and were used for total GSH, oxidized glutathione (GSSG), and protein determinations detailed below.

To determine intracellular ROS levels, the redox-sensitive fluorescent probe DCFH-DA was used as described previously (Royall and Ischiropoulos, 1993). When DCFH-DA enters the cells, its diacetate group is cleaved by an esterase, leaving a non-fluorescent molecule, which is amenable to oxidization to fluorescent dichlorofluorescein (DCF) in the presence of ROS (Kiani-Esfahani *et al.*, 2012). Yeast cells were incubated with 5 μM DCFH-DA for 30 min at 30°C. The treated cells were analyzed immediately using a microplate fluorometer.

Accumulated nitrite (NO_2^-), as an index of nitric oxide (NO), in the culture supernatants was determined using a colorimetric assay based on the Griess reaction (Sherman *et al.*, 1993). Supernatant aliquots (100 μl) were incubated with 100 μl of Griess reagent (6 mg/ml) at room temperature for 10 min, and then NO_2^- concentrations were determined by measuring the absorbance at 540 nm. A calibration curve was constructed using a range of known concentrations (0–160 μM) of sodium nitrite.

As described elsewhere (Nakagawa *et al.*, 1990), total GSH content in cellular extracts was determined using a GR enzymatic recycling assay. The reaction mixture (200 μl) contained 175 mM KH_2PO_4, 6.3 mM EDTA, 0.21 mM NADPH, 0.6 mM DTNB, 0.5 units/ml GR, and cellular extract at 25°C. Absorbance at 412 nm was monitored with a microplate reader. To assess GSSG only, reduced GSH was masked by derivatization with 2-vinylpyridine (Floreani *et al.*, 1997). Typically, 2 μl of undiluted 2-vinylpyridine was added to 98 μl of freshly prepared extracts, and the mixture was incubated at room temperature for 60 min. Total GSH content are reported in μg/mg protein. Protein content in cellular extracts was determined by Bradford's procedure (1976) with BSA as a standard.

The results are reported as means ± standard deviations (SDs). Statistical comparisons between experimental groups were performed using Kruskal-Wallis test, followed by Dunn's post hoc test for pairwise individual comparison. A *P* value less than 0.05 was considered to be statistically significant.

The *S. pombe* cells harboring pFMetSO were found previously to grow better than the vector control cells on minimal medium plates containing 25 μM Cd, suggesting the probable involvement of MsrB in the detoxifying response against Cd (Jo *et al.*, 2013). In the present work, the enhancement of the yeast growth by MsrB in the presence of Cd was further confirmed using medium-shift experiments. The *S. pombe* cells harboring pFMetSO and vector control cells, grown exponentially in rich medium, were transferred to fresh rich medium containing 0, 25, 50 or 100 μM Cd, and yeast growth was monitored by measuring absorbance at a wavelength of 600 nm. As shown in Fig. 1, both yeast cultures exhibited delayed specific growth rates in proportion to the Cd concentrations. The growth of the vector control cells was nearly arrested at 50 or 100 μM Cd, whereas the *S. pombe* cells harboring pFMetSO continued to grow, although at a slower rate, under these conditions (Fig. 1). The medium-shift experiment results in this study also support the growth-enhancing effect of MsrB in the absence of Cd documented previously in solid agar plates (Jo *et al.*, 2013). The growth-enhancing effect might be related to the desirable effects of MsrB on the ROS, NO and total GSH levels (Jo *et al.*, 2013). Taken together, these findings indicate that MsrB affects yeast growth rate in the presence of Cd.

At physiological concentrations, ROS participate in normal cellular functions such as intracellular signaling and redox regulation (Nordberg and Arnér, 2001). Excessive ROS, generated by abnormal metabolic reactions, produce a variety of damaging effects in macromolecules, resulting in genetic mutations and physiological dysfunction (Gutteridge, 1993). The intracellular ROS level is markedly elevated in the presence of diverse oxidative/nitrosative stress-inducing agents, which exert sequential damages of the integrity of various biomolecules. Although Cd^{2+} is a bivalent cation that is unable to generate free radicals directly, Cd-induced oxidative

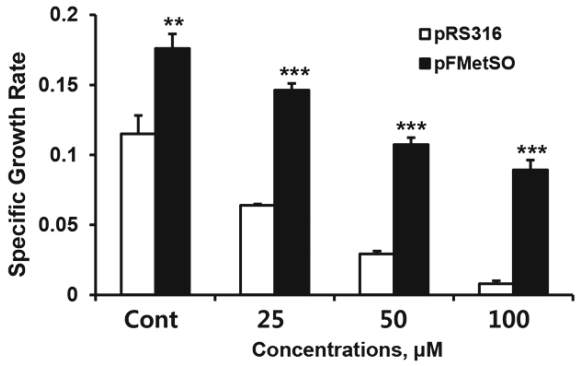

Fig. 1. Enhancing effects of MsrB on the *S. pombe* growth in the presence of cadmium. The *S. pombe* cells harboring pRS316 or pFMetSO, exponentially grown in the normal rich medium, were shifted to the fresh rich medium containing no Cd, 25 μM Cd, 50 μM Cd and 100 μM Cd . The yeast growths were monitored during 9 h after the shifts, and specific growth rates, expressed as ΔOD_{600}/h, were calculated from the three independent experiments. **, $P<0.01$; ***, $P<0.001$ versus the corresponding pRS316-containing cells.

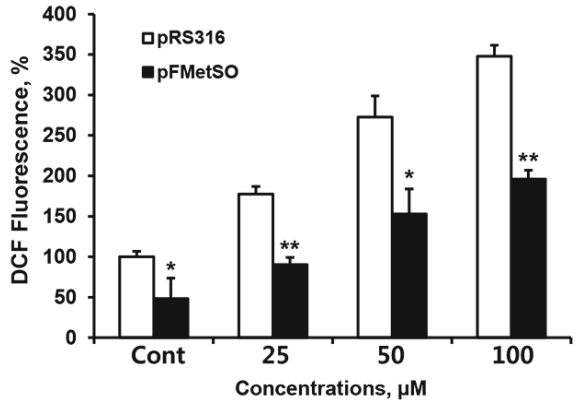

Fig. 2. Effects of MsrB on the reactive oxygen species (ROS) level in *S. pombe* under cadmium. The *S. pombe* cells harboring pRS316 or pFMetSO, exponentially grown in the normal rich medium, were treated with no Cd, 25 μM Cd, 50 μM Cd and 100 μM Cd for 9 h. The intracellular ROS levels were detected using DCFH-DA, and represented as relative DCF fluorescence. *, $P<0.05$; **, $P<0.01$ versus the corresponding pRS316-containing cells.

stress is a common phenomenon observed in many organisms (Hendry *et al.*, 1992; Bertin and Averbeck, 2006; Thévenod, 2009; Cuypers *et al.*, 2010). It has been proposed that Cd depletes GSH and protein-bound sulfhydryl groups, resulting in enhanced generation of ROS (Liu *et al.*, 2009). Cd-induced depletion of the reduced GSH pool disrupts the redox balance, leading to oxidative stress (Lopez *et al.*, 2006). ROS production is one of the first steps in Cd cytotoxicity, preceding mitochondrial damage characterized by the loss of the mitochondrial membrane potential, which leads to the activation of caspases and, consequently, to cell death by apoptosis (Chatterjee *et al.*, 2009; Cuypers *et al.*, 2010). Cd induces DNA damages and interferes with cell signaling through an induction of ROS formation (Filipič, 2012). When exposed to Cd, *S. cerevisiae* was shown to be under oxidative stress as evidenced by an enhanced accumulation of malondialdehyde, an oxidative stress marker (Liu *et al.*, 2005). Cd-induced oxidative stress in *S. cerevisiae* can be reversed by eliminating ROS (Liu *et al.*, 2005). Although both *Debaryomyces hansenii*, an osmotolerant and halotolerant yeast, and *S. cerevisiae* form ROS in response to Cd, *D. hansenii* is much more susceptible to Cd, possibly due to low GSH levels, low basal superoxide dismutase and peroxidase activities, low ATP levels produced in the presence of ROS inducers, and high levels of Cd accumulation (Navarrete *et al.*, 2009). Since MsrB was verified to affect yeast growth in the presence of Cd, the effect of MsrB on ROS levels in yeast cells in the presence of Cd was examined by exposing exponential-phase *S. pombe* cells harboring pFMetSO and vector control cells to varying concentrations of Cd for 9 h. The *S. pombe* cells harboring pFMetSO had lower ROS levels than vector control cells in the absence of Cd (Fig. 2, Cont), which is consistent with the notion that MsrB may have an ROS-scavenging effect under non-stressful growth conditions. The *S. pombe* cells harboring pFMetSO also exhibited markedly lower ROS levels than vector control cells in the presence of varying concentrations of Cd (Fig. 2). Although the ratios between the ROS levels in the non-treated and treated yeast cells seemed unchanged, the ROS levels remained much lower in the MsrB-overexpressed cells than in the vector control cells in the presence of Cd, which is still consistent with a protective effect of MsrB against Cd cytotoxicity. MsrB could play an ROS scavenging role in *S. pombe* cells independent of whether Cd is present or not.

NO (NO•) is a hydrophobic gaseous molecule that participates in a number of physiological processes in a variety of organisms. It possesses normal physiological effects when present in minute quantities, but has unfavorable effects when it is present in excessive quantities. Its direct effects come from chemical reactions in which it reacts directly with its biological target, whereas its indirect effects are mediated by reactive nitrogen species (RNS) which undergo further reactions with their respective biological targets, which include proteins, lipids, and DNA (Wink *et al.*, 2001). RNS play important roles in cellular signaling, but when generated at excessive concentrations they can subject cells to nitrosative stress, which may result in cell death. Cd raises the NO level in the livers and/or kidneys of mice (Li *et al.*, 2011); and NO content has also been shown to increase in plants upon exposure to Cd (Groppa *et al.*, 2008; Xiong *et al.*, 2009). Here, when the *S. pombe* cells harboring pFMetSO and vector control cells were subjected to varying concentrations of Cd for 9 h, both groups of cells had elevated NO levels in proportion to the Cd concentration applied (Fig. 3). However, the NO levels of the *S. pombe* cells harboring pFMetSO were significantly lower than those of the vector control cells (Fig. 3). These findings indicate that MsrB has a diminishing effect on NO levels in the *S. pombe* cells in the presence of Cd.

Ercal *et al.* (2001) demonstrated that cadmium causes oxidative stress through the depletion of intracellular GSH pools. In the proposed mechanism, cadmium, a redox inactive metal, is thought to deplete GSH by forming a complex with oxidized glutathione and inhibiting *de novo* GSH synthesis (Wang *et al.*, 2008). In *S. cerevisiae*, cadmium is detoxified by forming cadmium-phytochelatin complex, which is ultimately sequestered in the vacuole (Li *et al.*, 1997). Although the total GSH levels of the *S. pombe* cells harboring pFMetSO were

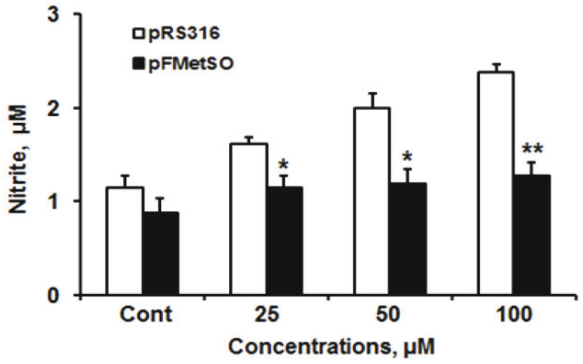

Fig. 3. Suppressive effects of MsrB on the nitric oxide (NO) level in *S. pombe* under cadmium. The *S. pombe* cells harboring pRS316 or pFMetSO, exponentially grown in the normal rich medium, were treated with no Cd, 25 μM Cd, 50 μM Cd and 100 μM Cd for 9 h. The levels of nitrite, as an index of NO, in the culture supernatants were determined based upon Griess reaction. *, $P<0.05$; **, $P<0.01$ versus the corresponding pRS316-containing cells.

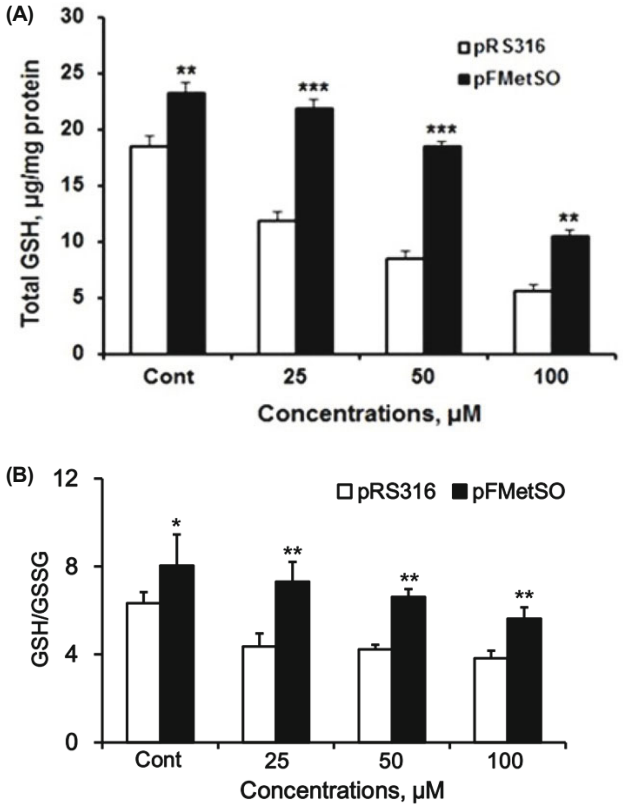

Fig. 4. Effects of MsrB on the total GSH (A) level and the reduced/oxidized GSH ratio (GSH/GSSG, B) in *S. pombe* under cadmium. The *S. pombe* cells harboring pRS316 or pFMetSO, exponentially grown in the normal rich medium, were treated with no Cd, 25 μM Cd, 50 μM Cd, and 100 μM Cd for 9 h. In **A**, the total GSH level was represented as μg/mg protein. *, $P<0.05$, **, $P<0.01$; ***, $P<0.001$ versus the corresponding pRS316-containing cells.

1.3-fold higher than those of the vector control cells in the absence of Cd, they were 1.8-, 2.2-, and 1.9-fold higher than those of the corresponding vector control cells in the presence of 25, 50, and 100 μM Cd, respectively (Fig. 4A). The reduced/oxidized GSH ratio (GSH/GSSG) was also 1.3-fold higher in the *S. pombe* cells harboring pFMetSO than in the vector control cells (Fig. 4B). In the presence of 25, 50, and 100 μM Cd, the ratios in the *S. pombe* cells harboring pFMetSO went up to 1.7-, 1.6-, and 1.5-fold, respectively, when compared to those in the corresponding vector control cells (Fig. 4B). Thus, MsrB appears to play a role in up-regulating the total GSH level and the GSH/GSSG ratio, either directly or indirectly, in *S. pombe* under both normal and Cd-treatment growth conditions.

Our findings suggest that MsrB is a major antioxidant enzyme responsible for cellular tolerance of Cd. Firstly, overexpression of MsrB enabled fission yeast cells survive at the relatively higher concentrations of Cd. This enhanced viability may be caused by diminished ROS levels in MsrB-overexpressing yeast cells, perhaps owing to MsrB's ability to keep intracellular ROS levels within tolerable levels in the fission yeast. Evidences for protective roles of Msr against oxidative damage and stress have been obtained in a variety of cells. In the microaerophilic food-borne pathogen *Campylobacter jejuni*, the *msrA*, *msrB* and *msrA*/*msrB* mutants exhibit increased sensitivity to hydrogen peroxide, organic peroxide, superoxide, and nitrosative and disulfide stress, and the *msrA*/*msrB* double mutant is markedly more sensitive to both oxidative and nitrosative stress, which suggests that MsrA and MsrB significantly protect the cells against these stress conditions (Atack and Kelly, 2008). Similarly, both *msrA* and *msrB* mutants of *Enterococcus faecalis*, an important nosocomial pathogen, are more sensitive to exposure to hydrogen peroxide and contain the reduced level of virulence in a systemic and urinary tract infection model than the wild-type parents (Zhao *et al.*, 2010). fRMsr, an *S. cerevisiae* enzyme specific for free Met-*R*-SO, is mainly responsible for the reduction of free Met-*R*-SO by proving that it is closely linked with growth of yeast cells on Met-*R*-SO and that its deletion increases sensitivity to oxidative stress and decreases life span (Le *et al.*, 2009). However, differences in defensive roles of MsrA and MsrB under various stress conditions currently remain unclear.

The accumulation of cadmium in *Rhodotorula* sp. Y11 and its higher tolerance to Cd were proved to be facilitated by induction of the activities of superoxide dismutase and catalase in the presence of cadmium (Li and Yuan, 2008). Cnt5, a member of the centaurin ADP ribosylation factor GTPase activating protein family, contributes to cadmium resistance by maintaining membrane integrity or by modulating membrane trafficking (Vashisht *et al.*, 2009). Ycg1, a component of the condensing complex involved in chromosome condensation during cell division, and Ydr520C having a zinc-finger domain confer strong cadmium resistance to yeast (Hwang *et al.*, 2009). Isoquercitrin, which belongs to the class of quercetin glycoside and exists in fruits and vegetables as well as several pharmaceutical plants and has a potent antioxidant activity, possesses scavenging abilities for superoxide anion, hydroxyl radical and nitrite induced by cadmium in mouse liver and kidney (Li *et al.*, 2011). Yap1-dependent thioredoxin and thioredoxin reductase in *S. cerevisiae* play an essential role in cadmium tolerance because strains lacking the corresponding genes are hypersensitive to cadmium (Vido *et al.*, 2001). The full functionality of the unfolded protein response and of other pathways activated in response to endoplasmic reticulum stress, caused by very low dose of cadmium, is required for cadmium tolerance thus identifying ER as a major target of cadmium toxicity in yeast (Gardarin *et al.*, 2010).

This work also demonstrates that MsrB participates in the maintenance of high level of total GSH pool in the fission yeast even in the presence of Cd. Considering the previous finding that Cd causes oxidative stress through the depletion of intracellular GSH pools (Ercal *et al.*, 2001), the *S. pombe* MsrB might exhibit its detoxifying activity against Cd through up-regulating the total GSH pool. However, how MsrB maintains the high intracellular GSH pool currently remains unknown. One possibility is that MsrB would enhance the GSH synthetic enzyme activities on a protein or activity level, which sequentially diminish the ROS and NO levels. Another possibility is that high intracellular GSH pool would be preserved due to the plausible capability of MsrB to diminish the ROS and/or NO levels irrespective of GSH. Whether either mechanism actually works or not, MsrB is deeply re-

sponsible for rescuing the fission yeast cells exposed to the high dose of Cd through the down-regulation of ROS and NO levels although the precise mechanism should be elucidated in the future approaches.

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