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Cu/Zn superoxide dismutase and the proton ATPase Pma1p of *Saccharomyces cerevisiae*

J. Allen Baron, Janice S. Chen, Valeria C. Culotta*

Department of Biochemistry and Molecular Biology, Johns Hopkins U. Bloomberg School of Public Health, 615 N. Wolfe St, Baltimore, MD 21205, USA

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ABSTRACT

In eukaryotes, the Cu/Zn containing superoxide dismutase (SOD1) plays a critical role in oxidative stress protection as well as in signaling. We recently demonstrated a function for *Saccharomyces cerevisiae* Sod1p in signaling through CK1γ casein kinases and identified the essential proton ATPase Pma1p as one likely target. The connection between Sod1p and Pma1p was explored further by testing the impact of *sod1Δ* mutations on cells expressing mutant alleles of Pma1p that alter activity and/or post-translational regulation of this ATPase. We report here that *sod1Δ* mutations are lethal when combined with the T912D allele of Pma1p in the C-terminal regulatory domain. This "synthetic lethality" was reversed by intragenic suppressor mutations in Pma1p, including an A906G substitution that lies within the C-terminal regulatory domain and hyper-activates Pma1p. Surprisingly the effect of *sod1Δ* mutations on Pma1-T912D is not mediated through the Sod1p signaling pathway involving the CK1γ casein kinases. Rather, Sod1p sustains life of cells expressing Pma1-T912D through oxidative stress protection. The synthetic lethality of *sod1Δ* Pma1-T912D cells is suppressed by growing cells under low oxygen conditions or by treatments with manganese-based antioxidants. We now propose a model in which Sod1p maximizes Pma1p activity in two ways: one involving signaling through CK1γ casein kinases and an independent role for Sod1p in oxidative stress protection.

1. Introduction

The copper and zinc containing superoxide dismutase (SOD1) of eukaryotes plays dual roles in oxidative stress protection and in signaling. This enzyme catalyzes the two-step disproportionation of superoxide to H_2O_2 and O_2, and by removing superoxide, SOD1 protects against the oxidative damage of this free radical. The role of SOD1 in signaling typically involves the H_2O_2 product of SOD1 catalysis [1–3]. For example, we recently reported that SOD1 of *Saccharomyces cerevisiae* and of humans can promote casein kinase 1-gamma (CK1γ) signaling by preventing the turnover of these kinases through a mechanism involving H_2O_2 [4]. *S. cerevisiae* expresses two CK1γ isoforms, Yck1p and Yck2p, that are tethered to the plasma membrane and signal repression of respiration and activation of the proton ATPase Pma1p in response to glucose [5,6]. Consistent with Sod1p control of Yck1p and Yck2p, we find that yeast *sod1Δ* mutants cannot repress respiration nor activate Pma1p with high glucose [4].

Pma1p is the major plasma membrane H^+-ATPase in *S. cerevisiae*. This transporter plays an essential role in maintaining the proton gradient that drives nutrient uptake and accordingly, null mutants in *pma1* are not viable [7]. Pma1p itself is regulated by a variety of pathways including the aforementioned CK1γ kinases [6] and is best known for its regulation by glucose availability [8].

The post-translational regulation of Pma1p by glucose involves a C-terminal region that with low glucose, interacts with the ATPase domain to inhibit Pma1p activity [9–11]. The addition of glucose stimulates phosphorylation of three residues (Ser-899, Ser-911, Thr-912) in the C-terminus triggering release from the ATPase domain coincident with an increase in the V_{max} for ATP hydrolysis and decrease in the K_m for ATP [11–14]. Much of what is known regarding Pma1p activation was elucidated through studies involving site-specific mutations and truncations in Pma1p that target its regulatory and ATPase domains [11–17].

Compared to glucose control of Pma1p, little is understood of how Sod1p affects Pma1p. Is Sod1p solely working through the CK1γ kinases or are alternative mechanisms involved? Here we investigate the Sod1p-Pma1p connection using a series of Pma1p

* Corresponding author. Dept of Biochemistry and Molecular Biology, Johns Hopkins U. Bloomberg School of Public Health, Room W8116, 615 N. Wolfe St, Baltimore, MD 21205, USA. Fax: +1 410 955 2926.
E-mail address: vculott1@jhu.edu (V.C. Culotta).

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mutants previously used to explore glucose control of Pma1p. We observed that one specific *pma1* mutant, namely T912D affecting the regulatory domain, is not viable when combined with *sod1Δ* null mutations. Sod1p is required to sustain the life of cells expressing Pma1-T912D and surprisingly, this is unrelated to the role of Sod1p in signaling through CK1γ. Instead Sod1p is acting as a potent anti-oxidant to protect Pma1-T912D cells from lethal oxidative stress. We propose a model in which Sod1p acts through both CK1γ signaling and oxidative stress protection to promote activity of the vital Pma1p ATPase.

2. Materials and methods

2.1. Yeast growth, yeast strains and plasmids

S. cerevisiae yeast strains were maintained at 30 °C either in an enriched YPD (yeast extract, peptone, 2% dextrose) or a minimal synthetic complete (SC) medium devoid of lysine where indicated [18]. Anaerobic growth was carried out using the GasPak EZ Anaerobe Container System (Becton Dickinson) on medium supplemented with 15 mg/L ergosterol and 0.5% Tween 80 (YPDE). Intermediate O_2 concentrations (i.e. 1, 5, 10%) used for growth assays were achieved with a Witt KM20-2 gas mixer and Almore Vacu-Quik jars by alternately vacuuming and saturating with the indicated O_2/N_2 mix 5 times.

The *S. cerevisiae* strains used in this study are either of the BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) or NY13 (*MATa ura3–52*) backgrounds and are described in Supplementary Table 1. Pma1p mutants in the NY13 background were generous gifts of Ken Allen and Carolyn Slayman, Yale U. *SOD1* was deleted in the various strains using *sod1::LEU2* or *sod1::KanMX* vectors as described [19,20]. Viable aerobic suppressors of the Pma1-T912D *sod1Δ* strain could be isolated at a frequency of 10^5 by plating 10^7 cells for two days in air on YPD media. It is noteworthy that in the BY4741 background, the single Pma1-T912A and Pma1-T912D strains developed *rho*-mutations (losses in mitochondrial DNA) at a high frequency (not shown). However, these *rho*-mutations did not accumulate in the background of isogenic *sod1Δ* or *akr1Δ* strains, demonstrating that losses in mitochondrial DNA are not responsible for the synthetic lethality of T912D *sod1Δ* strains. Tests for *rho*-mutations were conducted by plating cells on YP medium containing 3% glycerol and incubating at 5% oxygen that is sufficient to drive mitochondrial respiration but is not lethal to the oxygen-sensitive T912D *sod1Δ* strain.

Mutant *PMA1* alleles were all derived from pGW201 [21] using Quikchange site-directed mutagenesis (Stratagene). Cassettes of wild type or mutant *PMA1* were liberated by HindIII digestion and transformed into yeast; accurate *PMA1* gene replacement was verified by DNA sequencing.

2.2. Biochemical and microscopy analyses

For Pma1p activity assays, plasma membranes were isolated essentially as described [22] from a 1L YPD culture of cells grown to mid-log phase at 30 °C. Pma1p activity was determined measuring orthovanadate-sensitive phosphate release from ATP using published protocols [23,24] and values were normalized to that from WT cells.

Indirect immunofluorescence was performed largely as described [25] using cells grown in YPD to $OD_{600} = 1.0$ and fixed with 4.4% formaldehyde added directly to cultures for 30 min. The cells were then washed once with 100 mM potassium phosphate, pH 6.5 (KPhos buffer) and then fixed a second time overnight in KPhos buffer with 4% formaldehyde. Cells were washed once in KPhos buffer and spheroplasts were generated by incubation in 200 mM Tris–HCl, pH 8.0, 20 mM EDTA, 1% 2-mercaptoethanol for 10 min, followed by a second incubation in 1.2 M sorbitol, 100 mM potassium phosphate, pH 6.5 with 1.5 mg/ml Zymolyase 20T for 30–60 min. Cells were washed once in 1.2 M sorbitol and permeabilized in 1.2 M sorbitol, 2% SDS for 2 min. Cells were then washed twice in 1.2 M sorbitol, allowed to adhere to poly-lysine coated slides to form a monolayer, and then treated with primary (1:25 anti-Pma1p mouse monoclonal [40B7]; Abcam, ab4645) and secondary antibodies (1:250 Goat Anti-Mouse Alexa Fluor 488; Molecular Probes, A11001) in PBS with 1% BSA. After final aspiration, mounting solution containing 2.5% DABCO, 100 mM Tris HCl, pH8.8, 50% Glycerol, 0.2 ug/ml DAPI was added and slides sealed. Images were taken on a Zeiss Observer.Z1 fluorescence microscope with an Apotome VH optical sectioning grid (Zeiss, Gena, Germany) under 100X magnification.

3. Results and discussion

3.1. PMA1 mutants and Sod1p deficiency

We previously reported that Pma1p activity is low in glucose treated yeast when *SOD1* is deleted [4]. One possible mechanism might involve mis-sorting of Pma1p in the secretory pathway, as has been shown for a number of other yeast mutants with altered Pma1p activity [25,26]. By indirect immunofluorescence microscopy, Pma1p in WT cells is predominantly localized to the cell-surface and this precise pattern of Pma1p localization was mirrored in *sod1Δ* cells (Fig. 1A). Furthermore, the intensity of Pma1p staining was comparable in WT and *sod1Δ* cells (Fig. 1A), consistent with normal Pma1p polypeptide levels in *sod1Δ* cells [4]. Therefore, the poor glucose activation of Pma1p in *sod1Δ* cells [4] is not due to loss of protein or mis-trafficking of Pma1p.

A number of site specific and truncation mutants in Pma1p have been generated that impact Pma1p control by glucose [11–17]. We sought to determine the effects of expressing such Pma1p variants in cells lacking Sod1p. Null *sod1Δ* mutations were engineered into yeast that express Pma1p mutants targeting the C-terminal inhibitory and interacting ATPase domains, including Ala and phosphomimic Asp substitutions at the phosphorylation sites S899, S911, and T912, and Cys scanning substitutions in stalk segment 5 (summarized in Fig. 1B). Of all the strains engineered, one yielded a striking “synthetic” effect [27], meaning the combination of *pma1* and *sod1* mutations produced a unique trait not seen with the individual mutants alone. Specifically, Pma1-T912D exhibited synthetic lethality with *sod1Δ* - the double mutant was not viable under normal growth conditions (Fig. 1C and D). The results were reproducible in two distinct strain backgrounds and the synthetic lethality was only seen under atmospheric oxygen, as these same strains grew well under anaerobic conditions (Fig. 1C and D).

The lethal effect of combining *sod1Δ* and *PMA1* mutations appeared highly specific to Pma1-T912D. 13 out of 14 Pma1p mutants in the C-terminal inhibitory and ATPase domain showed no growth impairment when combined with *sod1Δ* (Fig. 1C and Supplementary Fig. 1), including the Pma1-T912A allele (Fig. 1C). This result was surprising in that T912D and/or T912A mutations have been reported with lowered ATPase activity [11,13,14,16] and both mutants have a high tendency to accumulate *rho*-mutations or losses in mitochondrial DNA (see *Materials and Methods*), but only Pma1-T912D is synthetically lethal with *sod1Δ*. Based on previous trypsin digestion experiments [11], Pma1-T912D is predicted to adopt a more open conformation compared to Pma1-WT or -T912A which may help explain the vulnerability of this mutant to loss of Sod1p (described in more detail below). For the purposes of this study, this synthetic lethal mutant will be referred to as T912D *sod1Δ*.

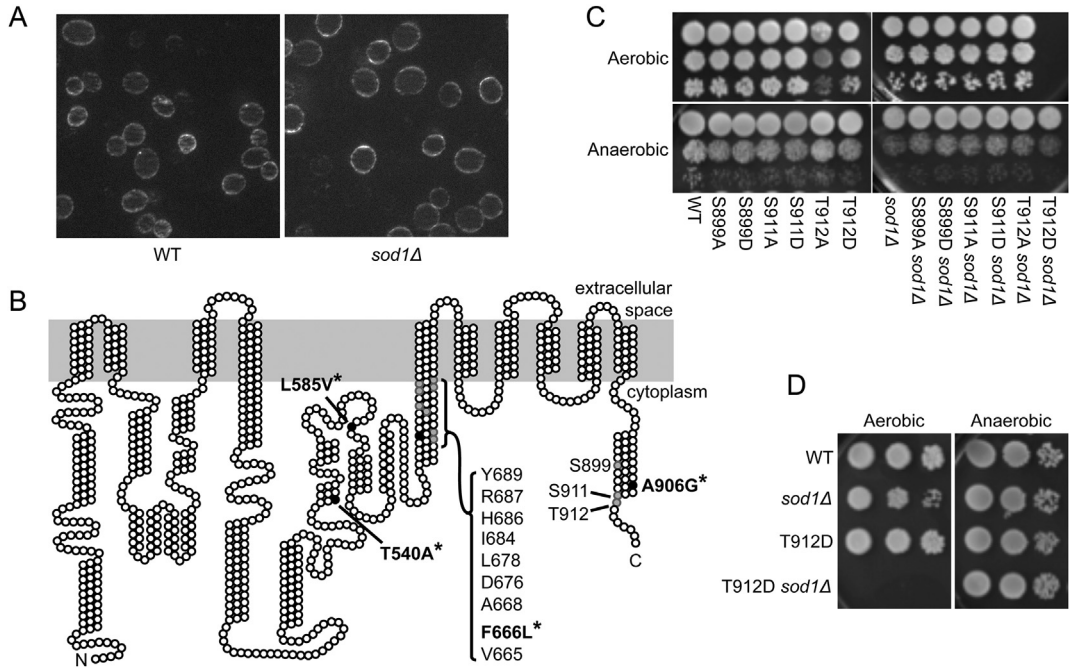

Fig. 1. Effect of *sod1Δ* on Pma1p localization and growth of Pma1p mutants with altered glucose regulation (A) Indirect immunofluorescence of endogenous Pma1p was performed on WT and *sod1Δ* cells in the BY4741 background as described in *Materials and Methods*. (B) Topology map of Pma1p based on data from Lecchi et al. [11]. Grey circles indicate mutants in stalk segment 5 and the C-terminus that were tested for synthetic lethality with *sod1Δ* in parts C, D, and Supplementary Fig. 1. Black circles and asterisks denote the secondary site mutations that suppress the synthetic lethality of T912D *sod1Δ*. (C, D) Plate-based growth tests were performed with strains in either the BY4741 (C) or NY13 (D) background where 10^4, 10^3, and 10^2 cells were spotted onto YPD (aerobic) or YPDE (anaerobic) and incubated at 30 °C for 5 days.

3.2. Suppressors of the T912D sod1Δ aerobic lethality

After prolonged incubation in air, viable colonies of T912D *sod1Δ* could be identified at a frequency of $\approx 10^5$. These represented stable genetic suppressors, or secondary mutations that bypass the synthetic lethality by either reversing the Sod1-or Pma1-deficiency. To discern between the two, we monitored aerobic lysine auxotrophy, a hallmark of Sod1p deficiency [28–30]. Mutants of *sod1Δ* cannot grow aerobically without lysine due to oxidative damage to the lysine biosynthetic pathway [28–30], and numerous genetic suppressors have been isolated that reverse this *sod1Δ* defect (e.g., *pmr1Δ, bsd2Δ*) [30–33]. However, none of the suppressors of T912D *sod1Δ* could grow aerobically without lysine (Fig. 2B), consistent with reversal of Pma1p deficiency, not of Sod1p.

It is known from previous studies that deficiencies in Pma1p mutants can be suppressed by intragenic mutations, i.e., second site *PMA1* mutations [13,14]. To test whether such intragenic mutations accounted for our suppressors of T912D *sod1Δ*, the entire *PMA1* gene was sequenced in four representative isolates that are shown in Fig. 2A. As depicted in Fig. 1B, suppression in each isolate correlated with a distinct missense mutation within *PMA1*. F666L lies within the aforementioned Pma1p stalk region and a similar mutation F666C was previously shown by Miranda and colleagues to result in constitutive activation of Pma1p [17]. The T540A, L586V mutations lie within the ATPase domain and A906G is within the C-terminal inhibitory domain (Fig. 1B). These findings are highly reminiscent of intragenic suppressors previously reported for the Pma1-S911A/T912A double mutant. Like T912D *sod1Δ*, the double Pma1-S911A/T912A is not viable and this lethality is rescued by mutations in the ATPase domain (P536L, A565T, G587N, G648S), the stalk domain (P669L, G670S) and the C-terminus (M907I) [13,14]. M907I was previously identified as a hyperactive Pma1p allele [14] and since this mutation is immediately adjacent to the A906G suppressor identified here, we chose Pma1-A906G for further analysis.

To confirm the suppression of T912D *sod1Δ* by Pma1-A906G, this allele was introduced in an independent strain background. As expected, A906G restored aerobic growth to the T912D *sod1Δ* strain in the BY4741 background (Fig. 2C top) but did not reverse aerobic lysine auxotrophy (Fig. 2C bottom), confirming suppression of Pma1p and not Sod1p-deficiency. To explore how A906G suppresses T912D, we examined its effect on the localization and activity of Pma1p. As seen in Fig. 3A, the A906G mutation significantly enhanced Pma1p ATPase activity without changing Pma1p localization or protein levels at the cell surface (Fig. 3B). In fact, the A906G mutation increases activity beyond that of WT Pma1p (Fig. 3A) and is a hyperactive allele of Pma1p, as has been reported for its close neighbor M907I [14]. Thus, the synthetic lethality of T912D *sod1Δ* can be rescued by hyper-activation of Pma1p. These findings strongly implicate profound loss of essential Pma1p activity as the cause of death in the T912D *sod1Δ* strain. Presumably, the lowered ATPase activity in Pma1-T912D (Fig. 3A and [14]) combined with the inhibitory effects of *sod1Δ* on Pma1p [4] diminish activity of this essential ATPase to a degree that is incompatible for life.

3.3. Oxidative damage, not loss of Yck1p signaling causes the synthetic lethality of T912D sod1Δ

We first surmised that the synthetic lethality of T912D *sod1Δ* was due to loss of Sod1p signaling to CK1γ for Pma1p activation [4,6]. If true, inhibiting the CK1γ kinases should likewise show synthetic lethality with Pma1-T912D. Yeast cells lacking both CK1γ kinases (Yck1p and Yck2p) are not viable, but their function in glucose signaling can be blocked by mutations in the Akr1p palmitoyl transferase that tethers these kinases to the cell surface [34]. Mutants in *akr1Δ* have reduced Pma1p activity [4], however we

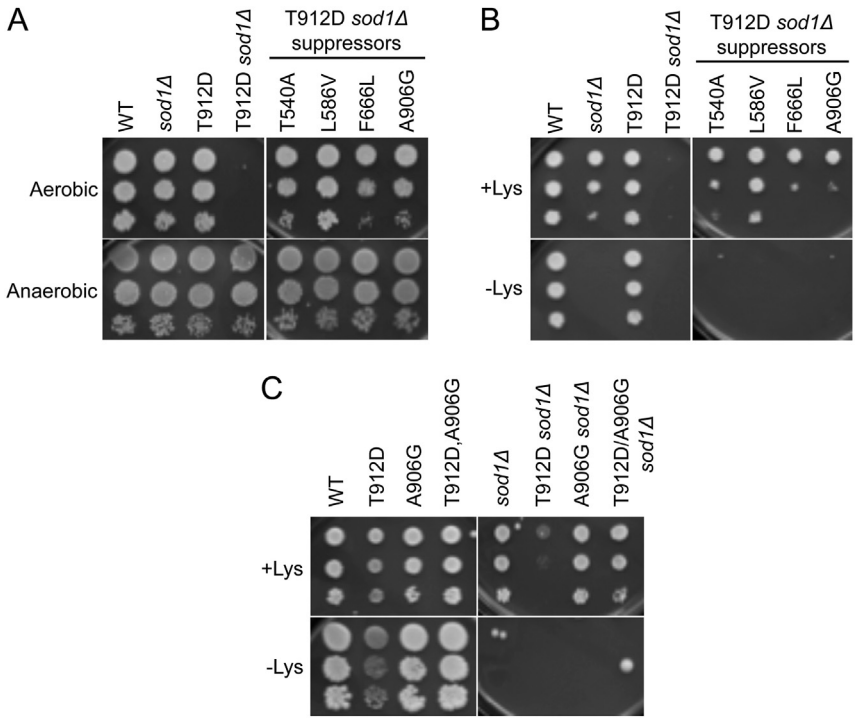

Fig. 2. T912D *sod1Δ* cells develop suppressors with secondary mutations in *PMA1* The indicated strains in the NY13 strain background (A, B) or in the BY4741 background (C) were tested for growth by spotting serial dilutions of cells as described in Fig. 1C,D. (A) Cells were spotted onto enriched YPD/YPDE medium and allowed to grow aerobically or under anaerobic conditions as in Fig. 1C,D. (B,C) Cells were spotted onto minimal SC media (+Lys) or SC media lacking Lys (-Lys) and incubated at 30 °C for 3 days in air. T540A, L586V, F666L and A906G indicate second site suppressor mutations in *PMA1* that permit aerobic growth of T912D *sod1Δ*.

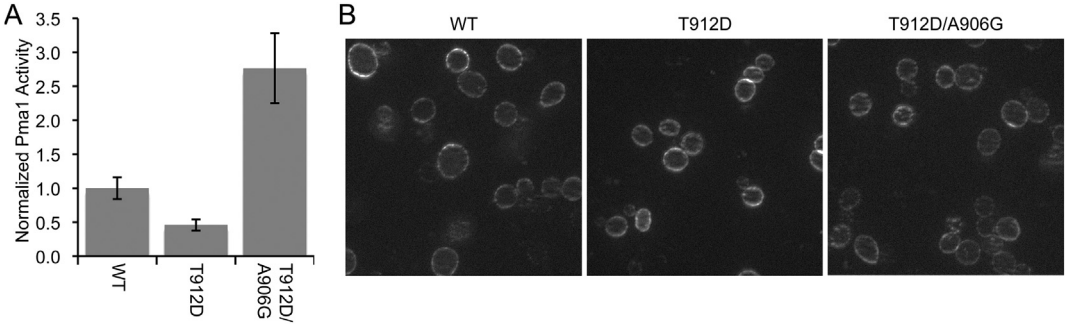

Fig. 3. The PMA1-A906G mutation increases Pma1p ATPase activity but does not alter Pma1p localization or protein level The indicated strains in the BY4741 background were grown at 30 °C shaking in YPD to mid-log phase and tested for: (A) Pma1p activity in isolated membranes as determined by the orthovanadate-sensitive ATPase activity as described in *Materials and Methods*. Values shown represent the average of two biological replicates normalized to WT; error bars indicate standard error; (B) Pma1p localization by indirect immunofluorescence microscopy as in Fig. 1A.

found that *akr1Δ* mutations are not synthetically lethal with Pma1-T912D (Fig. 4A). The unexpected viability of T912D *akr1Δ* cells did not reflect rapid accumulation of suppressors. We found no second site mutations in *PMA1*, as determined by whole gene sequencing, and cells retained the characteristic large cell size and elongated bud morphology that is the hallmark of *akr1Δ* mutations (data not shown) [35]). Since *akr1Δ* does not mimic *sod1Δ*, the synthetic lethality of T912D *sod1Δ* appears independent of the CK1γ kinases.

S. cerevisiae Sod1p not only regulates CK1γ, but plays an important role in oxidative stress protection [28,36–38]. Any oxidative damage of *sod1Δ* cells can be prevented by growing cells in low oxygen or by supplying high levels of antioxidants, such as reactive manganese complexes that scavenge superoxide [39–43]. These antioxidants will not correct the CK1γ deficiency of *sod1Δ* cells that is independent of oxidative stress [4]. However, the synthetic lethality of T912D *sod1Δ* strains was completely reversed by alleviating oxidative stress. The strain begins to grow when oxygen levels drop to 5% (Fig. 4B) and when cells are treated with high levels of manganese to form Mn-based antioxidants (Fig. 4C) [39–43]. Together, these observations strongly indicate that oxidative damage and not loss of CK1γ signaling, contributes to the synthetic lethality of T912D *sod1Δ*.

Why would cells expressing Pma1-T912D be particularly vulnerable to oxidative damage? As mentioned above, only the T912D and not T912A allele of Pma1p is synthetically lethal with *sod1Δ*. Pma1-T912D appears to adopt a more open conformation [11] and there are nine cysteines in Pma1p that may be susceptible to oxidative damage, including five in the catalytic domain [44]. Activity of WT Pma1p is lower in *sod1Δ* mutants, yet not sufficiently low to kill the cell [4]. However, in the case of Pma1-T912D, the more open conformation may increase vulnerability to oxidative modification. Such damage to the ATPase in combination with the already

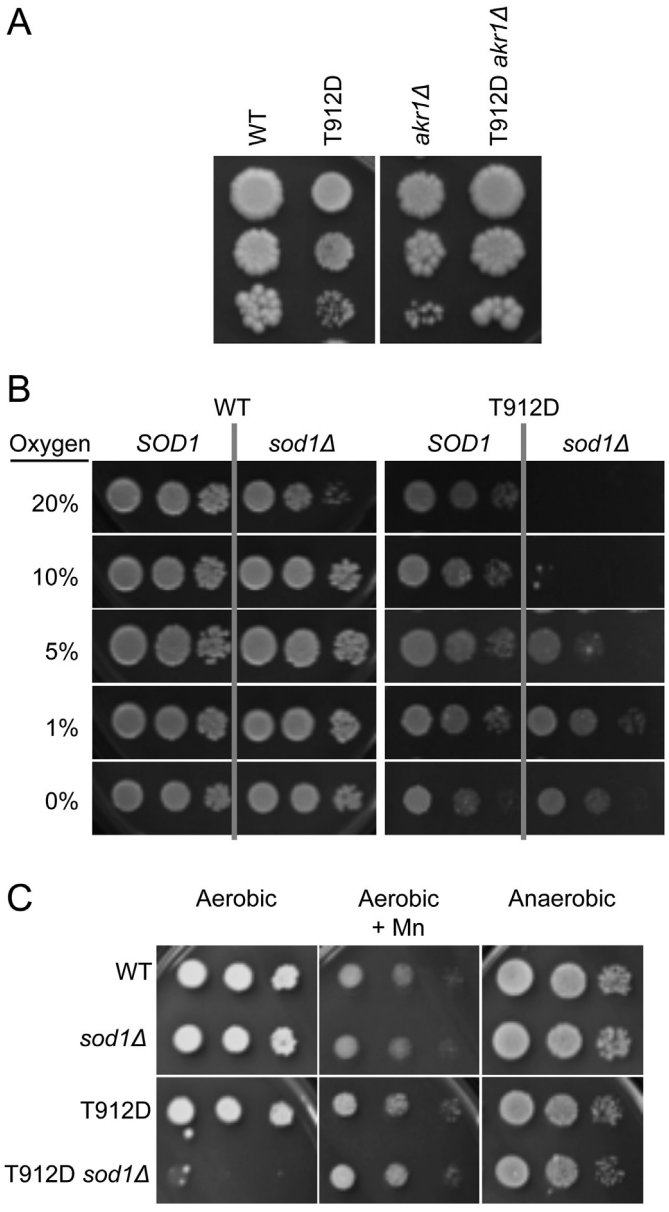

Fig. 4. T912D *sod1Δ* synthetic lethality is due to oxidative stress and not Yck1p and Yck2p mis-regulation (A) The indicated strains in the BY4741 background were tested for aerobic growth as described in Fig. 1C,D. (B,C) Serial dilutions of the indicated strains in the NY13 background were spotted as in Fig. 1C,D onto either YPDE (0% oxygen part B; Anaerobic part C) or YPD (1–20% oxygen) and incubated under the specified oxygen concentrations for 3 days at 30 °C. (C) "+ Mn" = YPD medium supplemented with 1 mM $MnCl_2$ to promote formation of Mn-based antioxidants.

diminished activity of Pma1-T912D could render the transporter essentially useless to support life. As an alternate possibility, the role of Sod1p in protecting the ATPase from oxidative damage may be indirect, through Pma1p regulators. For example, Pma1p is thought to be regulated by calcineurin [45] and yeast Sod1p has been shown to bind to and protect calcineurin from inactivation [46]. Regardless of whether Sod1p is protecting Pma1p directly or indirectly, these studies underscore the multiple ways this antioxidant enzyme can affect growth and metabolism of aerobic cells.

Conflict of interest

None declared.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.04.127.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.04.127.

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