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SOD1 Integrates Signals from Oxygen and Glucose to Repress Respiration

Amit R. Reddi[1] and Valeria C. Culotta[1,*]
[1]Department of Biochemistry and Molecular Biology, Johns Hopkins University Bloomberg School of Public Health, 615 North Wolfe Street, Baltimore, MD 21205, USA
*Correspondence: vculotta@jhsph.edu
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SUMMARY

Cu/Zn superoxide dismutase (SOD1) is an abundant enzyme that has been best studied as a regulator of antioxidant defense. Using the yeast *Saccharomyces cerevisiae*, we report that SOD1 transmits signals from oxygen and glucose to repress respiration. The mechanism involves SOD1-mediated stabilization of two casein kinase 1-gamma (CK1γ) homologs, Yck1p and Yck2p, required for respiratory repression. SOD1 binds a C-terminal degron we identified in Yck1p/Yck2p and promotes kinase stability by catalyzing superoxide conversion to peroxide. The effects of SOD1 on CK1γ stability are also observed with mammalian SOD1 and CK1γ and in a human cell line. Therefore, in a single circuit, oxygen, glucose, and reactive oxygen can repress respiration through SOD1/CK1γ signaling. Our data therefore may provide mechanistic insight into how rapidly proliferating cells and many cancers accomplish glucose-mediated repression of respiration in favor of aerobic glycolysis.

INTRODUCTION

Cellular energy production is mediated through the action of glycolysis and oxidative phosphorylation. In many cells, oxygen (O_2) availability dictates whether the end product of glycolysis, pyruvate, is oxidatively metabolized through the respiratory chain to produce ATP or is reductively metabolized through lactate or ethanol fermentation to regenerate NAD^+ for continued glycolysis. However, certain cells can be reprogrammed to undergo aerobic fermentation, a process characteristic of a variety of proliferating cells, including various cancers, lymphocytes, endothelial cells, and microorganisms such as Bakers' yeast (*Saccharomyces cerevisiae*) (Lunt and Vander Heiden, 2011).

Many factors contribute to the switch from respiration to aerobic fermentation, including transcriptional induction of glucose transport and glycolytic enzymes and repression of respiratory genes (Bensinger and Christofk, 2012; Diaz-Ruiz et al., 2011). In the case of Bakers' yeast, glucose itself signals repression of respiration in a process known as the Crabtree effect (Crabtree, 1929). Glucose activates a series of signaling pathways consisting of G protein coupled receptors, hexokinases, and transmembrane glucose receptors that work in concert to repress respiration and promote aerobic fermentation (Zaman et al., 2008). Interestingly, it was recently reported that yeast strains lacking the antioxidant enzyme Cu/Zn superoxide dismutase (SOD1 or yeast Sod1p) incompletely repress respiration in the presence of glucose (Sehati et al., 2011). It was not known whether this metabolic defect was due to oxidative damage or to an unknown role for SOD1 in signaling respiration repression.

SOD1 is well conserved throughout evolution and defends against oxidative stress by catalyzing the disproportionation of superoxide into peroxide (H_2O_2) and O_2 (McCord and Fridovich, 1969). Most eukaryotes express two intracellular SODs, a Mn-containing SOD2 in the mitochondrial matrix (Weisiger and Fridovich, 1973), and a highly abundant Cu/Zn SOD1 that is largely cytosolic but is also found in the mitochondrial inter membrane space (IMS) (Okado-Matsumoto and Fridovich, 2001; Sturtz et al., 2001). SOD1-deficient organisms experience various markers of oxidative stress (for example, higher incidences of liver cancer in SOD1$^{-/-}$ mice [Elchuri et al., 2005] and increased mutation frequencies and defects in lysine and methionine biosynthetic pathways in yeast *sod1*Δ mutants [Chang and Kosman, 1990; Gralla and Valentine, 1991; Sturtz and Culotta, 2002; Biliński et al., 1985]). SOD1 is a highly abundant protein in various organisms (Halliwell and Gutteridge, 2007; Pardo et al., 1995), but in studies that have been done in yeast, less than 1% of total SOD1 is required to protect the aforementioned amino acid biosynthetic pathways and to prevent toxicity from superoxide (Corson et al., 1998). The rationale for producing such large quantities of SOD1 has been enigmatic, and the enzyme may have as-of-yet undetermined functions in cell physiology.

Herein, we describe a role for SOD1 independent of its function in oxidative stress protection. Using a yeast model system, we demonstrate that Sod1p helps to integrate signals from glucose and O_2 to repress respiration. The mechanism involves Sod1p-stabilization of casein kinase 1-gamma (CK1γ) homologs, Yck1p and Yck2p. These kinases are essential for nutrient sensing (Liu et al., 2008; Moriya and Johnston, 2004), and we demonstrate here their role in SOD1-dependent respiration

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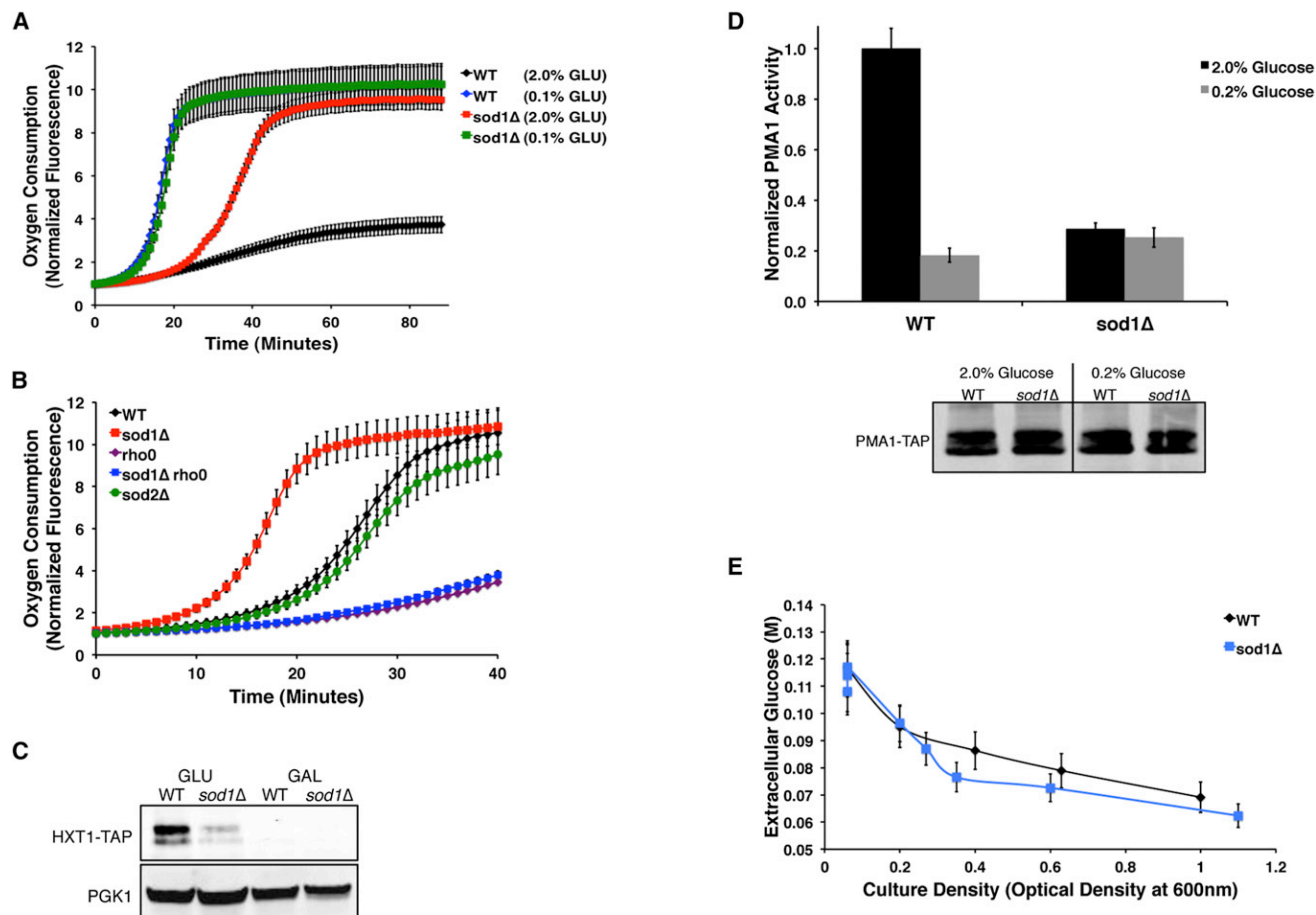

Figure 1. Defect in Glucose Repression of Respiration in *sod1*Δ Cells

(A and B) O_2 consumption analysis was carried out as described in Experimental Procedures, with the indicated yeast strains grown under the designated GLU conditions (A) or with 2% GLU (B). Data represent the mean ±SD of triplicate cultures.

(C and D) (C and D, bottom) Immunoblot analysis of TAP-tagged Hxt1p and Pma1p was carried out as described in Extended Experimental Procedures using an α-TAP antibody. In the Hxt1-TAP immunoblot, the highest molecular weight bands represent intact protein. PGK1 is the loading control detected by α-Pgk1p antibody. (D, top) Pma1p ATPase activity was measured in membranes from the indicated strains as described in Experimental Procedures. Results are normalized to WT cells cultured in 2.0% GLU. The data represent the mean ±SD of duplicate cultures.

(E) Extracellular glucose was monitored as described in Experimental Procedures as a function of yeast growth at an optical density of 600 nm. Data represent the mean ±SD of triplicate cultures.

See also Figure S1.

repression. Sod1p physically binds to a C-terminal degron in Yck1p, and the SOD enzymatic reaction prevents Yck1p degradation. The H_2O_2 product of the SOD reaction appears critical for preventing casein kinase turnover. Glucose and O_2 also stabilize Yck1p and Yck2p, apparently by controlling the amount of superoxide substrate for Sod1p. In this manner, in a single circuit, O_2, glucose, and reactive oxygen can repress respiration through SOD1/casein kinase signaling.

RESULTS

Cu/Zn SOD1 Is Needed for Glucose Control of Respiration

In yeast, O_2 consumption rates are lowest with abundant glucose (or GLU) and most rapid with low glucose (Figure 1A) or with an alternate carbon source such as galactose (or GAL) (Figure S1A available online). Consistent with previous studies (Sehati et al., 2011), this glucose repression of O_2 consumption appears defective in *sod1*Δ yeast. Cells lacking the largely cytosolic Cu/Zn Sod1p, but not the mitochondrial Mn Sod2p, exhibit elevated O_2 consumption in high glucose that is reflective of mitochondrial respiration, as it is obliterated in rho0 strains lacking mitochondrial DNA (Figure 1B). Additionally, a number of glucose signaling markers appear blocked by *sod1*Δ mutations, including glucose induction of the hexose transporter *HXT1* (Zaman et al., 2008) (Figure 1C). *sod1*Δ mutants are also defective in glucose activation of the H^+-ATPase Pma1p (Estrada et al., 1996) (Figure 1D), in spite of normal glucose consumption (Figure 1E).

Three major glucose sensing and signaling pathways are involved in respiratory repression in yeast, including the

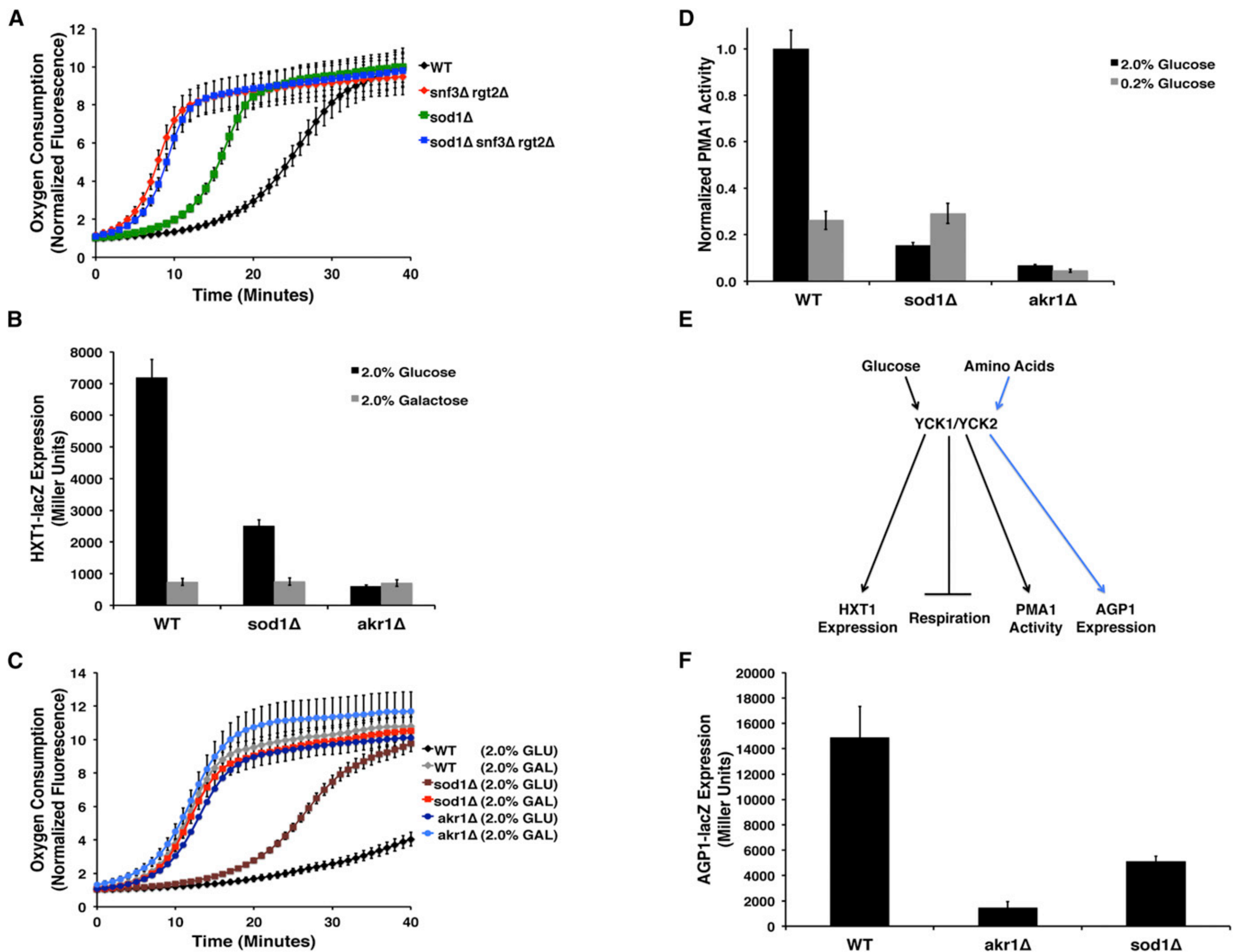

Figure 2. SOD1 Regulates Glucose Sensing in the SNF3/RGT2 Pathway through Yck1p

(A and C) O_2 consumption of the indicated strains was measured as in Figures 1A and 1B, with either 2% GLU (A) or in the designated carbon sources (C).

(B) The indicated strains expressing a *HXT1-lacZ* reporter were assayed for *HXT* promoter activity by β-galactosidase activity as described in Extended Experimental Procedures. Activity is reported as Miller units (Giacomini et al., 1992) and represents the mean ±SD of triplicate cultures.

(D) Pma1p ATPase activity was measured as in Figure 1D.

(E) Schematic illustrating downstream targets of Yck1p/Yck2p, where black and blue lines indicate glucose and amino acid signaling targets, respectively.

(F) *AGP1* promoter activity was measured in strains expressing a *AGP1-lacZ* fusion as in (B).

See also Figure S1.

GPA1/GPR2, HXK2, and SNF3/RGT2 pathways (Figure S1B) (Zaman et al., 2008). To determine which is affected by *sod1*Δ mutations, we screened for mutants that inhibit the *sod1*-linked increase in respiration. We observed that *sod1*Δ mutations still enhance respiration in *gpa2*, *gpr1*, and *ras2* mutants of the GPA2/GPR1 pathway, and in *hxk2* mutants of the HXK2 pathway (Figure S1C). By comparison, *sod1*Δ mutations had no effect on O_2 consumption in a strain lacking *SNF3* and *RGT2* (Figure 2A).

The SNF3/RGT2 pathway involves glucose activation of Yck1p and Yck2p, a pair of casein kinase 1 gamma isoforms (CK1γ) (Moriya and Johnston, 2004). Interestingly, Yck1p and Sod1p were previously noted to interact in a high-throughput mass spectrometry screen (Ho et al., 2002). To test whether loss of Yck1p/Yck2p mimicked the glucose-sensing defects of *sod1*Δ mutants, we employed *akr1*Δ mutants. The Akr1p palmitoyl transferase tethers Yck1p and Yck2p to the plasma membrane, and *akr1* mutations are often used to model loss of Yck1p/Yck2p (Pasula et al., 2010) because *yck1*Δ *yck2*Δ mutants are not viable (Robinson et al., 1992). Similar to *sod1*Δ strains, *akr1*Δ mutants cannot properly activate *HXT1* (Figure 2B) and exhibit high respiration (Figure 2C) and attenuated Pma1p activity (Figure 2D) in high glucose despite normal glucose uptake (Figure S1D). Yck1p and Yck2p affect multiple steps in glucose signaling that are also perturbed by *sod1*Δ mutations.

Yck1p and Yck2p are additionally involved in amino acid sensing, where extracellular amino acids signal through these casein kinases to induce amino acid permease genes such as

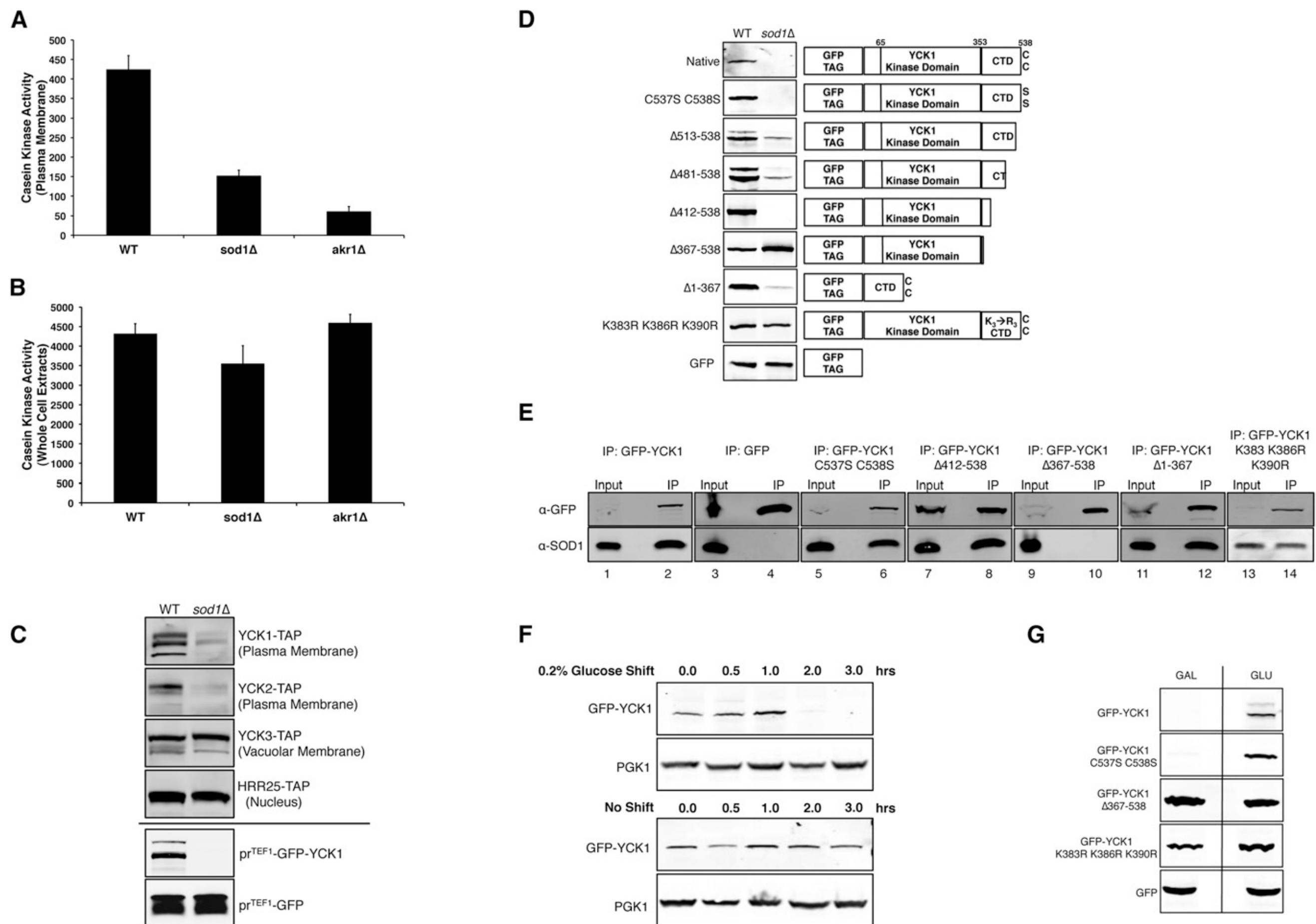

Figure 3. Glucose and SOD1 Regulate Yck1p Stability through a C-Terminal SOD1-Interacting Degron

(A and B) Casein kinase activity from 2% GLU-grown cells was carried out in isolated plasma membranes (A) or in whole cell lysates (B) as described in Experimental Procedures. Activity is reported as percent phosphorylation of fluorescent substrate per milligram protein. Data represent the mean ±SD of duplicate cultures.

(C) Immunoblot analysis of cells grown in 4% GLU and expressing (top) C-terminal TAP-tagged fusions of the designated casein kinases driven by their native promoters and detected by α-TAP, or (bottom) N-terminal GFP fusion to Yck1p (expressed from plasmid pAR113) or GFP alone (plasmid pAR118) driven by the *TEF1* promoter and detected by α-GFP as described in Extended Experimental Procedures.

(D) α-GFP immunoblot of cells grown in 4% GLU and expressing N-terminal GFP fusions to WT Yck1p ("Native" expressed from plasmid pGFPYCK1) or to Yck1p mutant derivatives of this construct. As diagrammed on right, these derivatives contain Yck1p C537S C538S or K383R K386R K390R mutations, or have truncations in the C-terminal domain (CTD) or contain the isolated CTD of Yck1p. C C, S S indicates Cys 537 Cys 538 or the double Ser substitutions, and $K_3 \rightarrow R_3$ indicates the K383R, K386R, K390R Yck1p mutant.

(E) Lysates from WT strains grown in 4% GLU and expressing the indicated GFP fusions to Yck1p (described in D) were subjected to immunoprecipitation with α-GFP. The input, corresponding to ~3% of total cell lysate, and the immunoprecipitated material (IP) were analyzed by immunoblot using α-GFP (top row) and α-SOD1 (bottom) antibodies as described in Extended Experimental Procedures. A total of 3% of the Sod1p input provides equivalent signals as 100% of IP Sod1p.

(F) WT cells transformed with pGFPYCK1 and grown in 4% GLU were switched to 0.2% GLU or maintained in high glucose for the indicated times in the presence of cycloheximide to inhibit new protein synthesis. Immunoblot analysis was conducted with α-GFP.

(G) WT cells expressing the indicated GFP fusions (described in D) were grown in 2% GLU or GAL and analyzed by immunoblot.

See also Figure S2.

AGP1 (Liu et al., 2008) (Figure 2E). If *sod1*Δ was specifically inhibiting Yck1p and Yck2p, then amino acid sensing should be impacted as well. As seen in Figure 2F and Figure S1E, *AGP1* promoter activity and protein expression levels, respectively, are markedly inhibited in *sod1*Δ cells, and the same is true for *akr1*Δ mutants. Loss of Sod1p affects the glucose- and amino acid-sensing pathways that require Yck1p and Yck2p.

SOD1 Interacts with and Stabilizes Casein Kinases Yck1p and Yck2p

Yck1p and Yck2p represent the only plasma membrane casein kinases of *S. cerevisiae*, and we find that *sod1*Δ cells have a marked reduction in their activity, but not of whole cell casein kinases, largely represented by vacuolar Yck3p and nuclear Hrr25p (Ho et al., 1997; Sun et al., 2004) (Figures 3A and 3B).

The same specificity for casein kinase inhibition was observed in *akr1*Δ mutants (Figures 3A and 3B). By immunoblot, C-terminal TAP-tagged Yck1p and Yck2p are virtually undetectable in a *sod1*Δ strain, whereas the vacuolar and nuclear Yck3p and Hrr25p are unaffected (Figure 3C). To address whether Yck1p/Yck2p loss occurred at the transcriptional or translational initiation level, we expressed an N-terminal green fluorescent protein (GFP) fusion to Yck1p under control of the *TEF1* promoter. This GFP-Yck1p fusion, but not GFP alone, was strongly downregulated by *sod1*Δ mutations (Figure 3C). Transcriptional and translational initiation cannot account for the *sod1*Δ loss in Yck1p, indicating protein stability effects.

Because *sod1*Δ specifically impacts plasma membrane Yck1p and Yck2p, we tested whether cell surface localization was key. A C537S C538S allele of Yck1p that disrupts the palmitylation site for membrane anchorage (Roth et al., 2011) is no longer plasma membrane localized (Figure S2A), yet its stability is still regulated by Sod1p (Figure 3D). Yck1p and Yck2p also have unique C termini outside of the catalytic region that are not present in Yck3p and Hrr25p (Figures S2B and S3). As seen in Figure 3D, deletion of Yck1p C-terminal residues 367–538 completely obliterated regulation by Sod1p and the protein remained stable in *sod1*Δ strains, whereas smaller C-terminal deletions to residue 412 had no effect. Moreover, a minimal fusion of the Yck1p C-terminal domain (CTD) to GFP was sufficient to induce GFP fusion degradation in *sod1*Δ strains (Figure 3D). Hence, residues 367–412 of the Yck1p CTD contain a SOD1-regulated degron. As shown in Figure S3, the C-terminal degron contains three lysine residues, K383, K386, and K390, also present in Yck2p that represent potential sites of regulation by ubiquitination or other posttranslational modifications. As shown in Figure 3D, mutation of these lysines to arginine stabilizes Yck1p in the absence of Sod1p. Hence, SOD1 regulation of Yck1p involves lysines in the Yck1p degron region.

To test whether Sod1p physically interacts with this degron region, coimmunoprecipitation studies were carried out. Sod1p was seen to coimmunoprecipitate with full-length Yck1p fused to GFP (Figure 3E, lane 2), but not with GFP alone (Figure 3E, lane 4). As was observed for Sod1p-stabilization of Yck1p (Figure 3D), Sod1p-Yck1p interactions do not require plasma membrane anchorage (no effect of Yck1p C537S C538S mutations, Figure 3E, lane 6) but do require the CTD of Yck1p. Interactions are disrupted upon deletion of the Yck1p CTD to position 367 (Figure 3E, lane 10), but not to 412 (lane 8), and the Yck1p CTD is sufficient to confer Sod1p binding (lane 12). Interestingly, the Yck1p C-terminal K383, K386, and K390 residues that are required for SOD1 stabilization of the kinase (as in Figure 3D) are not required for Sod1p binding to Yck1p (Figure 3E, lane 14). Hence, these lysines are specifically involved in Yck1p turnover, not Yck1p interaction with Sod1p.

The means by which glucose activates Yck1p and Yck2p are not well understood. We therefore tested whether glucose, like Sod1p, regulates Yck1p through protein stability. In the experiment of Figure 3F, cells grown in high glucose were switched to low glucose, and the stability of GFP-Yck1p was monitored in the presence of cycloheximide to inhibit new protein synthesis. This switch to low glucose led to destabilization of GFP-Yck1p within 2 hr (Figure 3F). Substituting glucose with the nonrepressing carbon source galactose also resulted in loss of GFP-Yck1p (Figure 3G). As with SOD1-control of Yck1p, the glucose stabilization of Yck1p does not require membrane anchorage (the C537S C538S Yck1p mutant is without effect) but requires the Yck1p C terminus and residues K383, K386, and K390 (Figure 3G). Therefore, during glucose repression of respiration, Yck1p is stabilized through a mechanism involving its SOD1-interacting domain and key lysines at the C terminus.

Enzymatically Active SOD1 Stabilizes CK1γ Homologs from Yeast and Mammals

We tested whether SOD enzymatic activity is required for Yck1p stability. Mutations H63A and H71A in yeast Sod1p disrupt the copper site and inactivate the enzyme, and these same Sod1p mutants no longer support Yck1p stability (Figure 4A), similar to effects of a *sod1*Δ deletion. Furthermore, yeast *ccs1*Δ mutants that lack the copper chaperone for Sod1p (Culotta et al., 1997) express inactive Sod1p, and these same mutants cannot stabilize Yck1p (Figure 4B). Thus, Yck1p stability requires Sod1p enzymatic activity.

In addition to Cu/Zn SOD1, most eukaryotes express a second unrelated SOD2 enzyme that localizes to the mitochondrial matrix and uses Mn as a cofactor (Weisiger and Fridovich, 1973). In *sod1*Δ *sod2*Δ cells lacking both SOD enzymes, Yck1p is absent as expected (Figure 4C, lane 1), and this is rescued by ectopic expression of *SOD1* (lane 3), but not of *SOD2* (lane 5). Because Yck1p and Sod2p localize to separate cellular compartments, we attempted to rescue loss of Yck1p by expressing Sod2p in the cytosol. Cytosolic Sod2p becomes active when cells accumulate high Mn (Luk et al., 2005) (Figure 4C, lane 8, bottom), but this is not sufficient to stabilize Yck1p. Another means for removing superoxide in yeast involves small molecule complexes of manganese, so-called Mn-antioxidants (Aguirre and Culotta, 2012; Barnese et al., 2012; Chang and Kosman, 1989; Reddi and Culotta, 2011), which are far more effective for removing superoxide in yeast than a variety of commonly used Mn-porphyrin SOD mimetics (Munroe et al., 2007). Supplements of high manganese can fully rescue O_2 sensitivity in the *sod1*Δ *sod2*Δ strain (Figure S4), but these same Mn-antioxidants do not rescue loss of Yck1p (Figure 4C, lanes 2, 6, and 8). Thus, only Sod1p can stabilize Yck1p.

Can the SOD1-stabilization of CK1γ kinases be extended to other eukaryotes? We expressed Cu/Zn SOD molecules from *Caenorhabditis elegans* and humans in *sod1*Δ strains and observed that these heterologous Cu/Zn SODs can also stabilize Yck1p (Figure 4D, lanes 3 and 4). We also tested whether yeast Sod1p can stabilize mammalian CK1γ. A bovine CK1γ isoform, CK1γ3, which has a high degree of sequence conservation (Figure S3) and complements yeast *yck1 yck2* mutants (Zhai et al., 1995), is stably expressed in *SOD1* wild-type (WT) yeast, but not in *sod1*Δ cells (Figure 4D, lanes 5 and 6). Moreover, human SOD1 expressed in yeast can stabilize this bovine CK1γ (see ahead, Figure 6C). Thus, mammalian CK1γ homologs are also stabilized by a variety of eukaryotic SOD1 molecules. This was addressed further using a human cell line. HEK293 cells were treated with the intracellular copper chelator tetrathiomolybdate (TTM), a treatment that readily inhibits SOD1 activity in cell culture (Alvarez et al., 2010; Juarez et al., 2008). TTM inhibits

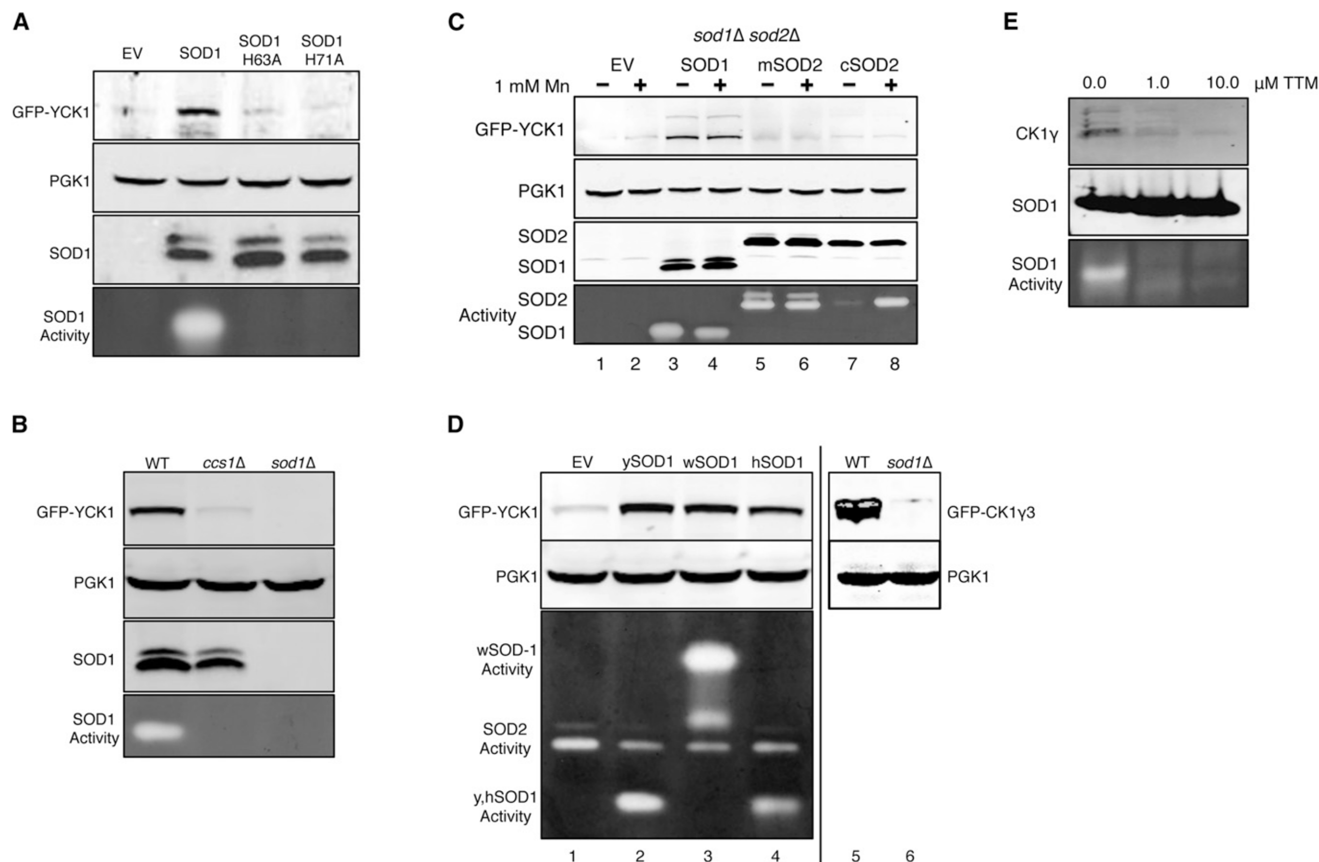

Figure 4. Enzymatically Active SOD1 Stabilizes CK1γ Homologs from Yeast and Mammals and in Cell Culture

(A–D) Immunoblots (top two to three panels) and native gel assays for SOD activity (bottom) were conducted on cells grown in 4% GLU and transformed with pGFPYCK1 or plasmid pAR124 for expressing GFP fused to bovine CK1γ3 (GFP-CK1γ3). As indicated, *sod1Δ* (A and D) and *sod1Δ sod2Δ* (C) strains also expressed the following SOD molecules: SOD1, ySOD1, H63A, H71A, *S. cerevisiae SOD1* or mutant derivatives; mSOD2 and cSOD2, mitochondrial and cytosolic *S. cerevisiae SOD2;* wSOD1, *C. elegans* Sod-1; hSOD1, human SOD1. These various SODs exhibit differential migration on native gels and their positions are denoted. "1 mM Mn" indicates manganese supplemented during entire growth period.

(E) Immunoblot of endogenous CK1γ and SOD1 and native gel assay for SOD activity were conducted on HEK293 cells treated with the indicated concentrations of the copper chelator TTM for 24 hr.

See also Figures S3 and S4.

SOD1 activity without changes in SOD1 protein, and this correlates with a drastic reduction in levels of human CK1γ (Figure 4E). These findings corroborate our yeast expression studies showing that yeast and mammalian CK1γ isoforms are stabilized by active SOD1 enzyme.

Levels of Cytosolic Cu/Zn SOD1 Required for Yck1p Stability

SOD1 is localized in both the cytosol and in the mitochondrial IMS (Sturtz et al., 2001). However, mitochondrial SOD1 is not relevant to Yck1p because expression of an IMS-specific Sod1p ("SCO2-SOD1" [Wood and Thiele, 2009]) does not stabilize Yck1p (Figure 5A). Thus, extramitochondrial Sod1p is required for Yck1p stability.

We sought to gauge how much cytosolic Sod1p is required to stabilize Yck1p. In our immunoprecipitation studies, we recover 3% of total Sod1p bound to Yck1p (Figure 3E and legend). This corresponds to ~8,000 Sod1p dimers, compared to the ~7,800 molecules of Yck1p estimated for yeast (Ghaemmaghami et al., 2003). However, we find that the actual level of total Sod1p required to maintain Yck1p stability is substantially greater than this 3% value. When *SOD1* expression is titrated down using a *MET25* repressible promoter, Yck1p protein is destabilized with a 5-fold reduction in Sod1p levels (Figure 5B, for quantitation, see figure legend). It is possible that the larger fraction of Sod1p (≥20% total Sod1p) that is required for stabilizing Yck1p is not all bound to the casein kinase at steady state, but is needed to feed the pool of kinase-bound Sod1p.

O_2 Regulation of Yck1p Stability

In various eukaryotes, SOD1 activity is downregulated as cells reach hypoxia and anoxia (Brown et al., 2004; Leitch et al., 2009; Leitch et al., 2012; White et al., 2009). *S. cerevisiae* Sod1p activity is virtually undetectable in cells grown under nitrogen, and we observe a dramatic loss in Yck1p under these conditions (Figure 6A, lane 2). Furthermore, bovine CK1γ3

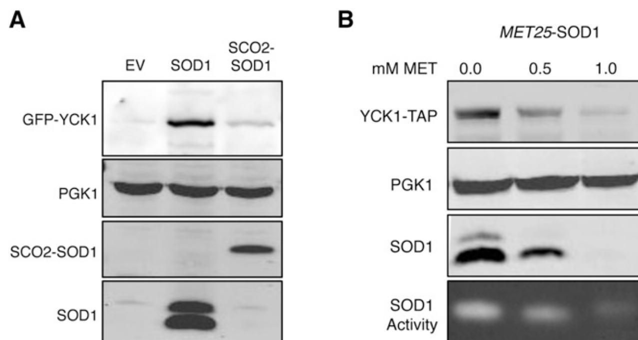

Figure 5. Cytosolic Sod1p Stabilizes Yck1p

(A) The *sod1Δ* strain grown in 4% GLU and expressing GFP-Yck1p was transformed with vectors for expressing yeast Sod1p (SOD1), or mitochondrial IMS targeted Sod1p (SCO2-SOD1) and analyzed by immunoblot. α-SOD1 was used to detect SCO2-SOD1.

(B) A *sod1Δ* strain expressing Yck1p-TAP and *MET25*-driven *SOD1* was grown in 4% GLU with increasing concentrations of methionine (MET) to repress *SOD1* expression. Upper three panels are immunoblots of the indicated proteins, and bottom panel is native SOD activity gel. Immunoblot quantitations were conducted using the software on the LIC-COR Odyssey imager and were normalized to PGK1 and are relative to the 0.0 MET control as follows: 0.5 mM MET, Yck1p-TAP = 0.3; Sod1p = 0.2; 1.0 mM MET, Yck1p-TAP = 0.10; Sod1 protein = 0.01.

expressed in yeast is similarly downregulated by low O_2 in cells that coexpress either yeast Sod1p (Figure 6B) or human SOD1 (Figure 6C). As with effects of low glucose or *sod1Δ* mutations, Yck1p instability with low O_2 does not require membrane anchorage (not affected by Yck1p C537S C538C mutations) but does require the Sod1p-interacting degron domain of Yck1p at the C terminus and residues K383, K386, and/or K390 (Figure 6B).

We tested whether loss of Yck1p with low O_2 could be reversed by reintroducing active SOD1 enzyme. Anaerobic cultures can be induced to produce mature Sod1p enzyme by placing *SOD1* under control of the constitutive *ADH1* promoter and by using a P144S allele of Sod1p that can acquire copper independently of Ccs1p and O_2 (Leitch et al., 2012). We were surprised to find that Yck1p is still downregulated under nitrogen in cells expressing P144S Sod1p (Figure 6D, lane 4). As a noteworthy caveat to this experiment, the mature P144S Sod1p enzyme should have little activity in vivo without O_2 due to limited superoxide substrate. Superoxide was monitored using the dihydroethidium (DHE) probe selective for superoxide over peroxide, peroxynitrite, and hypochlorous acid (Tarpey and Fridovich, 2001). Indeed, cells under nitrogen have low superoxide (Figure 6E). By providing the superoxide substrate for SOD1, O_2 may stabilize Yck1p.

Superoxide and Hydrogen Peroxide Effects on YCK1

SOD1 reactivity with superoxide could stabilize CK1γ kinases in two ways: either by preventing superoxide damage to CK1γ or by catalyzing superoxide conversion to H_2O_2, which in turn promotes kinase stability. Our studies with anaerobic cultures argue against the former. As seen in Figure 6A, the *sod1Δ* loss in Yck1p was not rescued under nitrogen (lane 4), even though superoxide levels are greatly reduced (Figure 6E). Moreover, when WT cells are shifted from O_2 to N_2, we observe a 2-fold drop in superoxide within 3 hr (Figure 7B), which closely parallels the kinetics of Yck1p turnover (Figure 7A). If anything, loss of superoxide correlates with Yck1p degradation, not stability.

It is noteworthy that this same loss in superoxide is seen in aerobic cultures that are shifted from glucose to galactose (Figure 7B). As with hypoxia, the kinetics of Yck1p turnover closely follow the decrease in superoxide with a switch to galactose (Figures 7A and 7B). In spite of the loss of superoxide substrate for Sod1p, the SOD enzyme remains bound to Yck1p during the switch to galactose (Figure S5). Loss of the superoxide substrate for Sod1p, rather than loss of Sod1p binding, seems to trigger Yckp1 turnover.

We were initially surprised that superoxide levels were higher in glucose conditions where mitochondrial respiration is repressed (Figure 1A). Nevertheless, the superoxide from glucose cultures is a substrate for Sod1p, as levels rise even further with *sod1Δ* mutations (Figure 7C). By comparison, the superoxide from galactose cultures may not be a substrate for Sod1p, as levels are unaffected by *sod1Δ* mutations (Figure 7C). Hence, cells grown in O_2 and glucose produce a superoxide substrate for Sod1p and Yck1p is stabilized (Figure 7D).

What is the source of superoxide relevant to Sod1p and Yck1p regulation? Part of the superoxide from glucose growth cells may originate from mitochondria because rho0 mutations eliminating respiration show a 2-fold decrease in cellular superoxide (Figure 6F). However, this loss of mitochondrial superoxide has no effect on Yck1p stability (Figure 6F). Extramitochondrial sources of superoxide can include NADPH-oxidases, and an intriguing candidate in yeast is the NADPH oxidase-like protein, Yno1p (Rinnerthaler et al., 2012). We observe that *yno1Δ* cells, which exhibit an ~25% reduction in superoxide levels, experience a marked loss in Yck1p stability (Figure 6F), suggesting that this NADPH oxidase-like protein is a superoxide source for SOD1/CK1γ signaling.

We next tested whether H_2O_2, the product of the SOD reaction, can affect Yck1p degradation. As a paradigm, we employed the switch to hypoxia and galactose (as in Figures 7A and 7B) where Yck1p turnover is easily monitored. (Yck1p turnover rates are not easily obtained from *sod1Δ* null cells where steady state Yck1p is undetectable.) As seen in Figure 7A, the addition of 0.5 mM exogenous H_2O_2 significantly stabilizes the Yck1p polypeptide during the switch to both hypoxia and galactose. As another means of increasing cellular peroxide, we added galactose oxidase (GO) to the growth medium. As seen in Figures S6A and S6B, GO produced a continuous level of peroxide that could be detected intracellularly using the reactive oxygen species (ROS) probe, 2,7-dichlorofluorescein diacetate (DCFDA) (Wu et al., 2009). Moreover, this GO-derived peroxide helped to stabilize Yck1p (Figure S6C). It is worth noting that the level of peroxide in both cases is relatively high. Stabilization of Yck1p requires either a one-time bolus of 0.5 mM H_2O_2 or a continuous flux of peroxide from GO (>0.11 mM/min), which generates 0.5 mM H_2O_2 within 5 min (Figures S6C and S6D). The rationale for needing such high non-SOD1 H_2O_2 can be explained by considering the numerous peroxide scavenging systems of the cell. Yeast express various catalases and

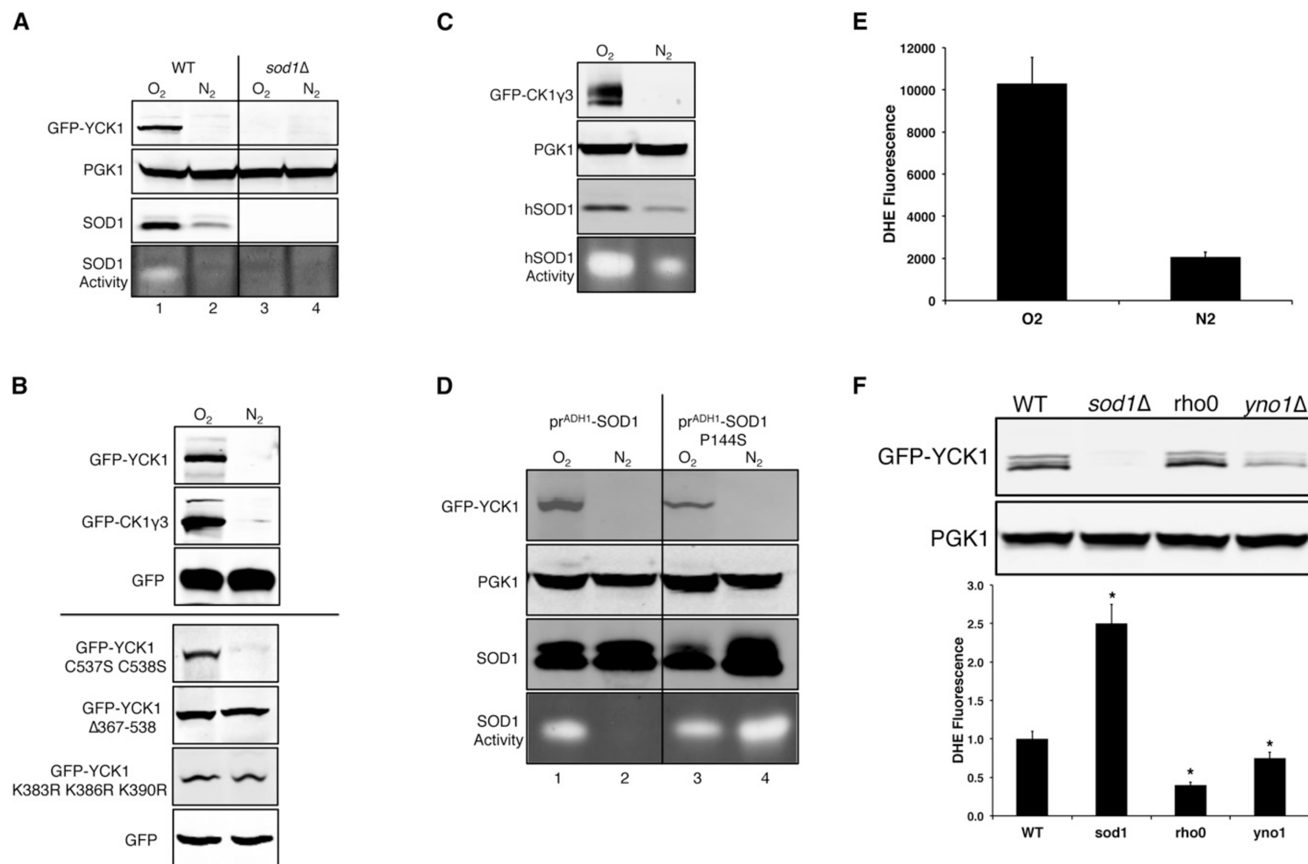

Figure 6. O_2 and Superoxide Regulate Yeast and Mammalian CK1γ Stability

The indicated cells were cultured in 4% GLU medium under either atmospheric oxygen (O_2) or nitrogen (N_2) conditions.

(A, C, and D) Immunoblots (top panels) and native SOD activity gels (bottom panel) were assayed from the indicated cells expressing GFP-Yck1p or GFP fused to bovine CK1γ3. hSOD1, pr^{ADH1}-SOD1, pr^{ADH1}-SOD1 P144S, *sod1*Δ cells expressing human SOD1 driven by the yeast *SOD1* promoter and *ADH1*-driven WT *SOD1* or the P144S derivative.

(B) Immunoblots of WT cells expressing GFP-Yck1p or the indicated Yck1p mutant derivatives or GFP fused to bovine CK1γ3.

(E) Superoxide levels were measured by DHE (dihydroethidium) fluorescence as described in Experimental Procedures. The data represent the mean ±SD of triplicate cultures.

(F) Immunoblots (top) and DHE fluorescence (bottom graph) of WT, *sod1*Δ, rho0, and *yno1*Δ cells expressing *TEF* driven GFP-Yck1p. Bottom graph data represent the mean ±SD of triplicate cultures, and asterisks indicate a $p < 0.05$ and reflect a statistically significant difference between the DHE fluorescence of WT cells and the indicated mutants.

peroxidases that can all contribute to peroxide removal, and indeed, we find that single mutations in the corresponding *cta1*, *ctt1*, *ahp1*, *dot5*, *prx1*, and *tsa1* genes were not sufficient to stabilize Yck1p under low glucose conditions (Figure S6E). In total, our data indicate local peroxide produced by Sod1p at the site of Yck1p not only escapes being quenched by the various peroxide scavenging enzymes in the cell, but also circumvents the requirement for high nonphysiological doses of H_2O_2 to stabilize Yck1p.

DISCUSSION

In many aerobic organisms, ambient O_2 promotes respiration, whereas loss of O_2 favors fermentation (Pasteur, 1861). However, in proliferating cells, aerobic glycolysis is frequently chosen over respiration as a source for energy irrespective of O_2 (Lunt and Vander Heiden, 2011). In many instances, glucose itself can repress respiration (Crabtree, 1929) through mechanisms that are not completely understood. In this study, we demonstrate that O_2 itself is a repressor of respiration. Our data demonstrate that O_2, glucose, and the antioxidant enzyme Cu/Zn SOD1 cooperate to maintain stability of Yck1p and Yck2p, two CK1γ casein kinases, for respiratory repression. SOD1 binds to a degron-containing region we have identified in Yck1p residues 367–412, and prevents degradation of the kinase through a mechanism involving lysines at the Yck1p C terminus. Our data support a model in which the superoxide generated during growth in the presence of glucose and O_2 feeds into cytosolic SOD1, and the concomitant production of H_2O_2 helps stabilize the CK1γ kinases for nutrient signaling (Figure 7D).

Glucose has long been known to repress respiration in proliferating cells, a phenomenon known as the Crabtree effect

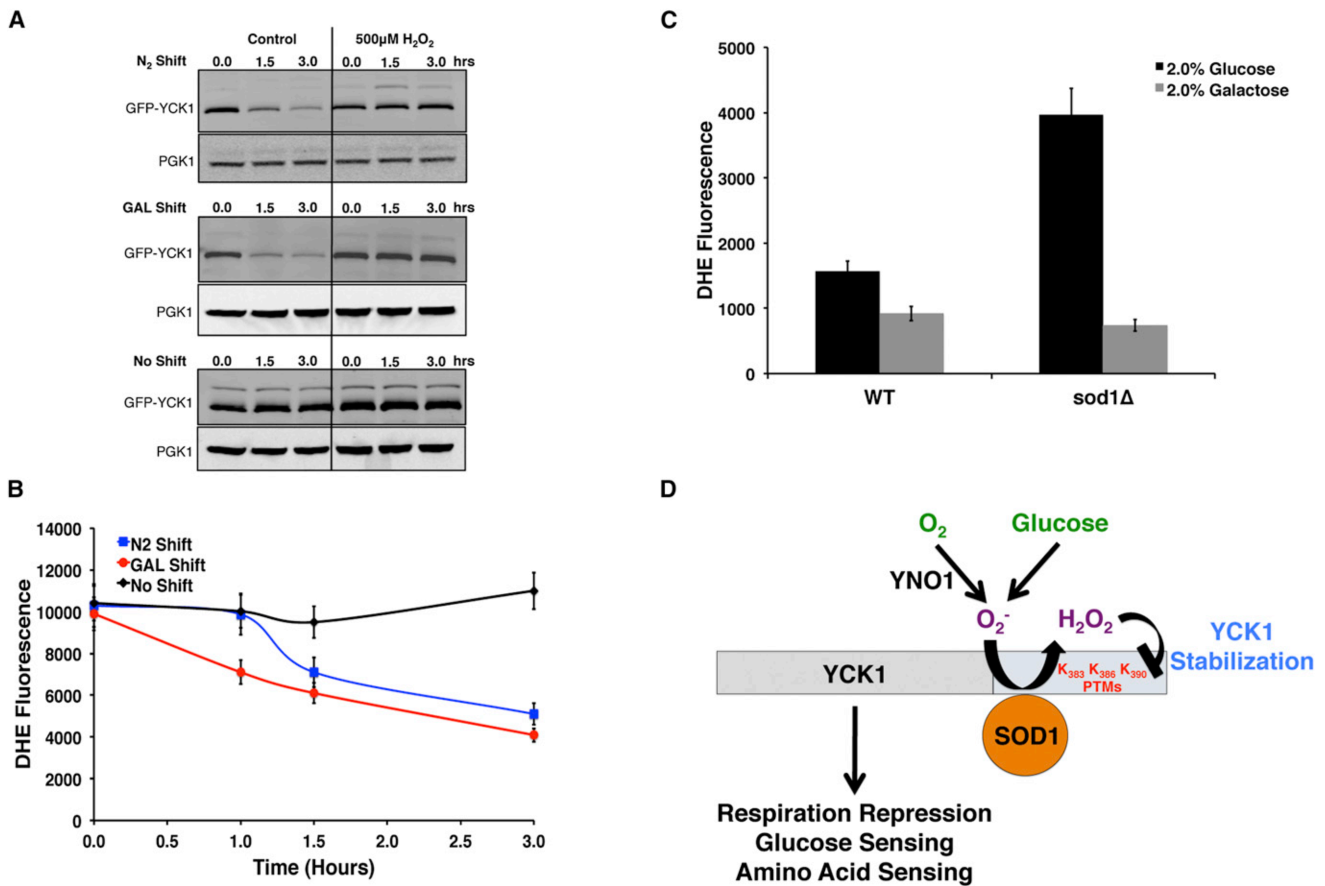

Figure 7. A Role for H_2O_2 in Stabilization of Yck1p under Aerobic Glucose Conditions

(A and B) WT cells expressing GFP-Yck1p under aerobic 4% GLU conditions were shifted either to anaerobic conditions (N_2 shift), or to aerobic 4% GAL conditions (GAL shift), or maintained as is (no shift) for the indicated times. (A) Turnover of Yck1p in the presence of cycloheximide was monitored by α-GFP immunoblot. Where indicated, 500 μM H_2O_2 was supplemented. (B) DHE detectable superoxide was monitored as described in Experimental Procedures. The data represent the mean ±SD of triplicate cultures.

(C) Superoxide levels were measured by DHE fluorescence in 2% GLU or 2% GAL cultures of WT and *sod1*Δ cells. The data represent the mean ±SD of triplicate cultures.

(D) A model for regulation of CK1γ isoforms by SOD1. Superoxide generated from growth in the presence of O_2 and glucose is converted by SOD1 to H_2O_2, which stabilizes a C-terminal degron on Yck1p, a casein kinase that regulates glucose and amino acid sensing and respiration repression, through a mechanism that involves peroxide-inhibition of posttranslational modifications (PTM) at lysines K383, K386, and/or K390.

See also Figures S5 and S6.

(Crabtree, 1929). Although the molecular basis of this effect is not well understood, it has previously been proposed that increased glycolytic flux plays an important role (Diaz-Ruiz et al., 2011). Indeed, many yeast mutants defective in glucose repression (such as the *snf3*Δ *rgt2*Δ mutant; Figure S1D; Ozcan et al., 1998) show a loss in glucose uptake correlating with the increase in cell respiration. However, the role of SOD1/CK1γ in glucose repression described here occurs independent of any changes in glucose uptake. The control of aerobic fermentation and respiration by Sod1p/Yck1p may represent a distinct element of the Crabtree effect.

The role of SOD1 in oxidative stress protection has been thoroughly characterized since its discovery more than 40 years ago (McCord and Fridovich, 1969). However, only a small fraction of total SOD1 enzyme appears required for protection against defined oxidative insults (Corson et al., 1998). SOD1 has also been shown to participate in the buffering of toxic copper ions (Culotta et al., 1995) and protecting the calcineurin phosphatase from oxidative damage (Wang et al., 1996). The role of SOD1 in casein kinase signaling shown here is independent of these effects and can be extended to mammalian cells. We find that Cu/Zn SODs from nematodes and humans can stabilize yeast Yck1p, and conversely, yeast Sod1p can stabilize bovine casein kinase 1 gamma-3 (CK1γ3). Furthermore, we find that SOD1 activity promotes CK1γ expression in a human cell line. As with yeast CK1γ, mammalian CK1γ isoforms are involved in regulating fermentative metabolism, but through the Wnt signaling pathway (Lee et al., 2012; Sethi and Vidal-Puig, 2010), suggesting a conserved role for SOD1 in regulating CK1γ isoforms and fermentative metabolism. Why was SOD1 selected for this purpose? The answer may lie in its superoxide substrate, an ideal candidate molecule to link O_2 and glucose to the repression

of respiration. Given that SOD enzymes are the only enzymatic receptors for superoxide in eukaryotic cells, the exploitation of cytosolic Cu/Zn SOD1 to transduce superoxide signals from O_2 and glucose is an intelligent repurposing of an antioxidant enzyme.

We provide evidence that the H_2O_2 product of the SOD1 reaction stabilizes the casein kinases. H_2O_2 produced by SOD1 has previously been shown to regulate tyrosine kinase receptor-mediated processes, and in this case, the targets are redox-sensitive cysteines (Juarez et al., 2008). The SOD1-responsive C-terminal degron in Yck1p does not contain any cysteines but does contain a number of lysines that, as we report here, are critical for SOD1/glucose/O_2 regulation of Yck1p stability. As one possibility, ubiquitination of these lysines by H_2O_2-sensitive E3 ligases may lead to Yck1p turnover. Cysteine-rich RING E3 ligases can be susceptible to oxidation (Meng et al., 2011), and E3 adaptors can also contain H_2O_2-reactive cysteines (Dinkova-Kostova et al., 2002; Zhang et al., 2004).

The implications for Sod1p regulation of casein kinases extend beyond repression of respiration. Yeast Yck1p/Yck2p participates in numerous nutrient signaling processes, such as amino acid sensing through the SPS pathway (Liu et al., 2008). We previously reported that the SPS pathway was downregulated by hypoxia (Gleason et al., 2011), and we can now explain this phenomenon by the hypoxia-induced loss of Sod1p activity and Yck1p. The exact rationale for downregulation of Yck1p/Yck2p under hypoxia is not clear but may involve its multifaceted roles in signaling, including amino acid sensing and other as-of-yet unknown targets of this regulatory kinase.

In conclusion, our studies demonstrate that SOD1 is not just for antioxidant defense. Through its ability to bind and protect key casein kinases from degradation in an O_2- and glucose-dependent manner, SOD1 is a vital component of nutrient-sensing pathways and is essential for repressing respiration. Taken together, our results suggest that SOD1 acts as a metabolic focal point for integrating O_2, nutrients (glucose), and reactive oxygen (superoxide) to direct energy metabolism.

EXPERIMENTAL PROCEDURES

General Considerations

All yeast strains and plasmids are listed, along with details on their genotype, source, and/or construction, in Extended Experimental Procedures. Yeast cultivation, cell culture, cell lysis, immunoblotting, immunoprecipitation, DCFDA determination of intracellular H_2O_2, and *lacZ* gene reporter assays, were conducted using standard methods and are described in detail in Extended Experimental Procedures.

Experiments involving anaerobic growth and manipulation were conducted under an atmosphere of 95% N_2 and 5% H_2 in a COY Chamber (COY Laboratory Products, Grass Lake, MI, USA). All absorption and fluorescence measurements were recorded on a Biotek HT Synergy plate reader.

Measurement of O_2 Consumption and Superoxide Levels

O_2 consumption was measured using the Becton Dickinson (BD) Oxygen Biosensor System according to the manufacturer's instructions, as described previously (Davidson et al., 2011), in mid-log-phase cells grown in media enriched with yeast extract, peptone (YP), with the indicated glucose (GLU) or galactose (GAL) concentrations. Briefly, triplicate cultures of cells were inoculated into YP 2% GLU media and grown to a density of OD_{600nm} = 1.0 (12–15 hr), rediluted into fresh media (initial OD_{600nm} = 0.15), and grown to a final OD_{600nm} = 1.0. Then, 200 μl of 5 × 10^7 to 2 × 10^8 cells/ml were collected and resuspended in fresh media, and O_2 consumption was recorded as the fold increase in fluorescence (λ_{ex} = 485 nm, λ_{em} = 620 nm) of wells containing cells relative to wells containing only media in BD Oxygen Biosensor System. Where indicated (Figure S1B), O_2 consumption rates were calculated from the maximum slopes of the sigmoidal O_2 consumption isotherms, and recorded as the change in the fold increase in fluorescence per minute, with the data normalized to WT cells. In cases where different GLU or GAL concentrations are utilized, cells were precultured in triplicate in YP 2% GAL to a density of OD_{600nm} ~1.0 (12–15 hr), and cultures were rediluted into fresh media containing the indicated GLU or GAL concentration (initial OD_{600nm} = 0.15) and allowed to grow to a final OD_{600nm} = 1.0. O_2 consumption was then measured as described above.

Superoxide levels were measured by monitoring the fluorescence of DHE-stained cells (λ_{ex} = 485 nm, λ_{em} = 620 nm) similarly to what was described previously (Neklesa and Davis, 2008). Briefly, 1 × 10^7 cells were harvested from duplicate or triplicate cultures, resuspended and incubated in 500 μl of fresh media containing 50 μM DHE for 20 min in the dark, washed twice with PBS solution, and fluorescence recorded. For measurements of anaerobic superoxide levels, staining and washing was carried out in an anaerobic COY Chamber, and then cells were rapidly removed for analysis on the plate reader (in air). The minimal superoxide detected in anaerobic cultures (as in Figure 6D) may reflect production during this short exposure to air. In instances where DHE staining was conducted on cells switched from O_2 to N_2 or GLU to GAL, cells were harvested at designated time points and subjected to DHE staining for 20 min prior to analysis.

Biochemical Assays

For Pma1p and casein kinase (CK) activity measurements, duplicate 1.0 l YP 2% or 0.2% GLU cultures were grown as described above for measuring O_2 consumption. Plasma membranes (PM) isolated according to published procedures (Perlin et al., 1989) were used for both Pma1p ATPase and CK activity measurements. CK activity was also assayed in whole cell lysates prepared as described in Extended Experimental Procedures. Pma1p ATPase activity was assayed in membrane fractions with or without a 5 min preincubation with 50 μM vanadate. ATPase activity was determined by quantitating the phosphate released during vanadate-sensitive ATP hydrolysis by molybdate reactivity (Perlin et al., 1989), and activity was normalized to WT cells grown in 2% GLU. CK activity was assayed using the CSNK1G3 Z′-LYTE Kinase Ser/Thr 5 Peptide Assay kit (Invitrogen) according to the manufacturer's instructions. Activity reflected the change in ratio of fluorescence emission (λ_{ex} = 360 nm, λ_{em1} = 460 nm, λ_{em2} = 528 nm) correlating with the degree of CK1 peptide substrate phosphorylation. Activity was recorded as % phosphorylation of substrate per milligram of whole cell or PM lysate protein.

SOD activity analysis was carried out by native PAGE and nitroblue tetrazolium staining as described previously (Flohe and Otting, 1984; Luk et al., 2005) on mid-log-phase cultures grown to a final OD_{600nm} = 1.0 in YP 4% GLU. Glucose consumption of triplicate mid-log-phase WT or *sod1Δ* cells was monitored by measuring extracellular glucose concentration as a function of culture density using the Quantichrome Glucose Assay Kit (BioAssay Systems).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2012.11.046.

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