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Cu/Zn Superoxide Dismutase (Sod1) regulates the canonical Wnt signaling pathway

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Abstract

Cu/Zn Superoxide Dismutase (Sod1) catalyzes the disproportionation of cytotoxic superoxide radicals ($O_2^{\bullet-}$) into oxygen (O_2) and hydrogen peroxide (H_2O_2), a key signaling molecule. In *Saccharomyces cerevisiae*, we previously discovered that Sod1 participates in an H_2O_2 -mediated redox signaling circuit that links nutrient availability to the control of energy metabolism. In response to glucose and O_2 , Sod1-derived H_2O_2 stabilizes a pair of conserved plasma membrane kinases - yeast casein kinase 1 and 2 (Yck1/2) - that signal glycolytic growth and the repression of respiration. The Yck1/2 homolog in humans, casein kinase 1- γ (CK1 γ), is an integral component of the Wntless and Int-1 (Wnt) signaling pathway, which is essential for regulating cell fate and proliferation in early development and adult tissue and is dysregulated in many cancers. Herein, we establish the conservation of the SOD1/YCK1 redox signaling axis in humans by finding that SOD1 regulates CK1 γ expression in human embryonic kidney 293 (HEK293) cells and is required for canonical Wnt signaling and Wnt-dependent cell proliferation.

Keywords

Cu/Zn Superoxide Dismutase; redox signaling; superoxide; hydrogen peroxide; reactive oxygen species; Wnt signaling; cancer

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Author Contributions.

BC, CMA, and AEC designed and performed experiments. JLA and YCJ designed experiments. BC, CMA, and ARR wrote the manuscript with input from all authors.

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Conflict of Interest

The authors do not have any conflicts of interest to declare.

Introduction.

Cu/Zn superoxide dismutase (Sod1) is a highly conserved antioxidant enzyme that detoxifies superoxide radicals ($O_2^{\bullet-}$) by catalyzing its disproportionation into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2). Sod1 is amongst the most abundant soluble proteins, accounts for > 80% of intracellular SOD activity [1], and its importance in oxidative stress protection is underscored by reduced proliferation, decreased lifespan, and metabolic defects when *SOD1* is deleted in various cell lines and organisms [2-14]. Most interestingly, using Baker's yeast as a model unicellular eukaryote, we found that only a small fraction (< 1%) of the total Sod1 pool is required to protect cells against cell-wide damage from $O_2^{\bullet-}$ [15,16]. Rather, we found that much larger quantities of Sod1 are required to produce H_2O_2 for the redox regulation of a pair of highly conserved plasma membrane yeast casein kinases, Yck1 and Yck2, that regulates nutrient-sensing [15].

Yck1 and Yck2 link extracellular glucose availability to repression of respiration and fermentative growth [16] (Figure 1a). Glucose binding to transmembrane glucose receptors, Snf3 and Rgt2, activates Yck1 and Yck2, which in turn phosphorylates transcriptional effectors Mth1 and Std1 [17] and signals their ubiquitination and proteasomal degradation by the Skp-Cullin-F-box (SCF) E3 ubiquitin ligase (UbL) Grr1. When glucose is limiting, Mth1 and Std1 are stable and activate the expression of genes required for the metabolic adaptation to low glucose and enhance respiratory metabolism. Sod1-derived H_2O_2 stabilizes Yck1/2, which leads to glucose-mediated repression of respiration and fermentative growth. When Sod1 or $O_2^{\bullet-}$ is diminished, Yck1/2 is degraded and respiratory metabolism is enhanced. Sod1 regulates Yck1 and Yck2 stability via the peroxide-dependent regulation of a still unknown post-translational modification (PTM) on conserved lysine (K) residues at the C-terminus of Yck1 and Yck2.

The Yck1/2 homolog in humans, casein kinase 1- γ (CK1 γ), is an integral component of the Wnt signaling pathway, which is essential for regulating cell fate and proliferation in early development and adult tissue and is dysregulated in many cancers [18]. In the canonical Wnt pathway, the transcriptional effector β -catenin activates the expression of Wnt target genes in control of cell proliferation, differentiation, migration, adhesion, and energy metabolism. Just as yeast Yck1/2 link extracellular glucose availability to energy metabolism (Figure 1a), human CK1 γ links extracellular signals from a family of Wnt proteins to control metabolism and physiology (Figure 1b). Wnt ligands bind to a heterodimeric receptor complex consisting of Frizzled (Frz) and low-density lipoprotein receptor-related proteins LRP5 and LRP6. This binding event activates CK1 γ , which then phosphorylates LRP5/6, ultimately leading to the sequestration of the β -catenin destruction complex. With the destruction complex unavailable, β -catenin is able to translocate to the nucleus where it can activate Wnt-target genes (Figure 1b, **left**). In the absence of Wnt ligands, the β -catenin destruction complex phosphorylates β -catenin, which in turn leads to its ubiquitination and proteasomal degradation by the SCF UbL β -Transducin Repeat-Containing Protein (β -TrCP) (Figure 1b, **right**).

Having demonstrated previously that Sod1 regulates Yck1/2 expression and glucose signaling in yeast [15,16], we sought to determine if Sod1 regulates CK1 γ expression and

Wnt signaling in mammalian cells. Herein, using RNA interference to silence *SOD1* in human embryonic kidney cell line (HEK293), we report that SOD1 regulates CK1 γ expression and Wnt signaling in mammalian cells.

Materials and Methods.

Chemicals, media components, and immunological reagents.

Dihydroethidium (Cat. # 50-850-563) was purchased from Thermo Fisher Scientific. Paraquat dichloride (Cat # 856177-1G) was purchased from Sigma-Aldrich. The mammalian nuclear isolation kit was purchased from Thermo Fisher Scientific (Cat. #78833). Rabbit polyclonal antibodies against GAPDH (VWR; Cat. # 89348-232), PGK1 (Thermo Fisher; Cat. # PA528612), LRP6 (Cell Signaling; Catalog # 2560), phospho- β -catenin S33/S37/T41 (Cell Signaling; Catalog # 9561), β -catenin (Abcam; Cat # 6302), Phospho-LRP-6 T1479 (Abnova; Cat. # PAB12632) [19], Lamin A/C (Cell Signaling; Cat. # 2032S), CK1 γ 3 (Thermo Fisher; Cat # PA5-99838) [20], Prdx1 (Millipore Sigma; Cat. # HPA007730-100UL), Trx1 (Cell Signaling; Cat. # 2429), Gpx1 (Abcam; Cat. # ab22604), and Prdx-SO₃ (Abcam; Cat. # Ab16830), and mouse monoclonal antibodies against Porin (Invitrogen; Cat. #459500) and Prdx2: PRDX2 (Fisher Scientific; Cat. # LFMA0144) were purchased from commercial sources as indicated. A previously described rabbit polyclonal antibody against Sod1 was obtained from the laboratory of Valeria Culotta (Johns Hopkins University) [16]. Goat α -rabbit (VWR; Cat # 89138-520) or α -mouse (VWR; Cat # 89138-516) secondary antibodies conjugated to a 680-nm emitting fluorophore were obtained from Biotium through VWR.

Cell culture, growth, gene silencing, Wnt stimulation, and nuclear fractionation.

Human embryonic kidney (HEK293) cells were obtained from (American Type Culture Collections. ATCC, Cat. # CRL-1573). Cells were cultured in Dulbecco's Minimal Essential Medium/ DMEM media (Thermo Fisher Scientific (Cat. # 3105328) containing Heat Inactivated Fetal Bovine Serum (HIFBS, 10 %, VWR Cat # 89510-188), supplemented with L-glutamine in 5% CO₂ and 37°C with 1% antibiotics (Penicillin-Streptomycin). Cells were plated in 6 well plates for immunoblotting or qPCR experiments (0.25 - 0.3 million cells/ well), or on 13 mm coverslips in 24 well plates for immunostaining. SOD1 siRNA (Cat. # 4390824) and Control siRNA (Cat. # 4390843) were purchased from (Thermo Fisher Scientific). SOD1 silencing was accomplished in reduced serum medium (Opti-MEM, Cat. # 11058021, Thermo Fischer Scientific) by transfection of SOD1 siRNA or scrambled control siRNA (for 72 hours) into 60-70% confluent HEK cells using Lipofectamine-2000 transfection reagent according to Manufacturer's protocols (Cat. # 11668019, Thermo Fischer Scientific).

Wnt conditioned media was produced by culturing L-Wnt-3A cells (ATCC, Cat. # CRL-2647) according to AtCC protocol, and media from L Cells (ATCC, Cat. # CRL-2648) was used as the corresponding negative control. Wnt stimulation was achieved by treating HEK293 cells with DMEM: Wnt3a conditioned media (1:1 v/v) for 3 hours.

Immunoblotting and SOD activity.

Immunoblotting was performed exactly as described previously with cells from 6-well dishes being harvested, washed in ice-cold Milli-Q water, and lysed in two pellet volumes of lysis buffer (10 mM sodium phosphate, 50 mM sodium chloride, 5 mM EDTA, 1.0% Triton X-100, 1 mM PMSF and a protease and phosphatase inhibitor cocktail (GBiosciences) [21]. For assessing peroxiredoxin oxidation, cell cultures were quenched with 10% trichloroacetic acid (TCA), followed by TCA-precipitating lysate protein and washing it in cold acetone. The dried pellet was resuspended in degassed resuspension buffer (6M urea, 10mM EDTA, 20mM Tris, 0.5%SDS, 10 μ M neocuproine, pH 8.5) containing 1mM PMSF and a protease inhibitor cocktail (GBiosciences) in an anaerobic chamber (Coy laboratories). Lysate protein concentrations were determined by the Pierce™ BCA protein assay kit (Thermo Scientific). SOD activity was measured using an in-gel assay based on native PAGE and nitroblue tetrazolium staining as described previously [16,22-24].

Immunofluorescence.

HEK293 cells plated on coverslips and transfected with control or Sod1 siRNA (72 h), were fixed using 4% paraformaldehyde in phosphate buffered saline (PBS) for 30 min and blocked with 10% FBS for 1 hour. The coverslips were then incubated with the β -catenin antibody for 2 h, followed by Cy3-conjugated secondary antibody for 1 hour. 4', 6-diamidino-2-phenylindole (DAPI, 1:10,000 dilution, Sigma) was used for nuclear staining. The mounted coverslips were imaged using Zeiss LSM510 confocal microscopy to assess cytoplasmic versus nuclear localization of β -catenin. Results were expressed as percent localization relative to the total number of DAPI positive cells. At least 10 fields were imaged from each condition from 3 independent experiments.

qRT-PCR.

RNA was isolated from HEK293 cells using the RNAeasy kit (Qiagen). cDNA was synthesized from 1 μ g of total RNA using iscript cDNA synthesis kit (Biorad). qRT-PCR was performed using SYBR Green PCR Master Mix (Biorad) and 7500 Real-Time PCR System (Applied Biosystems) using primers for β -catenin, Cyclin D1, c-myc, GAPDH, and SOD1.

Results.

Sod1 regulates the canonical Wnt signaling pathway.

In order to probe the role of Sod1 in the Wnt signaling pathway, we determined the effect of Sod1 silencing on CK1 γ expression and its impact on Wnt pathway activation in HEK293 cells upon stimulation with the Wnt3a ligand (Figure 1b). There are three plasma membrane tethered CK1 γ isoforms in humans: CK1 γ 1, CK1 γ 2, and CK1 γ 3. We chose to focus our efforts on Sod1-mediated regulation of CK1 γ 3 for the following reasons:

- a. in HEK293 cells, CK1 γ 3 protein levels are ~4-fold higher than CK1 γ 2 and > 10-fold higher than CK1 γ 1, which is undetectable [25];
- b. CK1 γ 3 (and CK1 γ 2) contains a conserved C-terminal K implicated in Sod1 and peroxide regulation of Yck1/Yck2 - K³⁸³ in Yck1 and K³⁵⁵ in CK1 γ 3 [16];

c. Sod1 regulates bovine CK1 γ 3 heterologously expressed in yeast [16].

SOD1 expression and activity is consistently reduced by ~60% using siRNA against SOD1 in HEK293 cells after 72 hours (Figure 2a and 2b). This degree of *SOD1* silencing results in a ~40% decrease in CK1 γ 3 expression (Figure 2a and 2c), as measured using a CK1 γ 3-specific antibody. These results are consistent with our previous studies in Baker's yeast that found that *sod1* cells exhibited a marked loss in Yck1/2 expression and heterologously expressed bovine CK1 γ 3. It is important to note that *SOD1* silencing does not affect the expression of other cytosolic peroxide metabolizing enzymes, including peroxiredoxins 1 and 2 (Figure S1a-d), thioredoxin 1 (Figure S1e and S1f), and glutathione peroxidase 1 (Figure S1e and S1g), thereby indicating that other antioxidant enzymes are not compensating for the loss of Sod1 function. Moreover, silencing of *SOD1* does not significantly impact steady-state peroxide levels and oxidative stress as indicated by the lack of significant changes to peroxiredoxin oxidation (Figure S1h-j).

SOD1 silencing and the concomitant decrease in CK1 γ 3 expression resulted in a significant decrease in multiple markers of Wnt pathway activation (Figure 1b). Wnt3a binding to its cell surface receptor activates CK1 γ which in turn phosphorylates LRP6 at T1479, ultimately leading to sequestration of the β -catenin destruction complex [18]. As a consequence, cytosolic β -catenin cannot be phosphorylated at residues S33, S37, and T41, allowing it to escape proteasomal degradation and translocate to the nucleus to regulate gene expression. Accordingly, we find that Wnt3a stimulation results in an increase in LRP6 phosphorylation (Figure 2a and 2g), a decrease in β -catenin phosphorylation (Figure 2a and 2e), an increase in β -catenin stability (Figure 2a and 2d), and increased nuclear β -catenin as measured by immunofluorescence (Figure 3a and 3b) and subcellular fractionation (Figure 3c and 3d). In contrast, Wnt3a stimulation in *SOD1* silenced cells *does not* increase LRP6 phosphorylation (Figure 2a and 2g), decrease β -catenin phosphorylation (Figure 2a and 2e), increase β -catenin stability (Figure 2a and 2d), or increase nuclear localization of β -catenin (Figure 3). Thus, *in total*, Sod1 is required for Wnt-dependent activation of the canonical Wnt pathway.

Sod1 regulates the Wnt-dependent activation of Wnt target genes and cell proliferation.

We next determined if Sod1 impacts the expression of Wnt target genes and Wnt-dependent cell proliferation. Once in the nucleus, β -catenin associates with transcription factors from the T cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors and drives transcription of hundreds of Wnt/ β -catenin target genes. Using qRT-PCR, we determined if silencing SOD1 in Wnt stimulated cells altered the expression of Wnt/ β -catenin target genes β -catenin, c-Myc, and Cyclin D1. Silencing SOD1 resulted in a 50% decrease in SOD1 mRNA (Figure 4d), consistent with the decrease in Sod1 polypeptide (Figure 2a and 2b). Moreover, silencing SOD1 in Wnt3a stimulated cells reversed the Wnt-induced increase in expression of β -catenin (Figure 4a), c-Myc (Figure 4b), and Cyclin D1 (Figure 4c) mRNA as expected.

Given that Wnt/ β -catenin target genes are associated with proliferative metabolism, we assessed if SOD1 silencing reversed Wnt-induced cell proliferation. Wnt stimulation results in a pronounced enhancement in cell proliferation by 48 hours (Figure 4e). However, in

SOD1 silenced cells, the Wnt-mediated stimulation in proliferation at 48 hours is completely reversed (Figure 4e). Notably, simply silencing *SOD1* in the absence of Wnt3a stimulation does not cause growth defects at any time point, indicating that the ~50% depletion in *SOD1* does not result in overt oxidative stress. Altogether, our data support a role for *SOD1* in regulating the Wnt signaling pathway.

Discussion.

Wnt signaling regulates cell fate, proliferation, motility, and differentiation during development and in adult tissues. Dysregulation of the Wnt pathway is associated with many diseases, including various cancers and neurodegenerative disorders [26]. The plasma membrane casein kinases, Yck1/2 in yeast and CK1 γ in humans, integrate extracellular ligand binding events to an intracellular signaling cascade that triggers changes in gene expression and cell physiology (Figure 1). Herein, we report that *SOD1* regulates CK1 γ expression in human embryonic kidney 293 (HEK293) cells and is required for canonical Wnt signaling and Wnt-dependent gene expression and cell proliferation. Our findings have a number of implications for the redox regulation of the Wnt pathway, redox signaling, and Sod1 biology.

H₂O₂ can both activate and inhibit the Wnt pathway [27,28]. Supplementation with high doses of exogenous peroxide (~100 μ M) inhibits Wnt signaling in HEK293 cells [29]. In contrast, low doses of H₂O₂ rapidly stabilize β -catenin and increase the expression of Wnt target genes [30]. The mechanism involves the H₂O₂ dependent regulation of nucleoredoxin (Nrx) [28], an inhibitor of Dishevelled (Dvl), which inhibits the GSK3 β mediated phosphorylation of β -catenin that leads to its proteasomal degradation [31] (Figure 1b). Thus, physiological redox signaling at multiple points in the Wnt pathway and pathological oxidative stress have the potential to modulate Wnt signaling.

A number of cancers are associated with hyperactive Wnt signaling and elevated Sod1 and CK1 γ expression [32-39]. Our results suggest that inhibiting Sod1 in such cancers may be a selective therapeutic strategy for suppressing growth in proliferative cells. Indeed, we found that silencing *SOD1* only inhibited cell proliferation in Wnt-stimulated HEK293 cells (Figure 4e). These results are consistent with a prior study that found that *SOD1* is essential for oncogene-driven mammary tumor formation but dispensable for normal development and proliferation [34]. Altogether, our results provide a strong foundation for exploring the role of *SOD1* in regulating redox gradients in control of Wnt signaling in health and disease and anti-*SOD1* based cancer therapeutics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Cu/Zn Superoxide Dismutase (Sod1) is a well conserved and abundant antioxidant enzyme.
- Sod1 plays important roles in redox signaling due to its production of H₂O₂.
- Sod1 regulates the canonical Wnt signaling pathway.

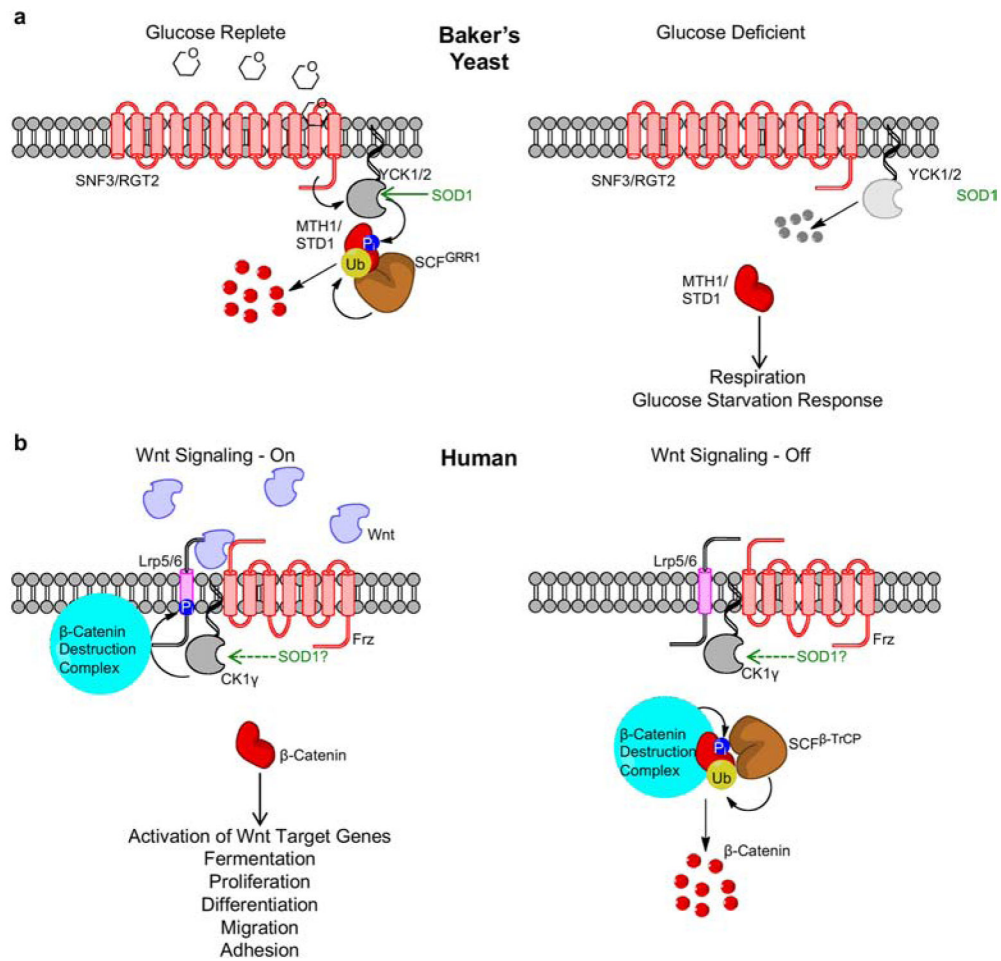
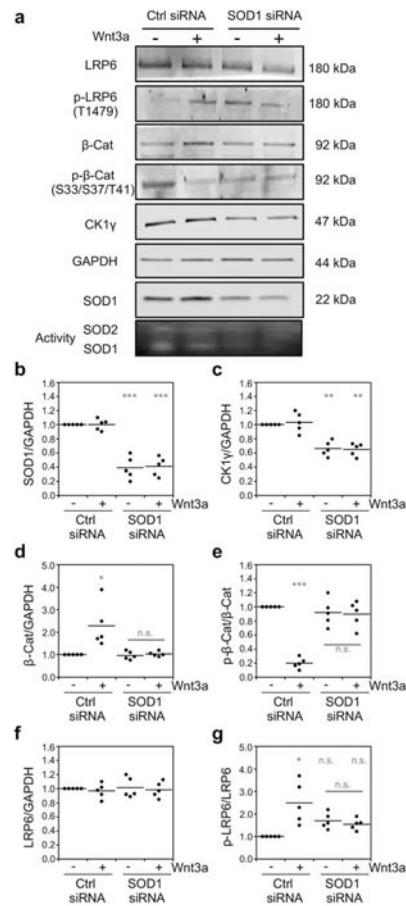
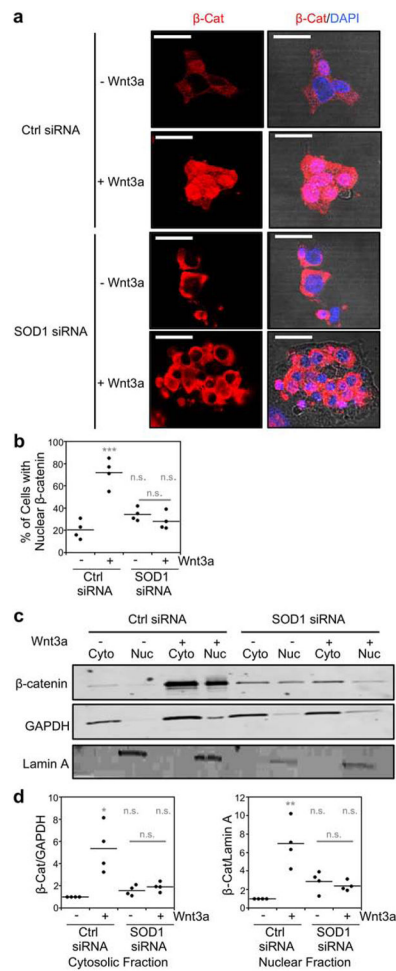


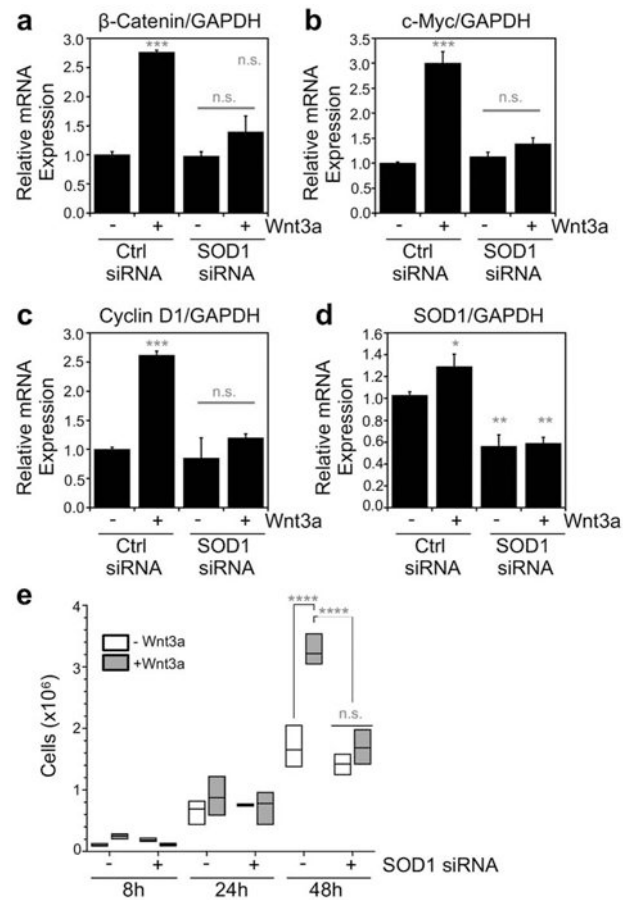
Figure 1. Comparison of (a) glucose sensing in Baker's yeast (*Saccharomyces cerevisiae*) and (b) Wnt signaling in humans, with an emphasis on the respective roles of Sod1 and plasma membrane casein kinase homologs, Yck1/Yck2 (yeast) and CK1 γ (humans). See text for a detailed description of the two pathways.

**Figure 2.**

Sod1 is required for the Wnt3a-dependent activation of the canonical Wnt signaling pathway. (a) Representative immunoblots and SOD activity gel of Wnt signaling pathway markers in response to Wnt3a activation and/or silencing of Sod1 with siRNA. (b-g) Quantification of the normalized levels of (b) Sod1, (c) CK1 γ 3, (d) β -catenin (β -Cat), (e) phosphorylated β -catenin at residues S33, S37, and T41 p- β -Cat, (f) LRP6, and (g) phosphorylated LRP6 at residue T1479 (p-LRP6) from five independent trials. p-LRP6 and p- β -Cat is normalized to total LRP6 or β -Cat, respectively. The statistical significance relative to – Wnt3a/Ctrl RNAi cells or for the indicated pairwise comparison is denoted by grey asterisks and determined by ordinary one-way ANOVA with the Bonferroni multiple-comparison post-hoc test. * P < 0.01, ** P < 0.001, *** P < 0.0001, n.s. = not significant.

**Figure 3.**

Sod1 is required for the Wnt3a-dependent nuclear localization of β -catenin. (a) Representative immunofluorescence of β -catenin localization relative to the nuclear marker, DAPI, in response to Wnt3a activation and/or silencing of Sod1 with siRNA. Scale bar is 20 μ m. (b) Quantification of β -catenin nuclear localization from four independent trials. ~200 cells were counted for each condition in each trial. (c) Representative immunoblots of β -catenin expression in cytosolic and nuclear fractions of HEK293 cells. (d) Quantification of the normalized levels of cytosolic and nuclear β -catenin (β -Cat) from four independent trials. Cytosolic β -Cat is normalized to the cytosolic marker GAPDH and nuclear β -Cat is normalized to the nuclear marker Lamin A. The statistical significance relative to – Wnt3a/ Ctrl RNAi cells or for the indicated pairwise comparison is denoted by grey asterisks and determined by ordinary one-way ANOVA with the Bonferroni multiple-comparison post-hoc test. * $P < 0.01$, ** $P < 0.001$, *** $P < 0.0001$, n.s. = not significant.

**Figure 4.**

Sod1 regulates Wnt3a-dependent gene expression and HEK293 cell proliferation. (a-d) Normalized mRNA expression of (a) β -catenin, (b) c-Myc, (c) Cyclin D1, and (d) SOD1 relative to GAPDH as measured by qRT-PCR in response to Wnt3a activation and/or silencing of Sod1 with siRNA. The indicated values represent the mean \pm s.d. from triplicate cultures. (e) Cell proliferation in response to Wnt3a activation and/or silencing of Sod1 with siRNA. The statistical significance relative to – Wnt3a/Ctrl RNAi cells or for the indicated pairwise comparison is denoted by grey asterisks and determined by ordinary one-way ANOVA with the Bonferroni multiple-comparison post-hoc test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, n.s. = not significant.