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Lipid Signaling via Pkh1/2 Regulates Fungal CO₂ Sensing through the Kinase Sch9

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ABSTRACT Adaptation to alternating CO₂ concentrations is crucial for all organisms. Carbonic anhydrases—metalloenzymes that have been found in all domains of life—enable fixation of scarce CO₂ by accelerating its conversion to bicarbonate and ensure maintenance of cellular metabolism. In fungi and other eukaryotes, the carbonic anhydrase Nce103 has been shown to be essential for growth in air (~0.04% CO₂). Expression of NCE103 is regulated in response to CO₂ availability. In Saccharomyces cerevisiae, NCE103 is activated by the transcription factor ScCst6, and in Candida albicans and Candida glabrata, it is activated by its homologues CaRca1 and CgRca1, respectively. To identify the kinase controlling Cst6/Rca1, we screened an S. cerevisiae kinase/phosphatase mutant library for the ability to regulate NCE103 in a CO₂-dependent manner. We identified ScSch9 as a potential ScCst6-specific kinase, as the sch9Δ mutant strain showed deregulated NCE103 expression on the RNA and protein levels. Immunoprecipitation revealed the binding capabilities of both proteins, and detection of ScCst6 phosphorylation by ScSch9 in vitro confirmed Sch9 as the Cst6 kinase. We could show that CO₂-dependent activation of Sch9, which is part of a kinase cascade, is mediated by lipid/Pkh1/2 signaling but not TORC1. Finally, we tested conservation of the identified regulatory cascade in the pathogenic yeast species C. albicans and C. glabrata. Deletion of SCH9 homologues of both species impaired CO₂-dependent regulation of NCE103 expression, which indicates a conservation of the CO₂ adaptation mechanism among yeasts. Thus, Sch9 is a Cst6/Rca1 kinase that links CO₂ adaptation to lipid signaling via Pkh1/2 in fungi.

IMPORTANCE All living organisms have to cope with alternating CO₂ concentrations as CO₂ levels range from very low in the atmosphere (0.04%) to high (5% and more) in other niches, including the human body. In fungi, CO₂ is sensed via two pathways. The first regulates virulence in pathogenic yeast by direct activation of adenylyl cyclase. The second pathway, although playing a fundamental role in fungal metabolism, is much less understood. Here the transcription factor Cst6/Rca1 controls carbon homeostasis by regulating carbonic anhydrase expression. Upstream signaling in this pathway remains elusive. We identify Sch9 as the kinase controlling Cst6/Rca1 activity in yeast and demonstrate that this pathway is conserved in pathogenic yeast species, which highlights identified key players as potential pharmacological targets. Furthermore, we provide a direct link between adaptation to changing CO₂ levels and lipid sensing in fungi.
conditions and lipid/Pkh1/2 signaling in yeast, thus establishing a new signaling cascade central to metabolic adaptation.

Carbon dioxide is key to life on earth. Besides being RuBisCO’s substrate in the carbon-fixing component of the Calvin cycle, it is the final product of cellular respiration (1). Consequently CO₂ plays a decisive role as a signaling molecule, and its levels are sensed by organisms as diverse as bacteria, plants, fungi, nematodes, insects, fish, and mammals (2, 3). In fungi, CO₂ impacts fundamental biological characteristics, including growth, morphology, and virulence (4). For pathogenic fungi, the ability to adapt to changing CO₂ is particularly relevant. When surviving as commensals on skin, fungi are exposed to atmospheric low CO₂ concentrations, but when they invade their host, CO₂ concentrations can rise to 150-fold higher levels (5% or more) (5–7).

In fungi, CO₂ is sensed via two distinct signaling pathways. In the first, HCO₃⁻/H⁺, which is in equilibrium with membrane-permeable CO₂ via action of the catalytic enzyme carbonic anhydrase (CA), directly activates adenylyl cyclases to signal via downstream protein kinase A (PKA) (8). This pathway was shown to trigger the yeast-to-hyphae switch of Candida albicans and capsule biosynthesis of Cryptococcus neoformans, representing well-characterized virulence factors of these major human pathogens (9, 10).

The second pathway is independent of adenylyl cyclase and much less characterized. This pathway directly affects cellular CO₂ fixation and HCO₃⁻ homeostasis and thus central metabolism. One readout of this pathway is regulation of expression of the β-class fungal CA Nce103p in response to environmental CO₂ (11, 12). CAs are ubiquitous zinc-containing metalloenzymes that accelerate the CO₂ to HCO₃⁻ interconversion and thus impact carboxylation reactions and pH homeostasis (13–15).

Notably yeast CA expression is strongly induced under CO₂-limiting atmospheric conditions and downregulated in elevated CO₂ (11). The overarching significance of this second CO₂-sensing pathway for fungal biology is emphasized by the fact that deletion of yeast CA abolishes growth in low CO₂ (16).

CO₂-dependent regulation of CA is conserved in Saccharomyces cerevisiae, C. albicans, and Candida glabrata. Some insight into the signaling events mediating CO₂-dependent regulation of CA expression in yeast was recently gained by identification of the transcriptional activator regulator of carbonic anhydrase 1 (Rca1). Loss of Rca1 in the pathogenic yeasts C. albicans and C. glabrata and loss of the Rca1 orthologue Cst6 in S. cerevisiae led to a common phenotype displaying lack of CA induction in low CO₂ (17, 18). Rca1 and Cst6 belong to the ATF/CREB transcription factor family and have a C-terminal basic leucine zipper domain (19). Comparison of the protein sequence of yeast Rca1/Cst6 revealed 3 conserved serine phosphorylation sites, and loss of serine 124 in the C. albicans orthologue abolished CO₂-dependent regulation of downstream CA (17). This supports our hypothesis that Rca1/Cst6 phosphorylation is a critical step in yeast CO₂ sensing.

To unravel this novel CO₂-sensing pathway, we used S. cerevisiae as a model. Using high-throughput kinase/phosphatase mutant library screening, we identified Sch9 as the Cst6-specific kinase candidate. We confirmed direct interaction and phosphorylation of Cst6 by the Sch9 kinase and show that Cst6/Rca1 signaling is conserved in C. albicans and C. glabrata CO₂ sensing. Finally, we provide evidence that Sch9 links CO₂ adaptation to lipid signaling via Pkh1/2.

RESULTS

High-throughput screening identifies potential Cst6 kinases. Previous work suggested that phosphorylation of the Cst6/Rca1 family of transcription factors is key to fungal CO₂ sensing, carbon fixation, and metabolism (17). To test this hypothesis, we opted for a high-throughput approach to identify candidate kinases/phosphatases. CO₂ regulation in the model organism S. cerevisiae was previously reported to be particularly pronounced in response to CO₂ variations of up to 20-fold between atmospheric
and high CO\(_2\) (11). Using quantitative reverse transcription-PCR (qRT-PCR), we confirmed this finding and found that CA mRNA levels are induced more than 23-fold when cultures are transferred from 5% CO\(_2\) (NCE103\(\text{CO}_2\)) to low-CO\(_2\) conditions (NCE103\(\text{air}\)), reaching maximum induction levels at 60 min (23.3 \pm 4.9-fold induction) (see Fig. S1A in the supplemental material). Furthermore, we established that induction of NCE103\(\text{air}\) was reduced in an S. cerevisiae cst6/\(H9004\) mutant (Fig. 1) and that CST6 expression itself was not affected by CO\(_2\) levels (Fig. S1B). Since CA expression by CO\(_2\) is conserved in these ascomycete yeasts, we decided to screen an S. cerevisiae kinase and phosphatase deletion mutant library of 155 strains (see Table S1 in the supplemental material) and quantify CO\(_2\)-dependent gene regulation (see Table S2 in the supplemental material for all data). Deletion of a Cst6 kinase should result in derepression of CA in high CO\(_2\) and consequently increased NCE103\(\text{CO}_2\) expression at levels comparable to growth in air (see Fig. S2 in the supplemental material) (17). Accordingly, mutants with mean NCE103\(\text{CO}_2\) expression levels twice as high (\(\geq 2.0\)-fold) as the wild type (WT) were considered putative kinase candidates. Of the 155 strains screened, 5 mutants met these criteria: the \(\text{tpd3}\Delta\), \(\text{tpf1}\Delta\), \(\text{bud3}\Delta\), \(\text{sch9}\Delta\), and \(\text{ptk2}\Delta\) strains (Fig. 1; see Table S2 and Table S3 in the supplemental material). Among those, the \(\text{sch9}\Delta\) mutant showed the highest upregulation of ScNCE103\(\text{CO}_2\) expression (3.55 \pm 1.55-fold), whereas ScNCE103\(\text{air}\) expression (6.12 \pm 2.98-fold) was similar to that of the WT. This deregulation pattern resulted in a low fold change between ScNCE103\(\text{air}\) and ScNCE103\(\text{CO}_2\) of 1.91 \pm 0.7-fold (Fig. 1; Table S2). Furthermore, the \(\text{sch9}\Delta\) strain did not display general growth defects, and \(\text{SCH9}\) encodes a kinase known to be involved in stress response via nutritional sensing in S. cerevisiae and adaptation to hypoxia in C. albicans (20–24), making Sch9 the most probable candidate as Cst6 kinase. To confirm our hypothesis, we analyzed the Nce103 protein level and fluorescence levels in a CA promoter-green fluorescent protein (GFP) fusion, then carried out immunoprecipitation (IP) experiments, and finally showed that Sch9 phosphorylates Cst6.

**FIG 1** Mutant library screening reveals putative Cst6 kinase. The S. cerevisiae wild type (WT) and mutants were grown in 5% CO\(_2\) to the exponential phase and were either maintained at 5% CO\(_2\) or transferred to air for 60 min. ScNCE103 expression was measured by qRT-PCR and normalized to WT expression under 5% CO\(_2\) (for all data, see Table S2). ScSch9 was the most promising Cst6 kinase candidate. The bars show means \(\pm\) standard deviations from \(\geq 5\) independent experiments. Significance against WT expression under the corresponding condition was calculated with a two-sided \(t\) test for unpaired samples and defined as \(P = 0.05\) (*) and \(P = 0.01\) (**).
Loss of Sch9 deregulates CO₂ sensing. Using a specific antibody against *S. cerevisiae* CA, we measured protein levels in the sch9Δ mutant cultivated under different CO₂ conditions. In contrast to the WT, Nce103 levels in the sch9Δ strain were elevated regardless of the strain being exposed to low or high CO₂ (Fig. 2A). Similar to NCE103 gene expression, the Nce103 protein level of the sch9Δ strain was not strongly affected by environmental CO₂. Next we transformed WT, cst6Δ and sch9Δ cells with a plasmid containing GFP under control of the ScNCE103 promoter and exposed to low and high concentrations of CO₂. GFP expression was analyzed using confocal microscopy (Fig. 2B). GFP fluorescence of WT cells under 5% CO₂ was constantly very low, but fluorescence increased after incubation in air (6-fold after 60 min and 9-fold after 120 min) (Fig. 2B). The persistent increase over time is likely due to the stability of GFP. In contrast, the cst6Δ strain showed nearly undetectable fluorescence levels under both conditions. The sch9Δ strain exhibited significantly higher fluorescence in 5% CO₂ compared to the WT, but there were also higher differences in intensity between cells.
Fluorescence in air was comparable to that of the WT. Noticeably, whereas NCE103 expression levels of the cst6Δ mutant in air and the sch9Δ mutant in 5% CO2 were not significantly different (Fig. 1), the GFP reporter signal for the cst6Δ mutant in air was lower than that for the sch9Δ mutant in high CO2. This could be due to the intrinsically high variation of NCE103 expression in air, which ranged from 3-fold to 10-fold when normalized to expression in 5% CO2. Furthermore, the experimental settings differ, with the GFP reporter being expressed from a plasmid rather than from its native locus. However, results for NCE103 levels in 5% CO2 are consistent in both assays and confirm that loss of Sch9 leads to a loss of CO2-dependent NCE103 repression.

**Sch9 is the Cst6 kinase.** To demonstrate physical interaction between Cst6 and Sch9, immunoprecipitation experiments were performed. Endogenous ScCst6 was precipitated from WT lysate using a specific anti-Cst6 antibody. Immunocomplexes were bound to Sepharose beads and incubated with recombinantly expressed Sch9-His. After extensive washing, Western blot analysis of bound proteins was performed (Fig. 3A). Beads without addition of anti-Cst6 antibody did not show a signal for either Cst6 or Sch9. Addition of Sch9-His without prior binding of Cst6 by antibodies also did not exhibit any signal, which excluded the possibility of unspecific binding. Importantly, when Cst6 was previously bound by anti-Cst6 antibody, we could detect specific binding of Sch9, thus demonstrating the ability of Sch9 to bind its expected substrate, ScCst6.

To provide further evidence that Sch9 is the Cst6 kinase, we carried out phosphorylation experiments. We overexpressed Sch9-His in *S. cerevisiae* under control of the inducible ScGAL1 promoter. Sch9 expression was induced by shifting the cells from glucose- to galactose-containing medium. *S. cerevisiae*-overexpressing cells were cultivated in 5% CO2, assuming that the kinase should be most active under this condition. ScSch9-His was purified from cell lysate via immunoprecipitation. Successful purification was followed by Western blot analysis resulting in a strong single band at ~120 kDa in the bead-bound fraction (Fig. 3B, left panel). Immunocomplexes with bound kinase were used for the radioactive kinase assay. The substrate Cst6-His was recombinantly expressed and purified using affinity chromatography. Purity was verified by Coomassie staining, represented by a single band at ~70 kDa (Fig. 3B, left panel). Sch9-His and Cst6-His were incubated in the presence of [γ-32P]ATP before separation by 4 to 20% SDS-PAGE. By means of autoradiography, incorporation of γ-32P was visualized. In control experiments, where the putative kinase ScSch9 was absent, no definite bands were visible, but a high background corresponding to nonincorporated radioactivity was seen (Fig. 3B, right panel). If Sch9 was present, two distinct bands of ~70 kDa and ~120 kDa were apparent, representing Cst6 and Sch9, respectively. These data demonstrate the phosphorylation of Cst6 by Sch9 in vitro and, furthermore, imply an extensive autophosphorylation of ScSch9, which was previously reported by Huber et al. (25).

**Cst6 phosphorylation analysis demonstrates that CO2 signals via phosphorylation of S266.** CO2 regulation of fungal CA expression is conserved, and *S. cerevisiae*
Cst6 is an orthologue to Rca1 in C. albicans and C. glabrata. Accordingly we hypothesized fungal CO₂-sensing signals via phosphorylation of defined Cst6/Rca1 amino acid residues. Comparison of the Cst6/Rca1 protein sequences identified three conserved serine residues at positions S124, S126, and S222 in C. albicans Rca1 corresponding to S266, S268, and S440 in S. cerevisiae Cst6. Previous work suggested that S124 but not S126 or S222 disrupts Ca regulation in C. albicans (17). Hence, we assumed that phosphorylation of S124 regulates fungal CO₂ sensing. In order to analyze the phosphorylation probability of the three serine residues, we overexpressed Cst6-His in the S. cerevisiae WT in 5% CO₂ assuming that phosphorylation status is at maximum. Cst6-His was affinity purified via immobilized metal ion affinity chromatography (IMAC) and subjected to 4 to 20% SDS-PAGE. Total protein was Coomassie stained, corresponding bands were excised, and trypsin-LysC, AspN, LysargiNase, or GluC digestion was performed to achieve high sequence coverage. In addition, an in-solution digestion with trypsin-LysC and GluC was done to also detect peptides of extended length. Phosphopeptides were enriched using TiO₂ and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Our results clearly showed that multiple sites of the Cst6 protein can be subject of phosphorylation. In the WT, we detected 19 different phosphorylation sites in at least two independent experiments (Fig. 4A). Thereof, 11 residues were previously identified by high-throughput approaches (26, 27). We identified another 8 sites, which were unknown to be phosphorylated, printed in boldface in Fig. 4A. In Cst6, the conserved serine residues correspond to S266, S268, and S440. Of those, only serine at position 266 was found to be phosphorylated in S. cerevisiae in 5% CO₂, while S268 and S440 were found to be unphosphorylated. In addition, the number of detected phosphorylations suggests the possibility of additional kinases being involved in regulation of Cst6, although we cannot draw any conclusions about biological relevance from the phosphorylation site mapping.

S266 is the only residue within ScCST6 matching the Sch9 consensus motif R-(R/K)-X-S (28). Hence, we mutated serine at position 266 to alanine (S266A) or aspartic acid (S266D) and cloned these sequences or WT CST6 into the cst6Δ mutant to prove influence on ScNCE103 expression. The cst6Δ mutant reverted with WT CST6 showed ScNCE103 expression comparable to that of the WT (Fig. 4B). Phosphoablative mutation of S266 to A led to a significant upregulation of ScNCE103CO₂ (2.73 ± 0.43-fold), while ScNCE103air (6.52 ± 2.12-fold) expression was unaltered. Interestingly, the pattern of expression of the cst6Δ + CST6S266D strain corresponds to the expression pattern of the sch9Δ mutant. This confirms our hypothesis and emphasizes S266 to be phosphorylated in 5% CO₂ in order to inhibit NCE103 expression. Phosphomimetic mutation of CST6 (CST6S266D) led to slightly decreased ScNCE103air expression in comparison to WT CST6, but differences were not statistically significant. This might point to alternative regulation mechanisms regulating NCE103 expression, especially under the air condition.

CO₂ sensing is conserved in yeast. Our findings and previous work suggest that CO₂-dependent regulation mediated via Cst6/Rca1 is conserved in some yeast species (17, 18). Due to the closer phylogenetic relationship between S. cerevisiae and C. glabrata, we first analyzed the influence of C. glabrata SCH9 (CgSCH9) on CgNCE103 expression under changing CO₂ conditions. Expression of CgNCE103 in 5% CO₂ and air after 60 min was quantified by qRT-PCR according to the previously described protocol for S. cerevisiae (Fig. 5A). The C. glabrata control strain, AFG1, revealed a 2-fold increase of CgNCE103air compared to CgNCE103CO₂ expression. This increase was completely absent in the rca1Δ mutant. In fact, CgNCE103 expression of the rca1Δ mutant in air was as low as in 5% CO₂. This is in contrast to the S. cerevisiae cst6Δ regulation pattern, where at least a small upregulation in air was observed. As expected, deletion of CgSch9 led to significantly elevated CgNCE103CO₂ levels (2.02 ± 0.43-fold) compared to the control. Noteworthy and in contrast to the pattern found in S. cerevisiae, CgNCE103air expression was also increased. However, despite these differences, Sch9 clearly affects NCE103 regulation in C. glabrata.
We also verified the influence of SCH9 on C. albicans NCE103 (CaNCE103) using similar protocols (Fig. 5B). The C. albicans WT exhibited a 4.6-fold increase of CaNCE103 expression in cells transferred to air conditions. In comparison, deletion of CaRCA1 resulted in significantly lower CaNCE103 expression. A C. albicans sch9Δ mutant strain (24) showed significantly increased CaNCE103 expression (2.61 ± 0.16-fold), similar to the effects of SCH9 deletion in S. cerevisiae and C. glabrata. Remarkably, also CaNCE103 levels were elevated, which coincides with what we found for C. glabrata, but not for S. cerevisiae. Taken together, these findings clearly indicate a conserved mechanism of CO2 adaptation in pathogenic and nonpathogenic yeast species and emphasize Sch9 is key to CO2 sensing in all three species.

**Lipid signaling via Pkh1/2 but not TORC1 regulates fungal CO2 sensing.** Previous work showed that in S. cerevisiae Sch9 is regulated by target-of-rapamycin (US...
approved name [USAN] sirolimus)-complex 1 (TORC1) and homologues of the mammalian 3-phosphoinositide-dependent protein kinase (mPDK1), Pkh1 and Pkh2, which phosphorylate Sch9 at specific sites (29, 30). TORC1 phosphorylates at least 6 residues of Sch9 (S711, T723, S726, T737, S758, and S765) (31), while ScPkh1/2 phosphorylates Sch9 at the PDK1 site T570 (32).

Analysis of LC-MS/MS data of overexpressed ScCST6-His allowed us to detect phosphorylated peptides specific for endogenous ScSch9. This enabled analysis of Sch9 phosphorylation in vivo to draw conclusions about putative activators. We detected the PDK1 site T570 and 3 of the phosphorylation sites addressed by TORC1 (S726, S758, and S765) to be phosphorylated in 5% CO\(_2\) (Fig. 6A). Therefore, we hypothesized that TOR and lipid signaling is involved in fungal CO\(_2\) sensing and that loss or inhibition of Sch9 upstream regulators mimics the \(sch9\)/H9004 mutation. Because activation of Sch9 is especially important under 5% CO\(_2\), we concentrated on changes of \(NCE103\) CO\(_2\) expression (see Table S2 and Table S4 in the supplemental material for \(NCE103\) air expression).

Notably, the \(tor1\)/H9004 mutant had been included in our initial qRT-PCR screening and showed slightly but not significantly increased CA expression in high CO\(_2\) (Fig. 6B). Catalytic subunits of TORC1 are either ScTor1 or ScTor2, and TORC1 is inhibited by sirolimus (33). Since Tor2 is an essential gene in \(S.\) cerevisiae, we investigated the impact of TORC1 signaling on fungal CO\(_2\) sensing via Sch9 by using sirolimus. Inhibition of TORC1 by sirolimus aggravated the effect of \(tor1\)/H9004 on CA gene expression and led to a significant upregulation of \(NCE103^{CO2}\) expression (1.85 \(\pm\) 0.46-fold) (Fig. 6B). However, it did clearly not reach the \(NCE103^{CO2}\) levels of the \(sch9\)\(\Delta\) mutant. \(PKH1/\)PKH2 single mutants did not show altered \(NCE103^{CO2}\) expression (Fig. 6B), probably because of Pkh1/2 redundancy in \(S.\) cerevisiae (34). As a conventional Pkh1/2 double deletion mutant is known to be lethal in \(S.\) cerevisiae (35), we used a temperature-sensitive double mutant to investigate the influence of Pkh1/2-mediated activation of Sch9 on \(NCE103^{CO2}\) expression. Indeed, expression levels of \(NCE103^{CO2}\) increased significantly to 2-fold in

![FIG 5 SCH9 function in CO\(_2\) signaling is conserved in pathogenic yeasts. C. glabrata (A) and C. albicans (B) strains were grown under different CO\(_2\) conditions, and expression of \(NCE103\) was measured by qRT-PCR. Cg\(NCE103\) expression was normalized to control expression (AFG1), and Ca\(NCE103\) expression was normalized to the \(C.\) albicans WT, cultivated in 5% CO\(_2\). Both species showed comparable regulation mechanisms. Deletion of \(RCA1\) led to significantly decreased \(NCE103^{air}\) expression. \(sch9\)\(\Delta\) mutants of \(C.\) glabrata and \(C.\) albicans showed increased \(NCE103^{CO2}\) expression, consistent with findings for \(S.\) cerevisiae. This suggests a conserved role of SCH9 in CO\(_2\) signaling in all three yeast species. The bars show means \(\pm\) standard deviations from \(\geq4\) independent experiments. Significance against control expression, calculated by two-sided \(t\) test for unpaired samples, was defined as \(P \leq 0.05\) (*), \(P \leq 0.01\) (**), and \(P \leq 0.001\) (***)].
the pkh1Δ pkh2Δ mutant compared to its control strain, 15 Dau. This implies that Sch9-mediated effects on CA expression could be dependent on both Sch9 regulators TORC1 and Pkh1/2.

To analyze the influence of Sch9 activation by TORC1 and Pkh1/2 more precisely, we investigated NCE103 expression of strains with site-specific mutations of known Sch9 phosphorylation sites addressed by TORC1 and Pkh1/2. As a control, the sch9Δ mutant reverted with WT SCH9 showed NCE103 expression levels in 5% CO₂ and air comparable to those of the WT strain. Surprisingly, also phosphoablative mutation of all 6 TORC1 sites (S711A, T723A, S726A, T737A, S758A, and S765A), referred to as SCH96A, resulted in WT-like NCE103 expression levels (Fig. 6C). In contrast, T570A mutation of the PDK1 site resulted in significantly elevated ScNCE103CO₂ expression (2.7 ± 0.59), while

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ScNCE103air expression was not changed. This is comparable with the deregulation pattern of the sch9A mutant. Additional S/T-to-A mutation of the 6 TORC1 sites within SCH9 (SCH9S570A, 6A) showed no additive effect on ScNCE103 expression (Fig. 6C).

Taken all together, we concluded that phosphorylation of Sch9 by Pkh1/2 at T570 is crucial for Sch9 activation in a 5% CO₂ environment in order to decrease ScNCE103 expression. Although phosphorylation sites addressed by TORC1 were detected in Sch9, CO₂-dependent activation of Sch9 and thus CO₂ adaptation seems to be independent of TORC1.

**DISCUSSION**

Sensing and adapting to alterations in ambient CO₂ are of paramount importance for all living organisms. Fungi adjust their metabolism to CO₂ availability, and CAs are essential in this process. CAs catalyze the reversible conversion of mobile CO₂ to HCO₃⁻, and disruption of their function using CA inhibitors or deletion distorts fungal metabolism or abolishes growth altogether.

Expression of the fungal CA NCE103 is regulated by the transcription factors Cst6 and Rca1, which belong to the ATF/CREB family (17). To identify enzymes responsible for Cst6/Rca1 phosphorylation and unravel fungal CO₂ sensing, we screened a kinase/phosphatase mutant library in the model organism S. cerevisiae. The library consisted of 111 kinase and 44 phosphatase mutants, and Sch9 was the most promising identified kinase candidate.

Sch9 regulates ribosome biogenesis, cell cycle progression, and life span and is involved in heat shock, osmotic and oxidative stress response by integrating nutrient signals (20–23, 36–38). Several lines of evidence support our hypothesis that Sch9 is indeed the Cst6 kinase and relays fungal CO₂ sensing: immunoprecipitation using endogenous Cst6 showed a physical interaction between Cst6 and Sch9. Furthermore, in vitro phosphorylation showed that Sch9 mediates the incorporation of radioactively labeled phosphate groups into Cst6, clearly demonstrating the kinase activity. Finally, the preferred consensus motif for Sch9 phosphorylation events is R-(R/K)-X-S (28).

Our work provides evidence that regulation of NCE103 in S. cerevisiae, C. glabrata, and C. albicans is conserved. Deletion of ScSCH9, CgSCH9, and CaSCH9 affected NCE103 expression in comparable ways as they lead to a significant upregulation of NCE103CO₂ (Fig. 1 and 5). Previous work provided some indirect evidence suggesting that S124 in C. albicans may be subject to phosphorylation impacting Rca1/Cst6 function (17). S124 is conserved in S. cerevisiae Cst6 (corresponding position S266) and C. glabrata Rca1 (S387). We now show proof that S266, but not other candidate residues, is phosphorylated in CST6 under elevated CO₂ and thus provide a molecular explanation for the conservation of CO₂ signaling in yeast (Fig. 4). Furthermore, we showed that phosphorylation of ScCST6 at S266 is crucial for repression of NCE103CO₂, because the phosphoablative mutation S266A resulted in increased NCE103CO₂ expression. This is in accordance with the results Cottier et al. found for S124 in C. albicans Rca1 (17).

Sch9 is a member of the AGC family (homologous to protein kinases A, G, and C) of proteins. It is conserved in S. cerevisiae, C. glabrata, and C. albicans but is also found in less-related fungal species like Fusarium graminearum (39). In mammalian cells, it has been hypothesized that PKB/Akt is the Sch9 homologue (40), although later studies suggest that Sch9 is more closely related to S6K1 (31), which acts downstream of TORC1. The best-characterized regulator of CA in human cells is HIF-1α, which induces human CA IX expression in response to hypoxia (41, 42). HIF-1α has been found to be regulated by mTOR, which suggested the assumption that Cst6/Rca1 regulation in yeast is also TOR dependent. Surprisingly, our experiments demonstrated that Sch9 phosphorylation by TORC1 does not contribute to CO₂-dependent NCE103 regulation (Fig. 6C). Indeed, inhibition of TORC1 by sirolimus showed effects on NCE103CO₂ (Fig. 6B), but these effects seem not to be dependent on Sch9 signaling. Furthermore, it was shown that addition of sirolimus is not always similar to inhibition of TORC1 (43). Instead of TORC1, phosphorylation by Pkh1/2 at T570 seems to be the driving force for Sch9 activation in order to repress NCE103 expression under 5% CO₂ (Fig. 7). It should
be considered that Pkh1/2 might not be the sole activator of Sch9. Deletion of SCH9 results in a 3.55-fold upregulation of ScNCE103, whereas increase following deletion of PKH1/2 was only 1.93-fold. Also T570A mutation of Sch9 leading to unresponsiveness to Pkh1/2 activation did not reach the high NCE103 levels. Thus, there is the possibility that Sch9 is directly influenced by CO2 levels and not only via Pkh1/2 (Fig. 7).

An influence of oxygen levels on Sch9 activity should also be considered: it has been previously reported that Sch9 inhibits hyphae formation in the fungal pathogen C. albicans under high-CO2 conditions (>1%) and hypoxia (<10%) but not under either condition alone (24).

It is noteworthy that another candidate identified in our screen was ScTpd3, which is a part of the protein phosphatase 2A complex. Although no kinase function has been reported, the protein phosphatase 2A complex is linked to nutrient signaling via Tor (44) and is suspected to dephosphorylate Sch9. Overexpression of ScTPD3 and deletion of SCH9 result in comparable phenotypes (45). Thus, ScTpd3 might indeed play a role in NCE103 regulation—maybe as a regulator of ScSch9.

The complexity of NCE103 regulation is further confirmed by our finding that despite the absence of ScCst6 as a transcriptional activator, we could observe upregulation of ScNCE103 air resulting in elevated ScNce103p, although this was clearly less pronounced than in the WT (Fig. 2A). Therefore, besides the ScSch9-ScCst6 pathway, there seem to be other mechanisms regulating ScNCE103, maybe another—so far unknown—transcription factor (Fig. 7B). This could be an explanation for the viability of cst6Δ mutants even under air conditions, whereas nce103Δ mutants are only able to grow in elevated CO2 environment (16). Only a moderate induction of NCE103 expression seems to be sufficient for fungal survival, but it will result in growth delay and, probably, affect other cell properties.

In this study, we provide insight into fungal CO2 sensing and show that lipid signaling activating Pkh1/2 regulates metabolic adaptation via Sch9. More work is needed to identify the precise molecular events and complex feedback mechanisms involved in CO2 signal reception and transduction to Sch9. However, the Sch9-Cst6/Rca1 cascade is conserved in yeasts and central to fungal metabolism, and Rca1 has
previously been reported to play a role in biofilm formation, which is instrumental in fungal pathogenesis (46). Thus, targeting fungus-specific components in organisms CO2 sensing, such as CA or Rca1/Cst6, constitutes a valid approach to new treatment option.

MATERIALS AND METHODS

Strains, plasmids, and primers. For the strains, plasmids, and primers used in this study, see Table S1.

ScNCE103 expression analysis. Exponential cultures of *S. cerevisiae* WT (BY4741) were cultivated at 37°C as duplicates in 5% CO2, and transferred to air for 10, 30, 60, 90, 120, or 180 min to induce ScNCE103 expression. Pellets were harvested, and RNA was extracted using a hot phenol-chloroform method (47). The quantity of RNA was measured with NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA), and 100 ng/μl RNA was utilized as the template for qRT-PCR using a Brilliant III Ultra Fast SYBR green qRT-PCR kit on a Stratagene Mx3005P (both Agilent Technologies, Santa Clara, CA). Gene expression was calculated by the ΔΔCt method (48) using ScACT1 as the housekeeping gene. The same procedure was done for the screening of *S. cerevisiae* kinase/phosphatase mutant library (Euroscarf, Frankfurt, Germany). Exponential cultures were transferred to air for 60 min to induce the highest ScNCE103 expression. ScNCE103 expression of mutants was determined by silver staining, and quantity was measured with a bicinchoninic acid (BCA) assay. At least 1 mg purified protein was used to produce specific rabbit antibodies against ScCst6-His and ScNce103-His according to the manufacturer’s protocol (Thermo Fisher Scientific). 1 mM stock solution in dimethyl sulfoxide (DMSO) was added. DMSO served as a solvent control. ScNCE103 expression of the 15 Dau and pkh1Δ pkh2Δ strains was measured with slight differences in the protocol due to temperature sensitivity of the pkh1Δ pkh2Δ mutant. Strains were preincubated overnight in 30°C and 5% CO2, and transferred to 37°C for 3 h to inhibit Pkh1 synthesis. Then samples were transferred to air for another hour to induce ScNCE103.

Expression and purification of His-tagged proteins from *E. coli*. *S. cerevisiae* CTS6, NCE103, and SCH9 were synthesized with a C-terminal 6×-His tag (GeneArt Gene Synthesis, Thermo Fisher Scientific) and cloned into *E. coli* expression vector p41. NCE103 and SCH9 were expressed in *E. coli* strain BL21(DE3) (New England Biolabs, Ipswich, MA). Bacteria were grown in LB medium (10 g/liter NaCl, 5 g/liter yeast extract, 10 g/liter tryptone [pH 7]) with 50 μg/ml ampicillin at 37°C. ScNCE103 expression was induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) at an optical density at 600 nm (OD600) of 0.2 and cultivated overnight until harvesting. Expression of ScSCH9 was induced at an OD600 of 0.8 for 4 h at room temperature. ScCTS6 expression was performed using *E. coli* Rosetta. Cells were cultivated in LB medium with 50 μg/ml ampicillin and 34 μg/ml chloramphenicol at 37°C until they reached an OD600 of 0.4. Expression was induced with 1 mM IPTG for 4 h at 37°C. Bacteria were harvested, resuspended in purification buffer (25 mM sodium phosphate [pH 7.4], 250 mM NaCl, 10 mM imidazole, 1 mM phenylmethylsulfonyl fluoride [PMSF], and disrupted by freezing and thawing for a total of 3 rounds. Recombinant proteins were purified by IMAC using Ni-nitrilotriacetic acid (NTA) Sepharose (Biozol, Eching, Germany). His-tagged proteins were eluted with purification buffer containing 250 mM imidazole. For further purification of ScCts6-His, size exclusion chromatography using an Äkta with a Sephadex 200 GL column (both GE Healthcare, Little Chalfont, United Kingdom) was performed. Protein purity was determined by silver staining, and quantity was measured with a bicinechonic acid (BCA) assay. At least 1 mg purified protein was used to produce specific rabbit antibodies against ScCst6-His and ScNce103-His (Thermo Fisher Scientific).

Western blot analysis. For detection of ScNce103 levels, 10 ml of *S. cerevisiae* exponential cultures was cultivated in 5% CO2, or transferred to air for 90 min. Cells were harvested, resuspended in 500 μl yeast lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.1% Triton X-100, 1 mM dithiothreitol [DTT], 10% glycerol, PhosStop, Complete EDTA-free protease inhibitor, PMSF), and lysed with glass beads (diameter, 425 to 600 μm) five times for 1 min each with incubation on ice between lyses. Lysates were centrifuged (16,000 × g, 10 min, 4°C), and total protein was measured using a BCA protein assay kit (Thermo Fisher Scientific). After addition of 5× SDS loading dye and heating at 95°C for 5 min, samples (1 mg total protein) were subjected to 4 to 20% SDS-PAGE. Transfer to polyvinylidene difluoride (PVDF) membrane was performed using the Bio-Rad Wet/Tank blotting system for 1 h. Three percent nonfat dry milk in Tris-buffered saline (TBS) was used for membrane blocking and antibody binding. Membranes were incubated with custom-made rabbit anti-ScNce103 (1:1,000 [Thermo Fisher Scientific]) and mouse anti-β-actin (AB8224; 1:1,000 [Abcam, Inc., Cambridge, United Kingdom]) overnight at 4°C with gentle rotation. After incubation with fluorophore-labeled secondary antibodies (Jackson ImmunoResearch, West Grove, PA) for 2 h at 4°C, signals were detected with the FluorChem Q Imager (Alpha Innotech, Kasendorf, Germany). Quantitative analysis was done using the AlphaView software (ProteinSimple).

**NCE103 promoter-driven GFP expression.** The *S. cerevisiae* WT, cts6Δ, and sch9Δ strains were transformed with pRS316 plasmid, containing GFP under control of the ScNCE103 promoter using the lithium-acetate (LiAc) method according to Gietz et al. (49). In brief, yeast cells of the early lag phase were harvested, washed, and resuspended in Tris-EDTA (TE) buffer with 100 mM LiAc. Fifty-microliter cell aliquots were incubated with heat-denatured salmon sperm DNA and 240 μl 50% polyethylene glycol (PEG) 3640, and then DNA was added. Samples were incubated at 30°C for 30 min. After addition of 45 μl DMSO, heat shock at 42°C for 15 min was performed. Centrifuged and resuspended cells were incubated for 2 h at 30°C and washed again before plating. PICKed colonies were cultivated at 30°C overnight in 5% CO2 in minimal medium supplemented with histidine, methionine, and leucine. After inoculation of duplicates, one culture was transferred to air, while the second one was further incubated under 5% CO2.
Fluorescence was detected with LSM 780 (Zeiss, Jena, Germany) at the time point of the switch from CO₂ to air and after 60 and 120 min of incubation. GFP fluorescence of 50 cells per time point was measured using ZEN software (Zeiss), and mean fluorescence was calculated.

**Protein expression in *S. cerevisiae*.** Overexpression of ScCST6-His and ScSCH9-His in *S. cerevisiae* was achieved using the ScGAL1 promoter. Yeast cells were cultivated in minimal medium, supplemented with histidine, methionine, and leucine at 30°C overnight. Overexpression of ScCST6 and ScSCH9 was induced by a shift from minimal medium to YEP medium (1% yeast extract, 2% peptone) containing 2% raffinose and after overnight culture to YEP with 2% galactose for an additional 6 h. Cells were harvested, resuspended in yeast lysis buffer, and lysed with glass beads as described. Cell extracts were tested by Western blotting for overexpression.

**Immunoprecipitation.** Exponential *S. cerevisiae* WT or ScSCH9-His-overexpressing cultures (50 ml) were harvested, resuspended in 500 μl immunoprecipitation (IP) buffer (20 mM sodium phosphate [pH 7], PhosStop, Complete protease inhibitor EDTA-free, PMSF), and lysed with glass beads as described. Extracts were incubated with either custom-made rabbit anti-Sc6, saturated with 5 μg 6×His tag peptide (Gentaur, Aachen, Germany), or anti-6×His antibody (GTX115045 [GeneTex, Hsinchu City, Taiwan]) overnight at 4°C. Fifty microliters of protein-G Sepharose beads (GE Healthcare) was washed with IP wash buffer (IP buffer plus 500 mM NaCl) and incubated with immunocomplexes for 1 h at 4°C. Beads were centrifuged at 2,500 rpm for 2 min, supernatant was taken off with a syringe and a 25-gauge needle, and beads were resuspended in wash buffer and incubated 1 min with shaking. This washing procedure was repeated four times. For coimmunoprecipitation (co-IP), beads were further incubated with 20 μg recombinant ScSch9-His lysate for 3 h at 4°C. After extensive washing, beads were boiled in SDS sample buffer, subjected to 4 to 20% SDS-PAGE, and analyzed by Western blotting. Rabbit anti-Sc6 (1:1,000) was used for detection of endogenous ScCst6 and fluoroecein isothiocyanate (FITC)-labeled mouse anti-6×His (MA1-81891; 1:250 [Thermo Fisher Scientific]) for detection of 6×His-tagged ScSch9.

**Radioactive kinase assay.** ScSch9-His was immunoprecipitated from *S. cerevisiae* ScSCH9-His-overexpressing culture. After washing of bead-bound immunocomplexes with IP wash buffer, an additional washing step with 1× kinase buffer (phosphate-buffered saline [PBS; pH 7.4], 4 mM MgCl₂, 10 mM DTT, 20% glycerol, 1 mM PMSF) was performed. One microgram of bacterially expressed and purified ScCst6-His was added to immunocomplexes in 50 μl kinase buffer. After addition of 100 μM ATP and 25 μCi[y-32P]ATP (BLU002250UC; specific activity, 10 Ci/mmol [PerkinElmer, Waltham, MA]), reaction mixtures were incubated at 30°C for 30 min. The kinase reaction was stopped by heating at 95°C for 5 min in 4× SDS loading dye, and samples were subjected to 4 to 20% SDS-PAGE. Gels were dried, and radioactive incorporation was detected by autoradiography.

**LC-MS/MS analysis and protein database search.** To detect CO₂-dependent phosphorylation in ScCst6, ScCST6-overexpressing *S. cerevisiae* cultures were harvested, and ScCst6-His was purified using IMAC. For mass spectrometry analysis, in-gel digestion with trypsin-LysC, AspN, and GluC (all Promega, Madison, WI) or LysC/Nase (50) and in-solution digestion with trypsin-LysC and GluC was performed. Peptides were extracted with trifluoroacetic acid and increasing concentrations of acetonitrile (50 to 90%) after in-gel digestion. Phosphopeptide enrichment was carried out using the TiO₂ method (TiO₂ Spin Tip sample prep kit, Protea Biosciences, Morgantown, WV), and the remaining peptides were purified with C₁₈ minicolumns (C₁₈ Spin Tip sample prep kit [Protea Biosciences, Morgantown, WV]). Dried peptides were solubilized in MS buffer (0.05% trifluoroacetic acid in 2% acetonitrile–98% H₂O) and applied to LC-MS/MS analysis, carried out on an Ultimate 3000 nano-rapid-separation liquid chromatography (nano-RSLC) system coupled to a QExactive Plus mass spectrometer (both from Thermo Fisher Scientific). Thermo raw files were processed via the Proteome Discoverer (PD) v1.4.0.288. Tandem mass spectra were searched against the NCBI protein database of *S. cerevisiae* using the algorithms of Mascot v2.4.1 (Matrix Science, Inc., London, United Kingdom), Sequest HT, and MS Amanda. For further information, see Text S1 in the supplemental material.

**Site-specific mutagenesis of ScCST6 and ScSCH9.** Phosphoablative (S266A) and phosphomimetic (S266D) sequences of ScCST6 (GeneArt Gene Synthesis) were inserted in the pRS316 plasmid using PacI/Ascl restriction sites. The ScCST6 promoter was cloned in pRS316 using SacI/Pacl. After transformation of the *S. cerevisiae* ctsΔ mutant, ScNCE103 expression in 5% and 0.04% CO₂, for construction of ScSCH9 with S/T-to-A mutation of all 6 TORC1 sites (*SCH9*Δ), the sequence of the *SCH9*Δ mutant was used as the template. A570 was remutated to T570 using QuikChange II site-directed mutagenesis kit (Agilent Technologies) with mutation-specific primers. The plasmid with the corrected sequence was checked via sequencing and used for transformation of the ctsΔ mutant.

**Construction of the *C. glabrata* sch9Δ mutant.** Deletion of *GscSCH9* was achieved by replacement of the *GscSCH9* open reading frame (ORF) with a *NAT1* cassette, which mediates resistance against nourseothricin. *NAT1* was amplified by PCR with EcoRI and SacI restriction sites and cloned into pBluescript. A homologous region upstream of the *GscSCH9* ORF with a length of 1,000 bp was amplified by colony PCR and cloned into pBluescript using Kpnl/Xhol. The same was done for a 1,000-bp fragment located downstream of *GscSCH9* ORF using restriction sites SacI and SacI. The whole construct, the *NAT1* cassette flanked by homologous regions, was restricted with Kpnl/SacI and purified in order to transform *C. glabrata*. AFG1, a strain lacking *SCH9*, was used for transformation because of its increased efficiency of homologous recombination (51). Transformation was performed using the lithium-acetate method, with cells plated on YPD with 100 μg/ml ClonNAT, and positive clones were selected. Integration of the *NAT1* cassette and deletion of *GscSCH9* were checked by colony PCR with specific internal primers.
Furthermore, deletion mutants were verified using external primers (see Fig. S3 in the supplemental material).

**NCE103 expression measurement of C. glabrata and C. albicans.** Measurement of CgNCE103 and CaNCE103 expression was performed according to the protocol for ScNCE103 expression described in the first section. As housekeeping genes, CgACT1 and CaACT1 were used.

**Statistical analysis.** Data are presented as arithmetic means ± standard deviation, and statistical significance (P < 0.05) was calculated using a two-sided t test for unpaired samples.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at https://doi.org/10.1128/mBio.02211-16.

**FIG S1, PDF file, 0.1 MB.**

**FIG S2, PDF file, 0.04 MB.**

**FIG S3, PDF file, 0.2 MB.**

**TABLE S1, PDF file, 0.1 MB.**

**TABLE S2, PDF file, 0.2 MB.**

**TABLE S3, PDF file, 0.1 MB.**

**TABLE S4, PDF file, 0.1 MB.**

**TEXT S1, PDF file, 0.1 MB.**

**TEXT S2, PDF file, 0.1 MB.**

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