The Zinc Cluster Protein Sut1 Contributes to Filamentation in Saccharomyces cerevisiae

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Sut1 is a transcriptional regulator of the Zn(II)$_{2}$Cys$_{6}$ family in the budding yeast Saccharomyces cerevisiae. The only function that has been attributed to Sut1 is sterol uptake under anaerobic conditions. Here, we show that Sut1 is also expressed in the presence of oxygen, and we identify a novel function for Sut1. SUT1 overexpression blocks filamentous growth, a response to nutrient limitation, in both haploid and diploid cells. This inhibition by Sut1 is independent of its function in sterol uptake. Sut1 downregulates the expression of GAT2, HAP4, MGA1, MSN4, NCE102, PRR2, RH03, and RH05. Several of these Sut1 targets (GAT2, HAP4, MGA1, RH03, and RH05) are essential for filamentation in haploids and/or diploids. Furthermore, the expression of the Sut1 target genes, with the exception of MGA1, is induced during filamentous growth. We also show that SUT1 expression is autoregulated and inhibited by Ste2, a key transcriptional regulator of filamentation. We propose that Sut1 partially represses the expression of GAT2, HAP4, MGA1, MSN4, NCE102, PRR2, RH03, and RH05 when nutrients are plentiful. Filamentation-inducing conditions relieve this repression by Sut1, and the increased expression of Sut1 targets triggers filamentous growth.

Sut1 of the budding yeast Saccharomyces cerevisiae is a member of the Zn(II)$_{2}$Cys$_{6}$ family of transcriptional regulators, also known as zinc cluster proteins (1, 2). The only known function of Sut1 is sterol uptake (3). Sterol biosynthesis in budding yeast can occur only when oxygen is available. Under these conditions, cells are unable to take up sterols from the extracellular medium. Conversely, in anaerobiosis, cells do not synthesize sterols and become capable of importing sterols (4). It has been reported that Sut1 is not expressed under aerobic conditions but induced in the absence of oxygen (3). Sut1 and other transcriptional regulators would then trigger the expression of genes such as DAN1 and AUS1, whose products mediate sterol import (5, 6). However, the underlying mechanisms of Sut1 action are not very well understood. It was suggested previously that Sut1 does not directly bind to the promoters of hypoxic genes such as DAN1 (6). Instead, Sut1 could induce the expression of these genes by relief from repression by the general corepressor Cyc8-Tup1. Sut1 activity is regulated by the Cdc42 effectors Ste20, Cla4, and Skm1 (7). These proteins can form a complex with Sut1, and they can translocate into the nucleus, where they control the expression of genes whose products are involved in sterol uptake.

Whereas little is known about Skm1, a wide range of signaling functions has been described for Ste20 and Cla4 (8). One of the best-characterized functions of Ste20 is the activation of distinct mitogen-activated protein kinase (MAPK) cascades that control filamentous growth, mating, and osmotic stress responses (9–14).

In some fungal pathogens such as Candida albicans, the transition between yeast and filamentous growth is critical for their virulence (15). Even though budding yeast does not form true hyphae, it has proved to be an excellent model system for filamentation in C. albicans, mainly because genetic manipulations that are easily carried out in budding yeast can be tedious in C. albicans. In budding yeast, filamentation is a response observed in both haploid and diploid yeast cells growing on a semisolid medium with limited nutrients (16). Filamentous growth in haploids is often called invasive growth and can be induced by the lack of a fermentable carbon source, such as glucose (17). In diploids, filamentous growth is also termed pseudohyphal growth and is triggered by low concentrations of nitrogen (18). During filamentation, cells undergo morphological changes from a yeast form to filamentous form. Cells become more elongated and switch from axial (haploids) or bipolar (diploids) budding to a unipolar budding pattern. In addition, cells do not separate following division and attach to and penetrate the semisolid medium on which they are growing. Together, these mechanisms allow the cells to forage for nutrients. Several signaling pathways regulate filamentous growth, including an Ste20-dependent MAPK cascade and a cyclic AMP (cAMP)-dependent protein kinase A (PKA) pathway. These pathways control the transcription factors Ste12, Tec1, Sok2, Pdh1, Flo8, and Mga1, which form a complex transcriptional network that governs filamentation (19).

Here, we demonstrate that the transcriptional regulator Sut1 is also involved in filamentation. High levels of SUT1 inhibit filamentous growth and decrease the expression levels of genes which are essential for filamentation and which are upregulated during filamentation. These data suggest that Sut1 partially represses the expression of these genes during vegetative growth when nutrients are plentiful. In filamentous growth, this inhibition is lost, resulting in increased expression of Sut1 target genes whose products contribute to filamentation.

MATERIALS AND METHODS

Yeast strains, plasmids, and growth conditions. All yeast strains used in this study are listed in Table 1. The strains are in the Σ1278b background. Yeast strains were constructed using PCR-amplified cassettes (22, 23). Yeast strains were grown in 1% yeast extract–2% peptone–2% dextrose (YPD) medium or in synthetic complete (SC) medium. Synthetic low-nitrogen medium was made by removing urea and amino acids (24). Yeast strains were transformed by the lithium acetate procedure (25). Yeast cultures were grown at 30°C unless otherwise indicated.

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ammonium dextrose (SLAD) medium for induction of pseudohyphal growth contains 0.67% yeast nitrogen base without amino acids and without ammonium, 2% glucose, and 50 μM (NH₄)₂SO₄. Synthetic high-ammonium dextrose (SHAD) medium, which was used as the reference medium, is the same as SLAD medium but with 50 mM (NH₄)₂SO₄.

All constructs used in this work are listed in Table 2.

**Filamentation assays.** For agar invasion assays, 10⁵ cells of a culture grown overnight were spotted onto YPD medium, selective medium, or galactose/raffinose medium and grown for 3 days (YPD medium) or 5 days (selective and galactose/raffinose medium) at 30°C. Plates were photographed before and after being rinsed under a stream of deionized water. For pseudohyphal growth assays, cells were grown overnight, and 100 cells were spread onto solid SLAD medium. Plates were incubated for 5 days at 30°C. Colonies were then examined with a Zeiss Axioskop 2 microscope equipped with a 5× objective, and images were captured using a ProGRes C12 camera (Jenoptik).

**β-Galactosidase assay.** Densities of cell cultures were measured by the optical density at 600 nm (A₆₀₀). A total of 0.1 to 10 ml of cells was harvested by centrifugation and resuspended in 1 ml Z buffer (100 mM sodium phosphate [pH 7.0], 10 mM KCl, 1 mM MgSO₄, 50 mM β-mercaptoethanol). Cells were permeabilized by the addition of 20 μl chloroform and 20 μl 0.1% SDS. After 15 min of incubation at 30°C, the reaction was started by the addition of 140 μl o-nitrophenyl-β-D-galactopyranoside (4 mg/ml in 100 mM sodium phosphate [pH 7.0]), the mixture was incubated at 30°C until the solution became yellow, and the reaction was stopped by the addition of 400 μl 100 mM Na₃CO₃. Samples were centrifuged, and the absorbance of the supernatant at 420 nm and 550 nm was determined. β-Galactosidase activity was calculated in Miller units as 1.000 × [A₄₂₀ − (1.75 × A₅₅₀)]/reaction time (min) × culture volume (ml) × A₆₀₀⁻¹.

Quantitative real-time PCR. Cells were grown to exponential phase in either SC (haploid cells) or SHAD (diploid cells) medium. Half of these cells were retained for RNA isolation. The remaining cells were washed with water, and 10⁵ cells were plated onto SC medium lacking glucose (haploids) or SLAD medium (diploids). Plates were incubated for 14 h at 30°C. Cells were then scraped from the plates, and RNA was immediately isolated by using a FastRNA Spin kit for yeast and a FastPrep-24 instrument (MP Biomedicals). Following DNA removal with a Turbo DNA-free kit (Applied Biosystems), 1 μg RNA was reverse transcribed to cDNA with the SuperScript III first-strand synthesis system using random hexamer primers (Invitrogen). PCR quantification was performed in triplicate from two biological samples using a 7900HT Fast real-time PCR system (Applied Biosystems). SYBR green JumpStart Taq ReadyMix (Sigma), and the primers listed in Table 3. The PCR thermal cycle was 94°C for 2 min, followed by 40 cycles of 94°C for 15 s and 57°C for 1 min. Data were analyzed by the ΔΔCₚ method using RQ Manager, version 1.2.1, software (Applied Biosystems). Expression levels were normalized to ACT1 transcript levels.

**Immunoblotting.** Exponential-phase cells were disrupted with glass beads in lysis buffer (20 mM Tris [pH 7.5], 100 mM NaCl, 10 mM EDTA, 1 mM EGTA, 5% glycerol, 1% Triton X-100, and protease inhibitor cocktail [Roche Diagnostics]) and clarified by centrifugation at 13,000 rpm for 5 min. Protein concentration was determined by using Bradford protein assay solution (Roth), and equal amounts were separated by SDS-PAGE and transferred onto nitrocellulose. Mouse monoclonal anti-Myc (9E10) and rabbit polyclonal anti-Cdc11 antibodies were obtained from Santa Cruz Biotechnology, and mouse monoclonal anti-hemagglutinin (HA) (clone 12CA5) antibody was obtained from Roche Diagnostics. Secondary antibodies were obtained from Jackson Research Laboratories.

**Chromatin immunoprecipitation.** Cultures (500 ml) were grown until they reached an optical density at 600 nm of 0.8 to 1.0. Cells were fixed

### Table 1 Yeast strains used in this study

| Strain | Genotype | Reference |
|--------|----------|----------|
| HUY30  | PPy966 hem1Δ::ATRP1 | This study |
| HUY32  | PPy966 hem1Δ::ATRP1 SUT1-9myc-His3MX6 | This study |
| HUY33  | PPy966 sur1Δ::His3MX6 | This study |
| IS9     | PPy966 rha1Δ::His3MX6 | This study |
| PC344   | MATa/MATa ura3-2//ura3-2 | 20 |
| PPy966  | MATa his3Δ::hisG leu2Δ::hisG rpl1Δ::hisG ura3-52 | 21 |
| SHY1    | PPy966 rha3Δ::His3MX6 | This study |
| SHY2    | PPy966 rha3Δ::KanMX6 | This study |
| SHY3    | PPy966 ngs1Δ::ATRP1 | This study |
| SHY4    | PPy966 prs1Δ::His3MX6 | This study |
| SHY13   | PC344 prs2Δ::hyphNT1/prs2Δ::KanMX6 | This study |
| SHY15   | PC344 ngalΔ::hyphNT1/ngalΔ::KanMX6 | This study |
| SHY16   | PC344 rho3Δ::hyphNT1/rho5Δ::KanMX6 | This study |
| SHY18   | PC344 rho3Δ::hyphNT1/rho3Δ::KanMX6 | This study |
| SHY24   | PC344 rho4Δ::hyphNT1/rho4Δ::KanMX6 | This study |
| SHY34   | PC344 SUT1/KanMX6-GAL1-3HA-SUT1 | This study |
| SHY39   | PPy966 gat2Δ::His3MX6 | This study |
| SHY40   | PPy966 nce102Δ::His3MX6 | This study |
| SHY42   | PPy966 msn14Δ::His3MX6 | This study |
| SHY47   | PPy966 hap4Δ::ATRP1 | This study |
| SHY62   | PC344 gat2Δ::hyphNT1/gat2Δ::KanMX6 | This study |
| SHY68   | PPy966 aos1Δ::His3MX6 prs1Δ::ATRP1 | This study |
| SHY90   | PC344 msn1Δ::hyphNT1/msn1Δ::KanMX6 | This study |
| SHY91   | PC344 hap1Δ::hyphNT1/hap1Δ::KanMX6 | This study |
| SHY92   | PC344 nce102Δ::hyphNT1/ncel012Δ::KanMX6 | This study |
| THY664  | PPy966 SUT1-9myc-His3MX6 | This study |
| THY706  | PC344 ste2Δ::hyphNT1/ste20Δ::KanMX6 | 20 |
| THY697  | PPy966 ste20Δ::hyphNT1 | 20 |
| THY762  | PPy966 KanMX6-GAL1-3HA-STE12 | This study |
| THY765  | PPy966 KanMX6-GAL1-3HA-PHD1 | This study |
| THY767  | PPy966 KanMX6-GAL1-3HA-TEC1 | This study |
| THY768  | PPy966 His3MX6-GAL1-3HA-FLO8 | This study |
| THY769  | PPy966 KanMX6-GAL1-3HA-MGA1 | This study |
| THY777  | PPy966 SUT1-9myc-His3MX6 ste12Δ::KanMX6 | This study |
| THY778  | PPy966 bas1Δ::ATRP1 | This study |

### Table 2 Plasmids used in this study

| Plasmid | Genotype | Reference |
|---------|----------|----------|
| B3782   | YEp355 carrying pFLOI1 | 24 |
| pHU35   | YEp367 carrying pHOS5 | This study |
| pHU36   | YEp367 carrying pMGAl | This study |
| pHU37   | YEp367 carrying pPR22 | This study |
| PM3C    | YEp355 carrying pHOS5 | This study |
| PM3C    | YEp367 carrying pGAT2 | This study |
| PMC7    | YEp367 carrying pHOS4 | This study |
| pNEV-N  | 2μm URA3 pDNA | 25 |
| pNF1    | pNEV-N carrying SUT1 | 1 |
| pSH13   | YEp367 carrying pFLOI1 | This study |
| pSH23   | YEp367 carrying pHPA4 | This study |
| pSH25   | YEp367 carrying pNE102 | This study |
| pTH387  | YEp367 carrying pHOS3 | This study |
| pTH388  | YEp367 carrying pSUT1 | This study |
| pTH391  | YEp367 carrying pMSN4 | This study |
| pTH395  | YEp355 carrying pHOS5(pSut1*) | This study |
| YEp355  | 2μm URA3 lacZ | 26 |
| YEp367  | 2μm LEU2 lacZ | 26 |

* The Sut1-binding sequence 5′-CCGCCGCCGCCGCCGC-3′ in the RHOS promoter located between positions −733 and −724 was mutated to 5′-GAGCTCTAGTCG-3′.

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February 2013 Volume 12 Number 2 ec.asm.org 245
TABLE 3 Primers used for quantitative real-time PCR

| Primer | Sequence (5′–3′) |
|--------|------------------|
| ACT1-F | GCTCTTGGACCTGCAAAAGA |
| ACT1-R | CCAACGCCAAGACAGAGGA |
| GAT2-F | TCTCCGATGGTCAAGGAGCT |
| GAT2-R | AAATGGCCTAGGAGTCAAGGTG |
| HAP4-F | GCTGACCGATGGTCAAGGACCAG |
| HAP4-R | TTTTCTGATGGTCAAGGGGT |
| MGA1-F | ATGGCAATGCTGTGGTGCTT |
| MGA1-R | TTGATCAGTGGTCAAGGGGT |
| MSN4-F | TACACACACACCCAAAGCAGG |
| MSN4-R | AGGCAACCAAAGACATGGT |
| NCE102-F | AGCTCAAGGGCATGTGCTT |
| NCE102-R | ACACCCATTTGGGAGTCTTC |
| PRR2-R | TGGGCGGTCAGTGGTCTTTCA |
| PRR2-R | ATCCCGAGGACCAAAAGGTC |
| RH03-F | TGCCCTAAAAGATGCGGGGT |
| RH03-R | GCCCGGTTTAAACGACTTTT |
| RH05-F | AAGTGTACGTGCTACCAAGGC |
| RH05-R | TTGGGTCTGGTTGCTGAGTC |

with 1% formaldehyde for 30 min at room temperature. The reaction was quenched with 125 mM glycine, and the cells were washed three times in cold phosphate-buffered saline (PBS). Cells were harvested and resuspended in chromatin immunoprecipitation (ChIP) lysis buffer (0.1% deoxycholate, 1 mM EDTA, 50 mM HEPES-KOH [pH 7.5], 140 mM NaCl, 1% Triton X-100, protease inhibitor cocktail). Cells were disrupted with glass beads. Lysates were collected by centrifugation and sonicated for 1 min by performing alternating cycles of 5-s pulses followed by a 15-s cool-down period using an Ultrasonic Processor XL (Hett System). After centrifugation, supernatants were immunoprecipitated by adding either anti-Myc or anti-HA antibody for 1 h at 4°C, followed by the addition of protein G Sepharose beads (GE Healthcare) for 1 h at 4°C. The beads were washed sequentially with ChIP lysis buffer, high-salt lysis buffer (0.1% deoxycholate, 1 mM EDTA, 50 mM HEPES-KOH [pH 7.5], 500 mM NaCl, 1% Triton X-100), LiCl buffer (0.5% deoxycholate, 1 mM EDTA, 250 mM LiCl, 0.5% NP-40, 10 mM Tris-HCl [pH 8.0]), and TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA). Immunoprecipitated protein-DNA complexes were eluted from the beads by incubation in elution buffer (50 mM Tris-HCl [pH 8.0], 10 mM EDTA, 1% SDS) for 20 min at 65°C. Following the reversal of cross-links overnight at 65°C, proteinase K (final concentration, 0.1 mg/ml) was added and incubated at room temperature for 1 h. DNA was then purified using a PCR purification kit (Macherey-Nagel). The SUT1 promoter region containing Sut1-binding sites was amplified by using primers 5′-GAGTTTCACCCAGAGAGA-3′ and 5′-AGAGAAACGATCATG-3′, and the region containing the Ste12-binding site was amplified by using primers 5′-TTCCCTGCAGTATGAGA-3′ and 5′-ACAGCAGTGTCGTCCACA-3′.

RESULTS

Sut1 negatively regulates filamentation. The Cdc42 effectors Ste20, Cla4, and Skm1 form a complex with Sut1, and they regulate the expression of Sut1 targets such as AUS1 and DAN1, whose gene products mediate sterol uptake under anaerobic conditions (7). Ste20 and Cla4 have important functions in signaling. These processes are usually studied under conditions of aerobiosis. We speculated that Sut1 and the Cdc42 effectors Ste20, Cla4, and Skm1 might have overlapping functions and that this would be in the presence and absence of oxygen. We therefore asked whether SUT1 is expressed under aerobic conditions. It was suggested previously that SUT1 is expressed only under anaerobic conditions (3). However, in our hands, a myc-tagged version of Sut1 under the control of its own promoter was readily detectable by immunoblotting in the presence of oxygen (Fig. 1). Since completely anaerobic conditions are difficult to maintain, most studies employ mutants in heme synthesis. Heme acts as an intermediary in regulating the expression of oxygen-responsive genes. Therefore, deficiency in heme biosynthesis, e.g., in a hem1Δ background, mimics anaerobic conditions in the presence of oxygen (27). Sut1-9myc protein levels in hem1Δ cells, mimicking anaerobic conditions, were only slightly higher than those in the wild-type strain (Fig. 1). We also determined SUT1 mRNA expression levels in wild-type and hem1Δ cells by quantitative real-time PCR. The SUT1 expression level in the heme-deficient strain was increased by 2.2 ± 0.13 when normalized to the level of the actin gene ACT1.

Since Sut1 is expressed in the presence and absence of oxygen, it might have functions under both conditions. Because of its interaction with Ste20, we examined the role of Sut1 in processes that are regulated by Ste20, such as mating and filamentation. A SUT1 deletion strain grew normally, had normal morphology, and exhibited no defects in mating and haploid invasive growth (data not shown). Likewise, diploid cells lacking both copies of SUT1 displayed normal pseudohyphal growth (data not shown).

We also examined whether increased SUT1 levels lead to a phenotype. Haploid cells overexpressing SUT1 under the control of the strong PMA1 promoter from a multicopy plasmid (1, 25) also had normal morphology, progressed normally through the cell cycle, and had normal cell growth (data not shown). In contrast, SUT1 overexpression strongly inhibited haploid invasive growth (Fig. 2A). Consistent with this phenotype, increased SUT1 levels reduced the expression of the filamentation marker FLO11 (Fig. 2B).

The only function attributed to Sut1 is sterol uptake (3). Since the medium was not supplemented with sterol, it seems very likely that the inhibition of invasive growth is independent of sterol import. To completely exclude a possible involvement of sterol import in this process, SUT1 was overexpressed in a strain lacking AUS1 and PDR11. These cells are unable to import sterol (28). As in the wild type, overexpression of SUT1 in aus1Δ pdr11Δ cells resulted in a loss of agar invasion (Fig. 2A).

Diploid cells overexpressing SUT1 from a plasmid also exhibited a normal growth rate (not shown), but pseudohyphal growth...
was completely absent (Fig. 2C). The expression level of the filamentation marker \textit{FLO11} was also strongly decreased in diploids with increased \textit{SUT1} levels (Fig. 2D). For this experiment, \textit{SUT1} was overexpressed from the \textit{GAL1} promoter integrated into the genome because the cells used have only one auxotrophic marker and could carry only the \textit{FLO11}-lacZ construct and not the \textit{SUT1} overexpression plasmid. Notably, pseudohyphal growth was also completely absent in the strain overexpressing \textit{SUT1} from the \textit{GAL1} promoter (data not shown).

These data strongly suggest that Sut1 has a function not only under anaerobic conditions but also in the presence of oxygen. It seems to negatively regulate filamentation in both haploid and diploid cells independently of sterol import. However, since we observed a filamentation phenotype only for cells overexpressing \textit{SUT1} and not for the \textit{SUT1} deletion strain, we wanted to know whether transcription factor genes that have synthetic genetic interactions with \textit{SUT1} play a role in filamentous growth. Synthetic genetic interactions between \textit{SUT1} and \textit{BAS1}, \textit{FKH1}, \textit{RIM101}, and \textit{YAP6} have been reported (29–31). Simultaneous deletion of \textit{FKH1} and its paralog \textit{FKH2} results in increased filamentation in haploids and diploids (32). A \textit{RIM101} deletion strain has a defect in haploid invasive growth (33), and \textit{YAP6} overexpression leads to increased filamentous growth (34). We also tested the effect of \textit{BAS1} deletion on filamentous growth because no such link has been described previously. Cells lacking \textit{BAS1} exhibit increased haploid invasive growth (Fig. 2E). These phenotypes further support the notion that Sut1 plays a role in filamentation under physiological conditions.

\textbf{Sut1 controls the expression of genes involved in filamentation.} How could Sut1 contribute to filamentous growth? A genome-wide screen to determine the genomic occupancy for transcriptional regulators revealed 24 binding sites for Sut1 in 16 different intergenic regions (35). Among these potential Sut1 targets are \textit{MGA1}, \textit{PRR2}, \textit{RHO3}, and \textit{RHO5}. Mga1 plays a role in pseudohyphal growth, and Rho3, Rho5, and Prr2 are involved in cell polarization (36–39). It is tempting to speculate that Sut1 could regulate filamentous growth by controlling the expression of these genes. We therefore analyzed these putative Sut1 targets. Four of the 16 genes (\textit{GAT2}, \textit{HAP4}, \textit{MSN4}, and \textit{NCE102}) were shown to be regulated by \textit{SUT1} in a previously reported DNA
microarray experiment (5). We decided to include these genes in our analysis as well.

First, we tested whether Sut1 controls the expression of its putative targets. To this end, the corresponding promoters were fused to the $\text{lacZ}$ gene. Deletion of $\text{SUT1}$ had no significant effect on levels of $\text{GAT2}$, $\text{HAP4}$, $\text{MGA1}$, $\text{MSN4}$, $\text{NCE102}$, $\text{PRR2}$, $\text{RHO3}$, and $\text{RHO5}$ (data not shown). In contrast, the expression level of all genes tested was decreased in cells overexpressing $\text{SUT1}$ (Fig. 3). The levels decreased between 3.1-fold ($\text{RHO3}$) and 10.1-fold ($\text{MGA1}$). Importantly, the observed downregulation is specific and not an artifact. Whereas $\text{SUT1}$ overexpression downregulated the expression of the Rho GTPases $\text{RHO3}$ and $\text{RHO5}$, it had no effect on the levels of $\text{RHO4}$, a related Rho GTPase (Fig. 3), demonstrating that the observed effect is specific for $\text{RHO3}$ and $\text{RHO5}$.

The effect of $\text{SUT1}$ overexpression was also examined in diploid cells, using $\text{RHO5}$ as an example. High $\text{SUT1}$ levels decreased $\text{RHO5}$ expression levels in diploids as well (Fig. 4A). Thus, the observed downregulation of expression by Sut1 does not seem to be restricted to haploid cells.

Sut1 target genes were also downregulated in the $\text{hem1}^{-}/\text{H9004}$ background.

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**FIG 3** SUT1 overexpression specifically reduces expression of its target genes. Cells harbored either a SUT1 overexpression construct (pNF1) or an empty plasmid (pNEV-N) in combination with the lacZ reporter fused to the indicated promoter regions. Shown is the mean with the standard deviation ($n \geq 6$).

**FIG 4** Characterization of downregulation of $\text{RHO5}$ expression by Sut1. (A) SUT1 overexpression reduces $\text{RHO5}$ levels in diploid cells. $\beta$-Galactosidase activity was determined for wild-type cells and cells overexpressing SUT1 from the GAl1 promoter, both carrying a $\text{RHO5-lacZ}$ plasmid (pMC3) ($n \geq 6$). (B) $\text{RHO5}$ expression is reduced in cells lacking HEM1. The wild-type strain and $\text{hem1}^{-}/\text{H9004}$ cells mimicking anaerobic conditions were grown in selective medium supplemented with ergosterol and Tween 80. Both strains carried a $\text{RHO5-lacZ}$ plasmid (pHU35). Shown is the mean $\beta$-galactosidase activity with the standard deviation ($n \geq 6$). (C) Mutation of the Sut1-binding site in the $\text{RHO5}$ promoter has no effect on the downregulation of $\text{RHO5}$ expression by SUT1 overexpression. Cells harbored plasmids on which lacZ was fused either to the wild-type promoter region of $\text{RHO5}$ (pHU35) or to the $\text{RHO5}$ promoter in which the Sut1-binding site 5\'-CCGGCCCCC-3\' located between positions −733 and −724 was mutated to 5\'-GAGCTCATGC-3' (pTH393). These cells also carried either a plasmid for SUT1 overexpression (pNF1) or an empty plasmid (pNEV-N). $\beta$-Galactosidase activity was determined for cells grown in selective medium ($n \geq 6$).
and indirectly via other transcriptional regulators that are under two mechanisms, directly by binding to the promoter of its target genes. Alternatively, Sut1 could affect gene expression by containing additional unidentified Sut1-binding sites in their sequences (data not shown). This could mean that all these genes were not affected by mutagenesis of the Sut1-binding site in the promoter (Fig. 4C). The Sut1-binding site in RHO5, MGA1, and PRR2 were also mutated. As for RHO5, the downregulation of expression of these genes was not affected by mutagenesis of the Sut1-binding sequences (data not shown). This could mean that all these genes contain additional unidentified Sut1-binding sites in their promoter regions. Alternatively, Sut1 could affect gene expression by two mechanisms, directly by binding to the promoter of its target and indirectly via other transcriptional regulators that are under the control of Sut1, such as Gat2, Hap4, Mga1, and Msn4.

Next, we tested whether the confirmed Sut1 target genes have a role in filamentous growth in haploid and/or diploid cells. Cells lacking either GAT2, RHO3, or RHO5 were defective in haploid invasive growth (Fig. 5A). The deletion of the other Sut1 target genes had no effect on invasive growth (Fig. 5A). Likewise, rho4Δ cells displayed normal haploid invasive growth (Fig. 5A), demonstrating that the defect observed for the rho3Δ and the rho5Δ mutants is highly specific.

In diploids, pseudohyphal growth was completely absent in cells lacking both copies of RHO3, and it was strongly reduced in the hap4, the rho5, and the mga1 mutants (Fig. 5B). Homozygous deletion of any other Sut1 target (GAT2, MSN4, NCE102, and PRR2) or RHO4 had no effect on pseudohyphal growth (Fig. 5B). In summary, the Sut1 target genes GAT2, HAP4, MGA1, RHO3, and RHO5 play an important role in filamentous growth.

Uregulation of Sut1 targets during filamentation. The fact that high levels of SUT1 inhibit filamentous growth and reduce the expression of genes that are essential for filamentation led us to the speculation that at least some of the Sut1 targets might be induced during filamentation. To test this model, we compared gene expression levels in cells grown on nutrient-poor plates and in cells in nutrient-rich liquid medium. In haploid cells, filamentation was induced by the lack of glucose, and in diploids, filamentation was induced during filamentation. To test this model, we compared gene expression levels in cells grown on nutrient-poor plates and in cells in nutrient-rich liquid medium. In haploid cells, filamentation was induced by the lack of glucose, and in diploids, filamentation was induced by low ammonium concentrations (17, 18). Since the cell number collected from plates was too low to determine expression levels of Sut1 targets by using β-galactosidase assays, we performed quantitative real-time PCRs. No significant changes of expression levels were observed for MGA1 in both cell types and for RHO3 in haploids (Fig. 6). In contrast, all other Sut1 targets were upregulated during filamentation. The induction ranged from 2.7-fold for RHO5 in haploid cells to 93-fold for GAT2 in haploid cells. For GAT2, HAP4, MSN4, and RHO5, the induction was similar in haploid and diploid cells (Fig. 6). For PRR2 and NCE102, the change of expression was more pronounced in haploids.

Regulation of SUT1 expression. The change in expression of Sut1 target genes during filamentation raises the question of how Sut1 itself is regulated. A binding site for Ste12, a key

FIG 5 Role of Sut1 target genes in filamentation. (A) Haploid invasive growth of Sut1 target gene deletion strains. Cells of the indicated strains were spotted onto YPD plates and were grown for 3 days at 30°C. Pictures were taken before (total growth) and after (invasive growth) rinsing with water. The ste20Δ strain was used as a control. (B) Diploid pseudohyphal phenotype of strains lacking Sut1 target genes. The indicated strains were grown on low-nitrogen SLAD medium for 5 days at 30°C. The ste20Δ/ste20Δ deletion strain served as a control.
transcription factor of filamentation (10, 14), has been predicted for the promoter of the SUT1 gene (40). We therefore tested whether Ste12 controls the expression of SUT1 using a SUT1-lacZ reporter construct. STE12 deletion had no effect on SUT1 expression (data not shown). In contrast, STE12 overexpression resulted in decreased SUT1 levels (Fig. 7A) and stronger invasive growth (Fig. 7B). This negative regulation of SUT1 expression is highly specific. Overexpression of other transcription factors that form a regulatory network during filamentation, such as FLO8, MGA1, PHD1, and TEC1 (19), also led to stronger invasive growth but did not affect SUT1 levels (Fig. 7A and B). We were unable to detect binding of endogenously expressed Ste12 to the SUT1 promoter by ChIP (data not shown). However, when overexpressed from the GAL1 promoter, Ste12 associated with the SUT1 promoter (Fig. 7C).

Several Sut1-binding sites have also been predicted within the SUT1 promoter, possibly allowing an autoregulation of SUT1 expression (40). Whereas SUT1 overexpression had no effect on the lacZ level, a decreased expression level was observed for cells lacking SUT1 (Fig. 7D). This suggests that Sut1 regulates its own expression in a positive manner. Sut1 binding with its own promoter was confirmed by ChIP (Fig. 7E). These data suggest that Sut1 might be downregulated during filamentation. Indeed, Sut1 protein levels are slightly decreased in haploid cells collected from plates lacking glucose compared to cells grown in liquid medium with high glucose levels (Fig. 7F). This reduction of Sut1 protein levels was also observed in the absence of STE12 (Fig. 7F). Other factors probably regulate expression in ste12Δ cells. This result is consistent with the observation that STE12 deletion does not affect SUT1-lacZ expression.

**FIG 7** Regulation of SUT1 expression. (A) STE12 specifically downregulates SUT1 expression. SUT1-lacZ expression was determined for the wild-type strain and cells overexpressing the indicated transcriptional regulators from the GAL1 promoter. Bars indicate the mean with the standard deviation (n ≥ 6). (B) Overexpression of STE12, FLO8, MGA1, PHD1, and TEC1, respectively, leads to increased haploid invasive growth. The wild-type strain and cells overexpressing the indicated transcription factor were spotted onto a yeast extract-peptone plate supplemented with galactose and raffinose and were grown for 5 days at 30°C. Pictures were taken before (total growth) and after (invasive growth) rinsing with water. (C) Ste12 binds to the SUT1 promoter. Cells overexpressing 3HA-tagged STE12 from the GAL1 promoter and wild-type cells without an HA tag were subjected to ChIP. The immunoprecipitates (IP) were tested for the presence of the SUT1 promoter region. As a positive control for the PCR, cell lysates were tested without any anti-HA precipitation. (D) SUT1 expression is autoregulated. The SUT1-lacZ expression level was determined for the indicated strains. Shown is the mean with the standard deviation (n ≥ 6). (E) Sut1 associates with its own promoter. The wild-type strain and cells expressing 9myc-tagged Sut1 were subjected to ChIP and tested for the presence of the SUT1 promoter region. Lysates without any anti-myc precipitation served as the control for the PCR. (F) Sut1 protein levels are reduced during filamentation. Cells expressing Sut1-9myc in the wild-type or ste12Δ background were grown either in liquid SC medium with 2% glucose, promoting yeast growth (YG), or for 14 h on SC plates that lack glucose, inducing filamentous growth (FG). Cells were lysed, and equal amounts of protein were analyzed by immunoblotting using antibodies against the myc epitope and Cdc11 (loading control).
targets under optimal growth conditions (Fig. 8A). Since these together, these data suggest that Sut1 represses the expression of its MGA1 Table 4). Furthermore, all Sut1 targets tested here, with the exception of NCE102 least a strong reduction of filamentous growth (summarized in work that controls the switch to filamentation (19). A global Phd1, Mga1, and Flo8, have been shown to form a complex net-
to filamentous growth (Fig. 8B).

Individual deletion of several of these genes leads to a loss or at least a strong reduction of filamentous growth (summarized in Table 4). Furthermore, all Sut1 targets tested here, with the exception of MGA1, are upregulated during filamentation. Taken together, these data suggest that Sut1 represses the expression of its targets under optimal growth conditions (Fig. 8A). Since these genes are expressed during normal growth, Sut1 seems to reduce transcription but not completely inhibit it. Stimuli that trigger filamentation would somehow relieve inhibition by Sut1, and the increased expression levels of Sut1 target genes would contribute to filamentous growth (Fig. 8B).

Other transcriptional regulators, including Ste12, Tec1, Sok2, Phd1, Mga1, and Flo8, have been shown to form a complex network that controls the switch to filamentation (19). A global screen for binding sites of these transcription factors revealed that only 20 promoters were bound by all six transcription regulators (19). Among them are the promoter regions of GAT2, HAP4, MGA1, RH03, and RH05 (Table 4). Here, we show that all these genes are essential for filamentation and are also regulated by Sut1. This overlapping specificity suggests that Sut1 is also a component of the transcriptional regulatory network for filamentation. Furthermore, since so many transcriptional regulators bind to the promoters of GAT2, HAP4, MGA1, RH03, and RH05, the corresponding genes seem to play a key role in filamentation. MSN4, NCE102, and PRR2 are also induced during filamentation, and not only Sut1 but also other transcriptional regulators involved in filamentation associate with their promoter regions. Sok2 binds to the promoters of PRR2 and MSN4, Phd1 binds to the MSN4 promoter, and Flo8 binds to the NCE102 promoter (Table 4) (19, 35).

Since MSN4, NCE102, and PRR2 are upregulated in filamentous growth and downregulated following SUT1 overexpression, and their promoters bind transcription factors that control filamentation, it seems very likely that they are also involved in filamentation, even though no mutant phenotype was observed for the corresponding deletion strains. The lack of a filamentation defect for the deletion strains could be explained by redundancy. For instance, it was reported previously that Msn4 has overlapping functions with the related transcription factor Msn2 (41). There is no obvious phenotype for cells lacking either MSN2 or MSN4, but the msn2Δ msn4Δ double mutant displays increased sensitivity to different stresses.

Mga1 has been shown to be involved in filamentation (37), and a global gene deletion analysis, which was published while the manuscript was in preparation, revealed a role for Rho3 and Rho5 in filamentous growth (42). The other Sut1 targets described here have not been associated with filamentation to our knowledge. The functions and the regulation of expression of at least some of these proteins are consistent with a role in filamentation. Mga1 is a transcription factor that is essential for pseudohyphal growth in diploid cells (37). We confirmed this result, but interestingly, we did not observe a filamentation defect in haploid cells. In diploids, Mga1 is considered to be a master regulator because MGA1 overexpression induced filamentation under noninducing conditions (19). Rho3 is a Rho GTPase that has at least three distinct functions in polarized growth (38, 43). It polarizes the actin cytoskeleton, mediates the transport of exocytic vesicles to the bud cortex, and is involved in the docking and fusion of these vesicles with the plasma membrane. It seems very likely that these processes are very important for filamentous growth because cells probably

### Table 4 Summary of Sut1 target gene characteristics

| Gene | Required for haploid filamentation | Required for diploid filamentation | Upregulated during haploid filamentation | Upregulated during diploid filamentation | Binding sites for filamentation transcription factors |
|------|-----------------------------------|-----------------------------------|----------------------------------------|----------------------------------------|--------------------------------------------------|
| GAT2 | +                                 | −                                 | +                                      | +                                      | Flo8, Mga1, Phd1, Sok2, Ste12, Sut1, Tec1        |
| HAP4 | −                                 | +                                 | +                                      | +                                      | Flo8, Mga1, Phd1, Sok2, Ste12, Sut1, Tec1        |
| MGA1 | −                                 | +                                 | −                                      | −                                      | Flo8, Mga1, Phd1, Sok2, Ste12, Sut1, Tec1        |
| MSN4 | −                                 | +                                 | −                                      | −                                      | Phd1, Sok2, Sut1                                 |
| NCE102 | −                              | −                                 | +                                      | +                                      | Flo8, Sut1                                          |
| PRR2 | −                                 | +                                 | −                                      | +                                      | Sok2, Sut1                                          |
| RH03 | +                                 | +                                 | −                                      | +                                      | Flo8, Mga1, Phd1, Sok2, Ste12, Sut1, Tec1        |
| RH05 | +                                 | +                                 | +                                      | +                                      | Flo8, Mga1, Phd1, Sok2, Ste12, Sut1, Tec1        |

* See references 19 and 35.

**DISCUSSION**

The only function that has been attributed to the zinc cluster protein Sut1 is the transcriptional control of genes whose products mediate sterol uptake under anaerobic conditions. Here, we show that Sut1 also contributes to filamentation. SUT1 overexpression inhibits filamentous growth in haploid and diploid cells, and it downregulates the expression of GAT2, HAP4, MGA1, MSN4, NCE102, PRR2, RH03, and RH05.

Individual deletion of several of these genes leads to a loss or at least a strong reduction of filamentous growth (summarized in Table 4). Furthermore, all Sut1 targets tested here, with the exception of MGA1, are upregulated during filamentation. Taken together, these data suggest that Sut1 represses the expression of its targets under optimal growth conditions (Fig. 8A). Since these genes are expressed during normal growth, Sut1 seems to reduce transcription but not completely inhibit it. Stimuli that trigger filamentation would somehow relieve inhibition by Sut1, and the increased expression levels of Sut1 target genes would contribute to filamentous growth (Fig. 8B).

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elongs by increased apical growth during filamentation. The Rho GTPase Rho5 and the transcription factor Msn4 are both involved in stress responses. Rho5 plays a role in stress responses such as the cell integrity pathway, oxidant-induced cell death, and the osmotic stress response (39, 44, 45). Msn4 controls the response to various stresses, such as glucose starvation, heat shock, and osmotic and oxidative stress (41, 46). Since nutrient limitation is also a stress signal, it is not surprising that these proteins are involved in filamentation. Gat2, a transcription factor of the GATA family, and Hap4, a transcriptional activator, are repressed by nitrogen and glucose, respectively (47–49). This regulation of their expression is consistent with a role in filamentation which is induced by low concentrations of glucose and nitrogen (17, 18). Very little is known about the serine/threonine protein kinase Prr2. Overexpression of PRR2 interferes with pheromone response signaling by an unknown mechanism (36). Since some proteins such as Ste20 are involved in filamentation and the mating pathway (10), this could also be the case for Prr2. Nce102 is required for the formation of eisosomes, large protein complexes that mediate the organization of the plasma membrane into specialized domains (50). While no link between eisosomes and filamentation has been demonstrated, membrane organization is important for polarized growth. It is therefore conceivable that eisosomes are involved in the regulation of polarized growth during filamentation.

Overall, the underlying molecular mechanisms of filamentation seem to be very similar in haploids and diploids (16). However, we found that GAT2 is required for haploid invasive growth but not for diploid pseudohyphal growth, whereas HAP4 and MGA1 are essential for filamentation in diploids but not in haploids. Furthermore, RHO3 expression changes upon filamentation in diploid cells but not in haploid cells. A recent genome-wide deletion analysis identified hundreds of genes that play a key role in either haploid or diploid filamentation, but only a relatively small number of genes are required for both processes (42). It therefore seems that filamentation in both cell types differs more than previously appreciated. This could be explained by the different stimuli used to trigger filamentation (lack of a fermentable carbon source in haploids and nitrogen depletion in diploids). Alternatively, the signaling and changes of gene expression might be slightly different in haploid and diploid cells.

Our model for the role of Sut1 suggests that during filamentation, the repression of Sut1 targets is lifted. Sut1 could be regulated posttranscriptionally or at the expression level. Previously, we have shown that Sut1 forms a complex with Ste20 (7), a protein that plays a key role in filamentation (10, 14). Ste20 controls the expression of Sut1 targets, such as AUS1 and DANI, that mediate sterol import under anaerobic conditions. This regulation requires an intact nuclear localization signal of Ste20, because Sut1 localizes exclusively to the nucleus (1, 7). In contrast, the nuclear localization signal of Ste20 is not required for its role in filamentation (7). This suggests that Ste20 does not play an important direct role in the regulation of the Sut1 protein during filamentation. Instead, Sut1 seems to be controlled at the transcriptional level. Binding sites for Sut1 and Ste12, a transcription factor that controls the switch to filamentous growth, have been predicted for the SUT1 promoter (40). Here, we show that SUT1 expression is regulated negatively by Ste12 and positively by Sut1. Sut1 indeed associates with its own promoter. Binding of Ste12 to the SUT1 promoter was detected only when STE12 was overexpressed but not when it was under the control of its endogenous promoter. Filamentous growth is markedly increased in cells overexpressing STE12. Ste12 seems to be hyperactive in these cells, probably occupying most of its binding sites, including the SUT1 sequence. We also show that Sut1 levels are reduced upon filamentous growth. Taking all our observations together, we propose the following model (Fig. 8). In filamentous growth, Ste12 becomes activated by a MAPK cascade (10, 51). This could result in decreased SUT1 expression levels. Decreased Sut1 levels could then also contribute to SUT1 downregulation. Reduced Sut1 concentrations in combination with other transcription factors that are regulated during filamentation (Ste12, Tec1, Sok2, Phd1, Mga1, and Flo8) would result in increased expression of the Sut1 targets described here. The combined activity of the Sut1 targets would then trigger filamentous growth. This model could also explain why no phenotype was observed for the sut1Δ mutant. All Sut1 target genes are regulated by at least another transcription factor. The loss of the repressor Sut1 in such a highly redundant system would not necessarily result in a clear phenotype.

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Regulation of Filamentation by Sut1

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