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The Zinc Cluster Protein Sut1 Contributes to Filamentation in *Saccharomyces cerevisiae*

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Sut1 is a transcriptional regulator of the $Zn(II)_2Cys_6$ 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*, *RHO3*, and *RHO5*. Several of these Sut1 targets (*GAT2*, *HAP4*, *MGA1*, *RHO3*, and *RHO5*) 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 Ste12, a key transcriptional regulator of filamentation. We propose that Sut1 partially represses the expression of *GAT2*, *HAP4*, *MGA1*, *MSN4*, *NCE102*, *PRR2*, *RHO3*, and *RHO5* 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 $Zn(II)_2Cys_6$ 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, Phd1, 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-

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TABLE 1 Yeast strains used in this study

Strain	Genotype	Reference
HUY30	PPY966 *hem1*Δ::*klTRP1*	This study
HUY32	PPY966 *hem1*Δ::*klTRP1 SUT1-9myc-His3MX6*	This study
HUY33	PPY966 *sut1*Δ::*His3MX6*	This study
ISY9	PPY966 *rho4*Δ::*His3MX6*	This study
PC344	*MAT***a**/*MAT*α *ura3-52/ura3-52*	20
PPY966	*MAT***a** *his3*::*hisG leu2*::*hisG trp1*::*hisG ura3-52*	21
SHY1	PPY966 *rho5*Δ::*His3MX6*	This study
SHY2	PPY966 *rho3*Δ::*KanMX6*	This study
SHY3	PPY966 *mga1*Δ::*klTRP1*	This study
SHY4	PPY966 *prr2*Δ::*His3MX6*	This study
SHY13	PC344 *prr2*Δ::*hphNT1*/*prr2*Δ::*KanMX6*	This study
SHY15	PC344 *mga1*Δ::*hphNT1*/*mga1*Δ::*KanMX6*	This study
SHY16	PC344 *rho5*Δ::*hphNT1*/*rho5*Δ::*KanMX6*	This study
SHY18	PC344 *rho3*Δ::*hphNT1*/*rho3*Δ::*KanMX6*	This study
SHY24	PC344 *rho4*Δ::*hphNT1*/*rho4*Δ::*KanMX6*	This study
SHY34	PC344 *SUT1*/*KanMX6-GAL1-3HA-SUT1*	This study
SHY39	PPY966 *gat2*Δ::*His3MX6*	This study
SHY40	PPY966 *nce102*Δ::*His3MX6*	This study
SHY42	PPY966 *msn4*Δ::*His3MX6*	This study
SHY47	PPY966 *hap4*Δ::*klTRP1*	This study
SHY62	PC344 *gat2*Δ::*hphNT1*/*gat2*Δ::*KanMX6*	This study
SHY68	PPY966 *aus1*Δ::*His3MX6 pdr11*Δ::*klTRP1*	This study
SHY90	PC344 *msn4*Δ::*hphNT1*/*msn4*Δ::*KanMX6*	This study
SHY91	PC344 *hap4*Δ::*hphNT1*/*hap4*Δ::*KanMX6*	This study
SHY92	PC344 *nce102*Δ::*hphNT1*/*nce102*Δ::*KanMX6*	This study
THY644	PPY966 *SUT1-9myc-His3MX6*	This study
THY706	PC344 *ste20*Δ::*hphNT1*/*ste20*Δ::*KanMX6*	20
THY697	PPY966 *ste20*Δ::*hphNT1*	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 ste12*Δ::*KanMX6*	This study
THY778	PPY966 *bas1*Δ::*klTRP1*	This study

TABLE 2 Plasmids used in this study

Plasmid	Genotype	Reference
B3782	YEP355 carrying *pFLO11*	24
pHU35	YEp367 carrying *pRHO5*	This study
pHU36	YEp367 carrying *pMGA1*	This study
pHU37	YEp367 carrying *pPRR2*	This study
pMC3	YEp355 carrying *pRHO5*	This study
pMC6	YEp367 carrying *pGAT2*	This study
pMC7	YEp367 carrying *pRHO4*	This study
pNEV-N	2μm *URA3 pPMA1*	25
pNF1	pNEV-N carrying *SUT1*	1
pSH13	YEP367 carrying *pFLO11*	This study
pSH23	YEp367 carrying *pHAP4*	This study
pSH25	YEp367 carrying *pNCE102*	This study
pTH387	YEp367 carrying *pRHO3*	This study
pTH388	YEp367 carrying *pSUT1*	This study
pTH391	YEp367 carrying *pMSN4*	This study
pTH393	YEp355 carrying *pRHO5*(Sut1*)[a]	This study
YEp355	2μm *URA3 lacZ*	26
YEp367	2μm *LEU2 lacZ*	26

[a] The Sut1-binding sequence 5′-CCGGCCCCCC-3′ in the *RHO5* promoter located between positions −733 and −724 was mutated to 5′-GAGCTCATGC-3′.

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 $(NH_4)_2SO_4$. Synthetic high-ammonium dextrose (SHAD) medium, which was used as the reference medium, is the same as SLAD medium but with 50 mM $(NH_4)_2SO_4$. For induction of the *GAL1* promoter, yeast cells were grown in medium with 3% raffinose instead of glucose. Galactose (final concentration, 2%) was added to induce the *GAL1* promoter. *hem1*Δ cells were grown in medium supplemented with 80 μg/ml ergosterol solubilized in Tergitol NP-40–ethanol (1:1) and 1% Tween 80.

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

Filamentation assays. For agar invasion assays, 10^5 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 by using a ProgRes C12 camera (Jenoptik).

β-Galactosidase assay. Densities of cell cultures were measured by the optical density at 600 nm (A_{600}). 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 $MgSO_4$, 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 1 M Na_2CO_3. 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 $1{,}000 \times [A_{420} - (1.75 \times A_{550})]$/reaction time (min) × culture volume (ml) × A_{600}.

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 10^5 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 $\Delta\Delta C_T$ 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 3 Primers used for quantitative real-time PCR

Primer	Sequence (5′–3′)
ACT1-F	GCCTTGGACTTCGAACAAGA
ACT1-R	CCAAACCCAAAACAGAAGGA
GAT2-F	TCTCCAATGGTGCAAACGCAGT
GAT2-R	AATGGCTGGAAGTGGTCAGCGT
HAP4-F	GCTGCACCGATGTGGAAACCAT
HAP4-R	TTTTTCGTGGGTGGTGCGTGA
MGA1-F	ATGGGCAGTCCCGTCCATTACT
MGA1-R	TCGCATCATGTTCACCGTGGGT
MSN4-F	TAGCACCACAAGGCAACAGCGT
MSN4-R	AGCGCACCAAAAGCATCGTCT
NCE102-F	AGCTCAAGCCGCTGTTGCAT
NCE102-R	ACACCGACTTGGCCAGTTCTTC
PRR2-F	TTGGCGCTTCCAGTGTCTTCCA
PRR2-R	ATCCCCACGGACCAAACGTCAA
RHO3-F	TGGCTAAAAAGATCGGTGCGCT
RHO3-R	GCCCGCGGTTAAAGCAACTCTT
RHO5-F	AATGTTCAGCTGCTACCCAAGC
RHO5-R	TTGGTGTTGGTTGTTGCAGTCG

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′-GAGTTGTCAGCAGAGAGA-3′ and 5′-AGAGAAACGATCATGATG-3′, and the region containing the Ste12-binding site was amplified by using primers 5′-TTCCCTCGAGTA TGAGA-3′ and 5′-ACAGCAGGATGGCTCACA-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 could 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 *hem1*Δ background, mimics anaerobic conditions in the presence of oxygen (27). Sut1-9myc protein levels in *hem1*Δ 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 *hem1*Δ 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 *aus1*Δ *pdr11*Δ 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

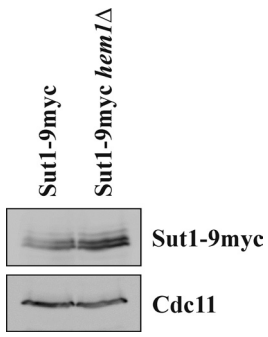

FIG 1 Sut1 is expressed under aerobic conditions. Cells of the indicated strains were grown in YPD medium supplemented with ergosterol and Tween 80 and lysed, and equal amounts of protein were analyzed by immunoblotting using antibodies against the myc epitope and Cdc11 (loading control). Sut1 protein detected by immunoblotting usually exhibits multiple bands. Following dephosphorylation of precipitated Sut1, these bands disappeared (data not shown), indicating that Sut1 is a phosphoprotein.

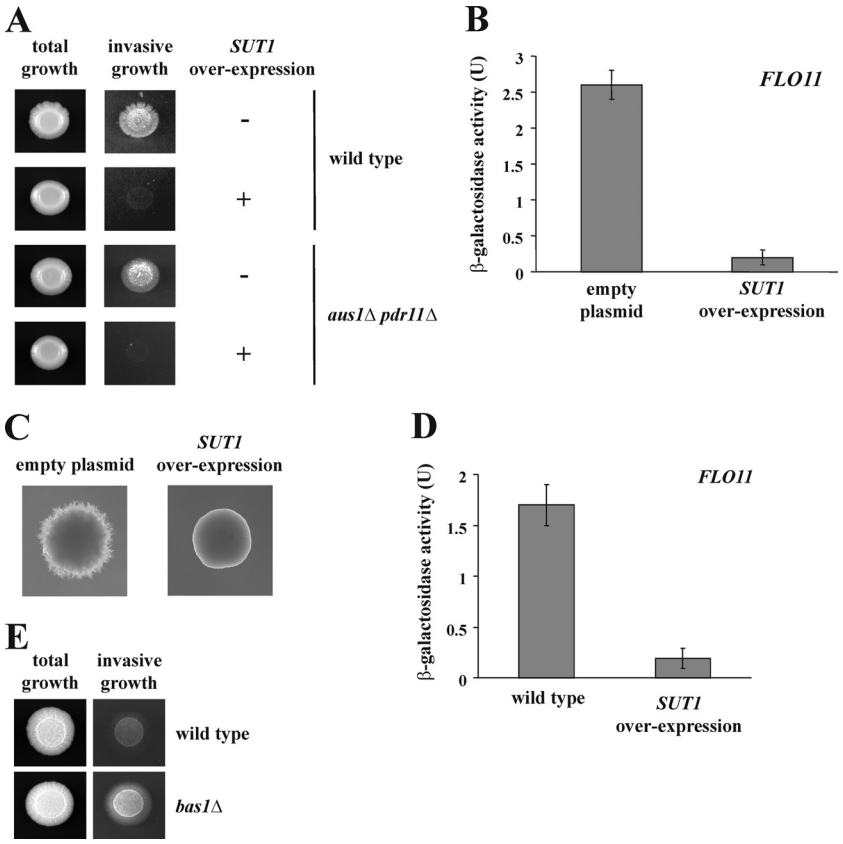

FIG 2 *SUT1* overexpression inhibits filamentous growth. (A) Cells overexpressing *SUT1* have a defect in haploid invasive growth. Cells of the indicated strains carrying either a *SUT1* overexpression construct (pNF1) or an empty plasmid (pNEV-N) were spotted onto a selective medium plate and were grown for 5 days at 30°C. Pictures were taken before (total growth) and after (invasive growth) rinsing with water. (B) *SUT1* overexpression results in reduced *FLO11* levels in haploid cells. Wild-type cells harboring a plasmid on which *lacZ* was fused to the promoter region of *FLO11* (pSH13) and either a *SUT1* overexpression construct (pNF1) or an empty plasmid (pNEV-N) were grown in selective medium. Shown is the mean β-galactosidase activity with the standard deviation ($n \geq 6$). (C) Cells overexpressing *SUT1* have a defect in diploid pseudohyphal growth. Wild-type cells carrying either an empty plasmid (pNEV-N) or a *SUT1* overexpression plasmid (pNF1) were grown on low-nitrogen SLAD medium for 5 days at 30°C. (D) *SUT1* overexpression in diploid cells results in decreased *FLO11* expression levels. β-Galactosidase activity was determined for wild-type cells and cells overexpressing *SUT1* from the *GAL1* promoter, both carrying a *FLO11-lacZ* plasmid (B3782), grown in selective medium supplemented with galactose and raffinose ($n \geq 6$). (E) Deletion of *BAS1* leads to increased haploid invasive growth. Cells were grown on YPD medium for 3 days. Images were taken before (total growth) and after (invasive growth) rinsing with water.

was completely absent (Fig. 2C). The expression level of the filamentation marker *FLO11* was also strongly decreased in diploids with increased *SUT1* levels (Fig. 2D). For this experiment, *SUT1* was overexpressed from the *GAL1* promoter integrated into the genome because the cells used have only one auxotrophic marker and could carry only the *FLO11-lacZ* construct and not the *SUT1* overexpression plasmid. Notably, pseudohyphal growth was also completely absent in the strain overexpressing *SUT1* from the *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 *SUT1* and not for the *SUT1* deletion strain, we wanted to know whether transcription factor genes that have synthetic genetic interactions with *SUT1* play a role in filamentous growth. Synthetic genetic interactions between *SUT1* and *BAS1*, *FKH1*, *RIM101*, and *YAP6* have been reported (29–31). Simultaneous deletion of *FKH1* and its paralog *FKH2* results in increased filamentation in haploids and diploids (32). A *RIM101* deletion strain has a defect in haploid invasive growth (33), and *YAP6* overexpression leads to increased filamentous growth (34). We also tested the effect of *BAS1* deletion on filamentous growth because no such link has been described previously. Cells lacking *BAS1* exhibit increased haploid invasive growth (Fig. 2E). These phenotypes further support the notion that Sut1 plays a role in filamentation under physiological conditions.

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 *MGA1*, *PRR2*, *RHO3*, and *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 (*GAT2*, *HAP4*, *MSN4*, and *NCE102*) were shown to be regulated by *SUT1* in a previously reported DNA

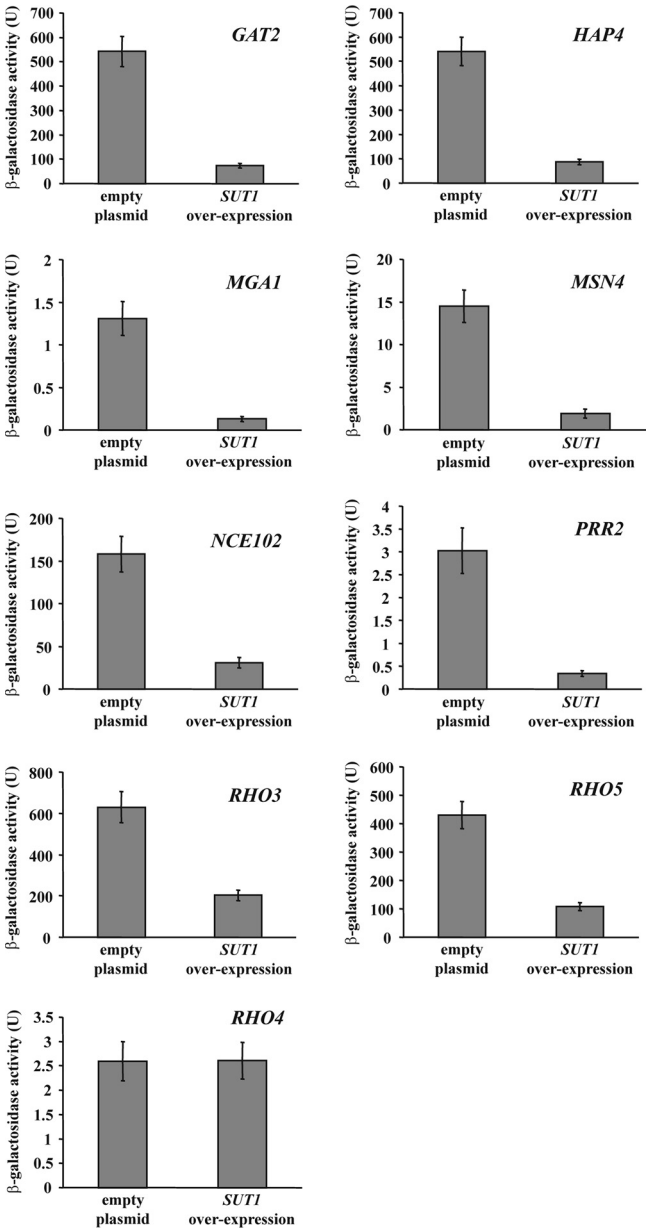

FIG 3 *SUT1* overexpression specifically reduces expression of its target genes. Cells harbored either a *SUT1* overexpression construct (pNF1) or an empty plasmid (pNEV-N) in combination with the *lacZ* reporter fused to the indicated promoter regions. Shown is the mean with the standard deviation ($n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

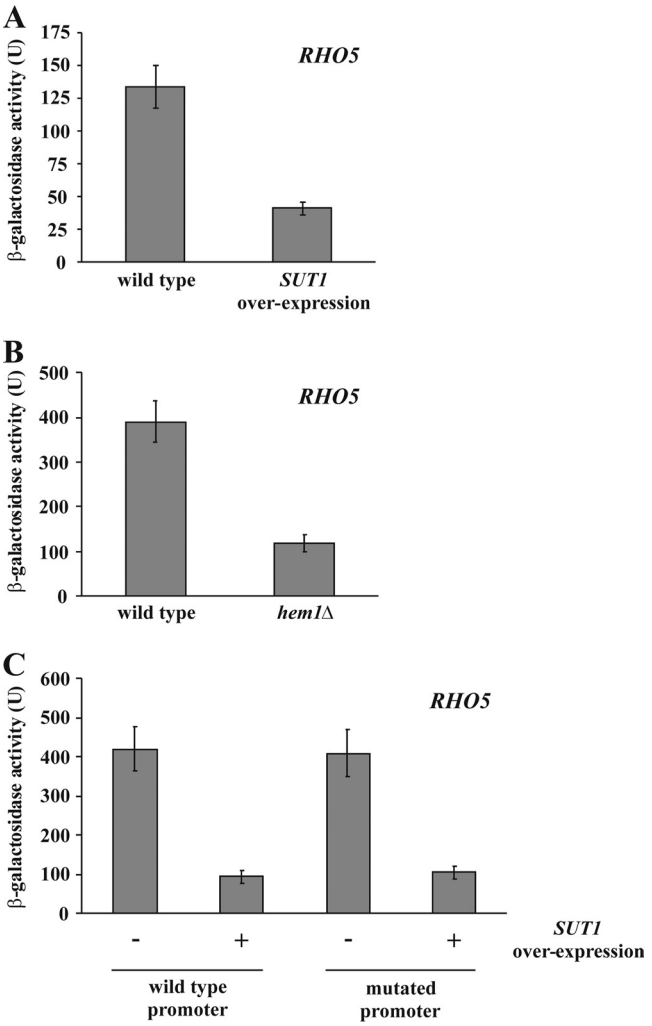

FIG 4 Characterization of downregulation of *RHO5* expression by Sut1. (A) *SUT1* overexpression reduces *RHO5* levels in diploid cells. β-Galactosidase activity was determined for wild-type cells and cells overexpressing *SUT1* from the *GAL1* promoter, both carrying a *RHO5-lacZ* plasmid (pMC3) ($n \geq 6$). (B) *RHO5* expression is reduced in cells lacking *HEM1*. The wild-type strain and *hem1*Δ cells mimicking anaerobic conditions were grown in selective medium supplemented with ergosterol and Tween 80. Both strains carried a *RHO5-lacZ* plasmid (pHU35). Shown is the mean β-galactosidase activity with the standard deviation ($n \geq 6$). (C) Mutation of the Sut1-binding site in the *RHO5* promoter has no effect on the downregulation of *RHO5* expression by *SUT1* overexpression. Cells harbored plasmids on which *lacZ* was fused either to the wild-type promoter region of *RHO5* (pHU35) or to the *RHO5* promoter in which the Sut1-binding site 5′-CCGGCCCCCC-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). β-Galactosidase activity was determined for cells grown in selective medium ($n \geq 6$).

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 *lacZ* gene. Deletion of *SUT1* had no significant effect on levels of *GAT2*, *HAP4*, *MGA1*, *MSN4*, *NCE102*, *PRR2*, *RHO3*, and *RHO5* (data not shown). In contrast, the expression level of all genes tested was decreased in cells overexpressing *SUT1* (Fig. 3). The levels decreased between 3.1-fold (*RHO3*) and 10.1-fold (*MGA1*). Importantly, the observed downregulation is specific and not an artifact. Whereas *SUT1* overexpression downregulated the expression of the Rho GTPases *RHO3* and *RHO5*, it had no effect on the levels of *RHO4*, a related Rho GTPase (Fig. 3), demonstrating that the observed effect is specific for *RHO3* and *RHO5*.

The effect of *SUT1* overexpression was also examined in diploid cells, using *RHO5* as an example. High *SUT1* levels decreased *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 *hem1*Δ back-

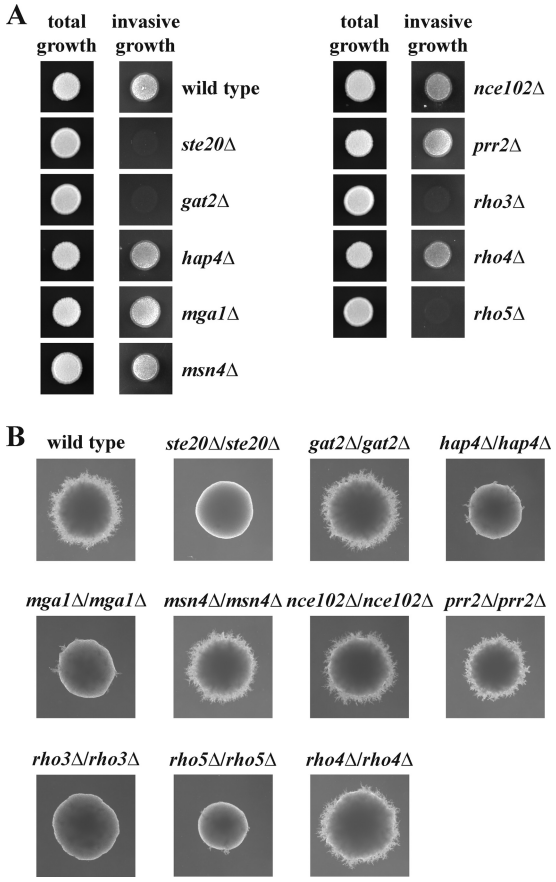

FIG 5 Role of Sut1 target genes in filamentation. (A) Haploid invasive growth of Sut1 target gene deletion strains. Cells of the indicated strains were spotted onto YPD plates and were grown for 3 days at 30°C. Pictures were taken before (total growth) and after (invasive growth) rinsing with water. The *ste20*Δ 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 *ste20*Δ/*ste20*Δ deletion strain served as a control.

ground, shown here for *RHO5* in Fig. 4B. As mentioned above, these cells have increased Sut1 levels (Fig. 1) (3). This result is consistent with the data from the *SUT1* overexpression experiments. Importantly, the expression of Sut1 target genes correlates with the Sut1 levels in the cell and is not an overexpression artifact.

We next wanted to know whether the downregulation of Sut1 targets is mediated directly by Sut1 binding to the corresponding promoters. Using again *RHO5* as an example, the Sut1-binding site in the *RHO5* promoter was mutated. *RHO5* levels expressed from the mutated promoter with and without *SUT1* overexpression were indistinguishable from *RHO5* levels transcribed from the wild-type *RHO5* promoter (Fig. 4C). The Sut1-binding sites in the promoter regions of *RHO3*, *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.

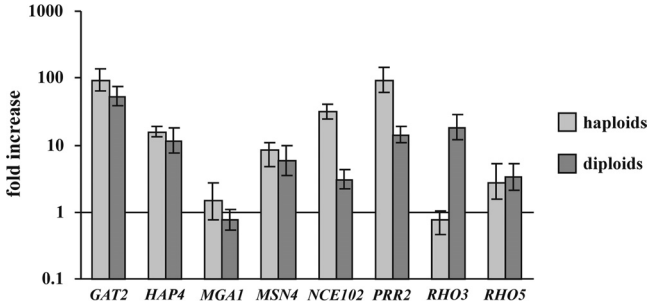

FIG 6 Expression of Sut1 target genes during filamentation Relative expression levels of the indicated genes in cells grown on nutrient-poor (no glucose for haploid cells and low nitrogen for diploid cells) plates for 14 h were determined by quantitative real-time PCR. Cells grown in liquid nutrient-rich medium served as a reference. Three biological replicates were performed. Levels were normalized to the *ACT1* gene expression level. Relative gene expression was calculated using the comparative $\Delta\Delta C_T$ method. Shown is the mean relative quantity (RQ) value, which represents the relative expression level (fold change) compared to the reference sample. The RQ minimum and RQ maximum levels are displayed on the error bars and represent the standard error of the mean relative expression level at a 95% confidence level.

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, *rho4*Δ cells displayed normal haploid invasive growth (Fig. 5A), demonstrating that the defect observed for the *rho3*Δ and the *rho5*Δ 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.

Upregulation 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 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

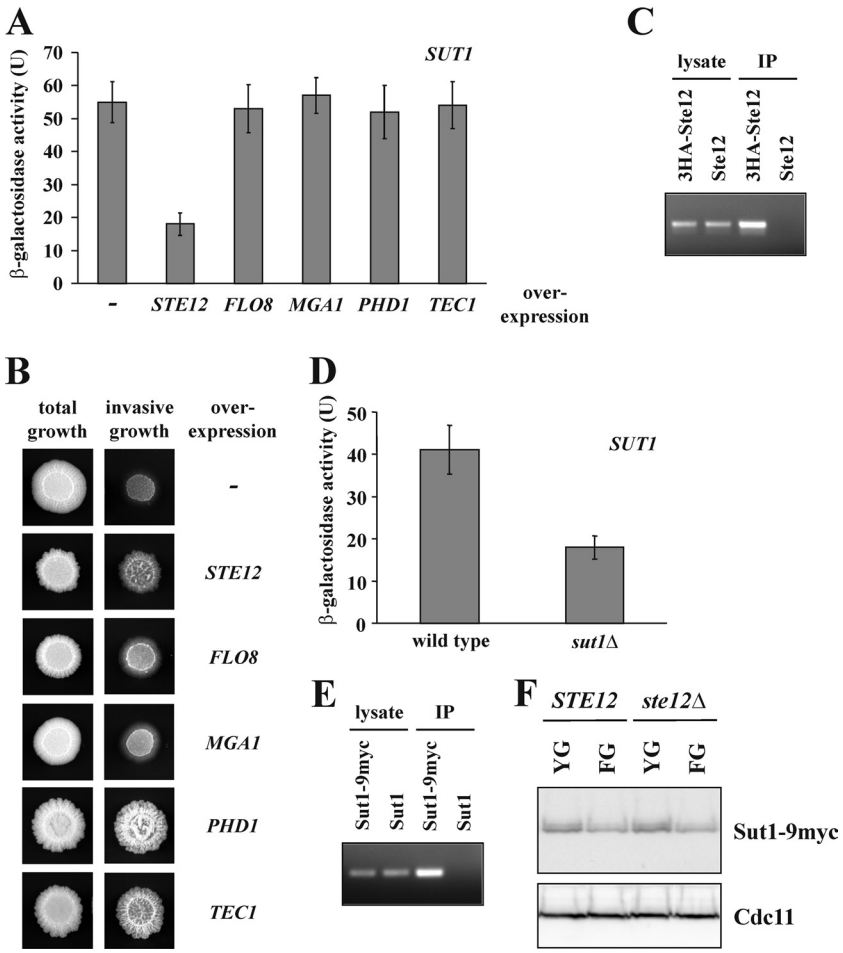

FIG 7 Regulation of *SUT1* expression. (A) *STE12* specifically downregulates *SUT1* expression. *SUT1-lacZ* expression was determined for the wild-type strain and cells overexpressing the indicated transcriptional regulators from the *GAL1* promoter. Bars indicate the mean with the standard deviation ($n \geq 6$). (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 ($n \geq 6$). (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 *ste12*Δ 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).

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 *ste12*Δ cells. This result is consistent with the observation that *STE12* deletion does not affect *SUT1-lacZ* expression.

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[a]
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
RHO3	+	+	−	+	Flo8, Mga1, Phd1, Sok2, Ste12, Sut1, Tec1
RHO5	+	+	+	+	Flo8, Mga1, Phd1, Sok2, Ste12, Sut1, Tec1

[a] 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*, *RHO3*, and *RHO5*.

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*, *RHO3*, and *RHO5* (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*, *RHO3*, and *RHO5*, 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).

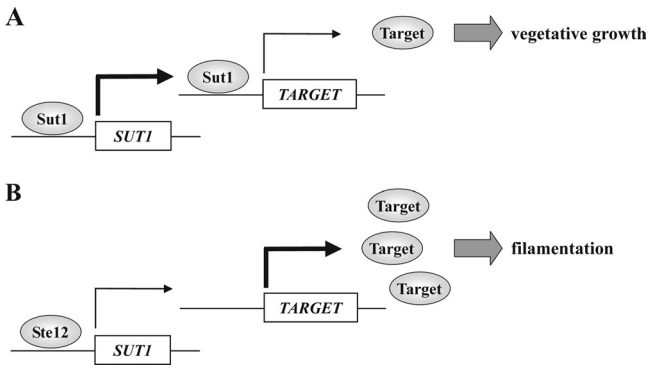

FIG 8 Model for the role of Sut1 in filamentous growth. (A) Under optimal growth conditions, Sut1 binds to its own promoter, allowing relatively high expression levels of *SUT1*. Binding of Sut1 to the promoters of various target genes, such as *HAP4*, *MSN4*, *NCE102*, *PRR2*, *RHO3*, and *RHO5*, partially represses their expression. (B) When cells are grown on semisolid medium and nutrients are scarce, the transcription factor Ste12 becomes activated and reduces *SUT1* transcription. Reduced Sut1 protein levels further reduce *SUT1* expression and increase transcription of Sut1 target genes. Expression of these genes is also under the control of the transcription factors Flo8, Mga1, Phd1, Sok2, Ste12, and Tec1, which are not shown here. The increased expression levels of all these factors together might trigger filamentous growth.

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 *msn2*Δ *msn4*Δ 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

elongate 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 posttranslationally 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 *DAN1*, 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 *sut1*Δ 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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