# The Cdc42 Effectors Ste20, Cla4, and Skm1 Down-Regulate the Expression of Genes Involved in Sterol Uptake by a Mitogen-activated Protein Kinase-independent Pathway

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In *Saccharomyces cerevisiae*, the Rho-type GTPase Cdc42 regulates polarized growth through its effectors, including the p21-activated kinases (PAKs) Ste20, Cla4, and Skm1. Previously, we demonstrated that Ste20 interacts with several proteins involved in sterol synthesis that are crucial for cell polarization. Under anaerobic conditions, sterols cannot be synthesized and need to be imported into cells. Here, we show that Ste20, Cla4, and Skm1 form a complex with Sut1, a transcriptional regulator that promotes sterol uptake. All three PAKs can translocate into the nucleus and down-regulate the expression of genes involved in sterol uptake, including the Sut1 targets *AUS1* and *DAN1* by a novel mechanism. Consistently, deletion of either *STE20*, *CLA4*, or *SKM1* results in an increased sterol influx and PAK overexpression inhibits sterol uptake. For Ste20, we demonstrate that the down-regulation of gene expression requires nuclear localization and kinase activity of Ste20. Furthermore, the Ste20-mediated control of expression of sterol uptake genes depends on *SUT1* but is independent of a mitogen-activated protein kinase signaling cascade. Together, these observations suggest that PAKs translocate into the nucleus, where they modulate expression of sterol uptake genes via Sut1, thereby controlling sterol homeostasis.

## INTRODUCTION

The small Rho GTPase Cdc42 plays a central role in the regulation of cellular polarity in eukaryotic cells (Etienne-Manneville, 2004; Jaffe and Hall, 2005). In the budding yeast *Saccharomyces cerevisiae*, Cdc42 regulates different types of polarized growth during various phases of its life cycle, including budding during vegetative growth, mating between haploid cells of opposite mating types, and filamentous growth upon nutrient limitation (Park and Bi, 2007). Cdc42 promotes polarized growth through multiple pathways, including polarization of the actin cytoskeleton and directed vesicle trafficking. Among the Cdc42 effectors that trigger these pathways are Ste20, Cla4 and Skm1, members of the p21-activated kinase (PAK) family of serine/threonine protein kinases (Hofmann *et al.*, 2004; Park and Bi, 2007).

Cdc42 recruits Ste20 and Cla4 from the cytoplasm and activates them at the plasma membrane at sites of polarized growth. Therefore, Ste20 and Cla4 are enriched at tips of buds and mating projections (Peter *et al.*, 1996; Leberer *et al.*, 1997; Holly and Blumer, 1999). The recruitment of Ste20 and Cla4 to these sites is governed by protein–protein as well as

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Abbreviations used: BR, basic-rich; CRIB, Cdc42/Rac-interactive binding; PAK, p21-activated kinase; PH, pleckstrin homology; TLC, thin layer chromatography.

protein–lipid interactions. PAKs carry a conserved Cdc42/Rac-interactive binding (CRIB) domain that mediates binding to Cdc42 and regulates their activity (Cvrckova *et al.*, 1995; Peter *et al.*, 1996; Leberer *et al.*, 1997; Lamson *et al.*, 2002; Ash *et al.*, 2003). Ste20 and Cla4 also bind to Bem1, a scaffold protein that brings Cdc42, its activator Cdc24 and either Ste20 or Cla4, into proximity (Leeuw *et al.*, 1998; Gulli *et al.*, 2000; Bose *et al.*, 2001; Winters and Pryciak, 2005; Yamaguchi *et al.*, 2007). In addition, phosphoinositide-binding domains promote the association with membrane lipids. Cla4 carries a pleckstrin homology (PH) domain and Ste20 contains a short basic-rich (BR) region domain, both of them binding to membrane lipids.

Ste20 regulates multiple mitogen-activated protein kinase (MAPK) pathways that control mating, filamentous growth, and osmotic stress response, and it is also involved in exit from mitosis and hydrogen peroxide-induced apoptosis (Leberer *et al.*, 1992; Ramer and Davis, 1993; Liu *et al.*, 1993; Roberts and Fink, 1994; O'Rourke and Herskowitz, 1998; Raitt *et al.*, 2000; Höfken and Schiebel, 2002; Ahn *et al.*, 2005). Probably the best characterized function of Cla4 is the assembly of the septin ring, which plays a fundamental role in cytokinesis and cell compartmentalization (Weiss *et al.*, 2000; Schmidt *et al.*, 2003; Kadota *et al.*, 2004; Versele and Thorner, 2004). In addition, Cla4 regulates mitotic entry and exit (Höfken and Schiebel, 2002; Seshan *et al.*, 2002; Sakchaisri *et al.*, 2004). Very little is known about Skm1, and no clear function has been attributed to this PAK (Martín *et al.*, 1997). Interestingly, Ste20 and Cla4 interact with Erg4, Cbr1 and Ncp1, which are all involved in sterol biosynthesis, and the deletion of the corresponding genes results in various polarity defects, suggesting that sterol biosyn-

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thesis is crucial for cell polarization (Ni and Snyder, 2001; Tiedje *et al.*, 2007).

Many aspects of sterol homeostasis are conserved between yeast and human, and ergosterol, the predominant sterol of yeast, is structurally and functionally related to sterols of higher eukaryotes (Sturley, 2000). Ergosterol biosynthesis can occur only when oxygen is available. Although sterol synthesis is an energy-consuming process, cells do not take up significant amounts of exogenous sterol under aerobiosis. The physiological significance of this phenomenon, termed aerobic sterol exclusion, is poorly understood (Lewis *et al.*, 1985). It may be a way for cells to ensure that only the best fitting sterols accumulate in its membranes (Parks and Casey, 1995). In contrast, under anaerobic conditions, when sterol biosynthesis is compromised, cells become capable of importing sterols whose presence in the medium is then required for growth. Because completely anaerobic conditions are difficult to maintain, most studies used sterol auxotrophs or 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, and as a consequence these cells accumulate sterol from the medium (Gollub *et al.*, 1977). The molecular mechanisms of sterol import are poorly understood. Sterol uptake is controlled by the transcriptional regulators Sut1, Upc2, and Ecm22, which are members of the Zn(II)2Cys6 family of DNA-binding proteins (Schjerling and Holmberg, 1996). Under anaerobic conditions, Upc2 and Sut1 up-regulate the expression of the ATP-binding cassette transporter *AUS1* and *PDR11*, and the putative cell wall mannoprotein *DAN1* (Figure 1) (Régnacq *et al.*, 2001; Wilcox *et al.*, 2002; Alimardani *et al.*, 2004). These proteins then mediate sterol influx. Presumably, other proteins contribute to this process as well (Wilcox *et al.*, 2002; Alimardani *et al.*, 2004).

Here, we characterize the link between proteins regulating sterol uptake and the PAKs Ste20, Cla4 and Skm1. Our data suggest a model (Figure 1), according to which PAKs shuttle into the nucleus, where they interact with Sut1 and possibly other transcriptional regulators and down-regulate the expression of *AUS1*, *DAN1*, and *PDR11*. As a consequence, sterol influx is also negatively regulated. Thus, PAKs seem to play an important role in the control of sterol uptake.

## MATERIALS AND METHODS

### Yeast Strains, Plasmids, and Growth Conditions

All yeast strains are listed in Supplemental Table S1. The strains used in this study were in the YPH499 background with the exception of strains used for filamentous growth and sterol uptake. For filamentous growth, the Σ1278b background was used (MLY48, MLY49, PPY966, and THY696). Cholesterol uptake experiments were performed with the BY4742 background (Winzeler *et al.*, 1999) (MLY180, MLY181, MLY185, THY725, THY734, THY737, YRS1707, and YRS1962). Yeast strains were grown in yeast extract, peptone, dextrose (YPD) or synthetic complete (SC) medium. For induction of the *GAL1* promoter, yeast cells were grown in yeast extract, peptone (YP) or SC medium with 3% raffinose instead of glucose. Galactose (final concentration 2%) was added to induce the *GAL1* promoter. *hem1Δ* cells were grown in medium supplemented either with 40 $\mu$g/ml δ-aminolevulinic acid or with 80 $\mu$g/ml ergosterol solubilized in Tergitol NP-40/ethanol (1:1) and 1% Tween 80. Yeast strains were constructed using polymerase chain reaction (PCR)-amplified cassettes (Longtine *et al.*, 1998; Knop *et al.*, 1999; Janke *et al.*, 2004). All constructs used in this work are listed in Supplemental Table S2.

### Split-Ubiquitin Technique

The split-ubiquitin screen using *STE20* as bait is described in Tiedje *et al.* (2007). For the interaction assays, $10^5$ *ste20Δ* cells carrying the split-ubiquitin
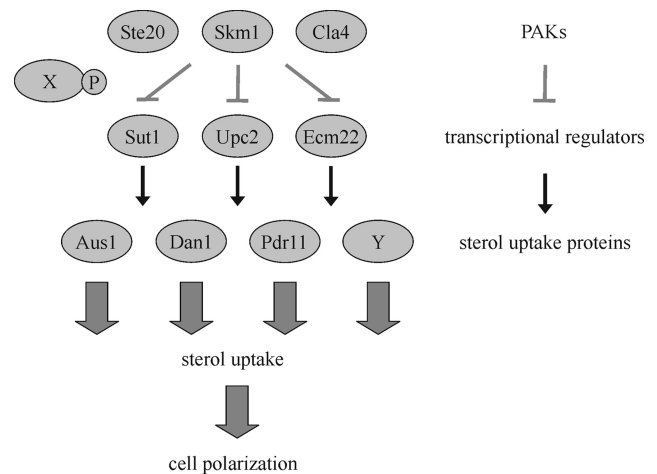


**Figure 1.** Transcriptional regulation of genes mediating sterol uptake. In the presence of oxygen, yeast cells synthesize sterols and are unable to take up sterols from the extracellular medium. In contrast, under anaerobiosis cells rely on sterol uptake, because sterol synthesis requires oxygen. Under these conditions, the transcriptional regulators Sut1, Upc2, and Ecm22 promote the expression of *AUS1*, *DAN1*, and *PDR11* and less well characterized genes (Y), which mediate sterol import. The underlying mechanisms of this transcriptional regulation are largely unknown. Here, we show that the PAKs Ste20, Cla4, and Skm1 can translocate into the nucleus, where they bind to the transcriptional regulator Sut1. The fact that the PAKs negatively regulate expression of *AUS1*, *DAN1*, and *PDR11*, suggests that PAKs inhibit Sut1 and/or Upc2 and Ecm22 by a novel mechanism. As demonstrated for Ste20, the down-regulation of gene expression requires its kinase activity. However, since there is no evidence for Sut1 phosphorylation by PAKs, an unknown protein (X), phosphorylated by PAKs, may be involved in the control of transcriptional regulators. Because sterol synthesis is crucial for cell polarization under aerobic conditions, it seems likely that sterol uptake plays an equally important role in the absence of oxygen.

plasmids were spotted on SC-His/Leu and SC-His/Leu/Ura plates and were grown for 2 d at 30°C.

### Pull-Down Assays and Antibodies

Glutathione transferase (GST), GST-Sut1, His$_6$-Sec6, and His$_6$-Sut1 were expressed in *Escherichia coli* BL21 (DE3) and purified using glutathione-Sepharose (GE Healthcare, Chalfont St. Giles, Buckinghamshire, United Kingdom) and nickel-nitrilotriacetic acid (Ni-NTA) agarose (QIAGEN, Valencia, CA), respectively. These immobilized recombinant proteins were presented to yeast lysates of *3HA-STE20*, *3HA-CLA4*, and *3HA-SKM1*, respectively, for 90 min at 4°C in immunoprecipitation (IP) buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 10 mM EDTA, 1 mM EGTA, 5% glycerol, 1% NP-40, and 1% bovine serum albumin). After five washes with IP buffer, the associated proteins were eluted with sample buffer and analyzed by immunoblotting.

Monoclonal mouse anti-hemagglutinin (HA) (12CA5) was obtained from Roche Diagnostics (Indianapolis, IN). Rabbit polyclonal anti-Cdc11 and goat polyclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA), and rabbit polyclonal anti-green fluorescent protein (GFP) was from Fitzgerald Industries (Acton, MA). Secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA).

### Microscopy

For fluorescence microscopy, cells were fixed with 4% formaldehyde for 10 min, washed twice with phosphate-buffered saline (PBS), and resuspended in water or in 0.25 $\mu$g/ml 4,6-diamidino-2-phenylindole (DAPI) in 50% glycerol. Cells were examined with an Axiovert 200M fluorescence microscope (Carl Zeiss, Jena, Germany) equipped with a 100× Plan oil-immersion objective, and images were captured using an AxioCam MRm charge-coupled device camera (Carl Zeiss).

### β-Galactosidase Assays

Densities of cell cultures were measured by OD$_{600}$. One to 10 ml of cells was harvested by centrifugation and resuspended in 1 ml of Z buffer (100 mM

sodium phosphate, pH 7.0, 10 mM KCl, 1 mM MgSO₄, and 50 mM β-mercaptoethanol). Cells were permeabilized by addition of 20 $\mu$l of chloroform and 20 $\mu$l of 0.1% SDS. After 15-min incubation at 30°C, the reaction was started by addition of 160 $\mu$l of *o*-nitrophenyl-β-D-galactopyranoside (4 mg/ml in 100 mM sodium phosphate, pH 7.0) and incubated at 30°C until the solution became yellow, and then the reaction was stopped by addition of 400 $\mu$l of 1 M Na₂CO₃. Samples were centrifuged, and the OD₄₂₀ and OD₅₅₀ of the supernatant was determined. β-Galactosidase activity was calculated in Miller units as $1000 \times [OD_{420} - (1.75 \times OD_{550})]$/reaction time (min) × culture volume (ml) × OD₆₀₀.

## Invasive Growth and Pheromone Response Assays

For agar invasion assays, $10^5$ cells of an overnight culture were spotted on YPD and grown for 2 d at 30°C. Plates were photographed before and after being rinsed under a gentle stream of deionized water.

For examination of the formation of a mating projection, logarithmically growing cells were incubated with 1 $\mu$g/ml α-factor for 3 h. These cells were fixed with formaldehyde for morphological examination.

## Sterol Uptake

Sterol uptake was essentially analyzed as described previously (Reiner *et al.*, 2006). *Hem1*Δ mutant cells were cultured in δ-aminolevulinic acid-containing media, washed, and diluted in minimal media supplemented with Tween 80 (5 mg/ml), cholesterol (20 $\mu$g/ml), and 0.025 $\mu$Ci/ml [¹⁴C]cholesterol (American Radiolabeled Chemicals, St. Louis, MO). After 2, 4, 8, and 24 h, equal OD units of cells were collected and washed with 0.5% Tergitol. [³H]Palmitic acid was added to the cell pellet as internal standard. Cells were disrupted with glass beads in the presence of chloroform/methanol (1:1), and lipids were extracted into the organic phase. Lipids were separated by thin layer chromatography (TLC) (Merck, Darmstadt, Germany), with the solvent system petroleum ether:diethylether:acetic acid (70:30:2, per vol.), and free cholesterol and cholesteryl esters were quantified by scanning with a Tracemaster 40 Automatic TLC-Linear Analyzer (Berthold Technologies, Bad Wildbad, Germany). TLC plates were then exposed to a phosphorimager screen and visualized using a phosphorimager (Bio-Rad Laboratories, Hercules, CA).

## RESULTS

### PAKs Interact with the Transcriptional Regulator Sut1

Previously, we described a screen to identify novel interactors of Ste20 by using the split-ubiquitin technique (Tiedje *et al.*, 2007). This method is based on the ability of N- and C-terminal halves of ubiquitin to assemble into a quasi-native ubiquitin (Johnsson and Varshavsky, 1994; Wittke *et al.*, 1999). If two proteins, which are attached to the N- and C-terminal halves, respectively, interact, the ubiquitin peptides may be forced into proximity and a ubiquitin-like molecule is reconstituted (Figure 2A). Ubiquitin-specific proteases recognize this molecule, but not its halves, and cleave off the enzyme Ura3, that carries an additional arginine at the extreme N terminus (RUra3). The freed RUra3 reporter is rapidly degraded by proteases of the N-end rule, resulting in uracil auxotrophy. Conversely, growth on 5-fluoroorotic acid (5-FOA) indicates protein interaction as well, because 5-FOA is converted by Ura3 into 5-fluorouracil, which is toxic for the cell. By using this technique, we identified Sut1 as a putative Ste20-interacting protein (Figure 2B). *SUT1* encodes a transcriptional regulator involved in sterol uptake under anaerobic conditions (Bourot and Karst, 1995; Ness *et al.*, 2001). To confirm the interaction between Ste20 and Sut1 by an independent approach, recombinant GST and GST-Sut1 purified from *E. coli* were bound to glutathione-Sepharose beads, which were then incubated with a yeast extract of *STE20-3HA* cells. Ste20-3HA interacted with GST-Sut1 but not with GST alone (Figure 2C). Next, it was tested whether Cla4 and Skm1, which are related to Ste20, also interact with Sut1 using a pull-down assay. 3HA-Cla4 and 3HA-Skm1, both expressed from yeast, bind specifically to recombinant His₆-Sut1 but not to the unrelated His₆-Sec6, which was used as a negative control (Figure 2D).
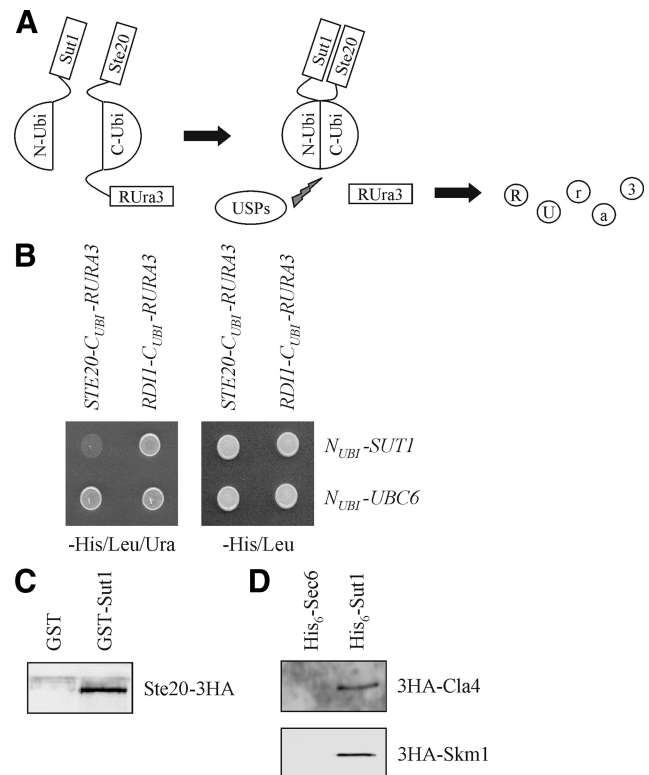


**Figure 2.** PAKs form a complex with Sut1. (A) The split-ubiquitin technique. See text for details. USPs, ubiquitin-specific proteases. (B) Ste20 interacts with Sut1 using the split-ubiquitin system. Cells ($10^5$) of the indicated plasmid combinations in a *ste20*Δ background were spotted onto media either lacking histidine and leucine to select for the plasmids or lacking histidine, leucine, and uracil to monitor protein interactions. The unrelated proteins Rdi1 and Ubc6 served as negative controls. (C) Ste20 interacts with Sut1 in vitro. Purified GST and GST-Sut1 bound to glutathione-Sepharose beads were incubated with an extract from cells expressing *STE20-3HA* from the plasmid pKA86. Eluted proteins were analyzed by immunoblotting using anti-HA antibodies. (D) Cla4 and Skm1 bind to Sut1. Recombinant His₆-Sut1 and His₆-Sec6 bound to Ni-NTA beads were incubated with lysates from cells expressing *3HA-CLA4* and *3HA-SKM1*, respectively. Eluted proteins were analyzed by immunoblotting using anti-HA antibodies.

### Nuclear Localization of PAKs

Sut1 localizes to the nucleus (Ness *et al.*, 2001), whereas Ste20 can be found in the cytosol and at the plasma membrane at sites of polarized growth (Peter *et al.*, 1996; Leberer *et al.*, 1997) (Figure 3B). Therefore, it is not clear where the interaction between Sut1 and Ste20 takes place. Analysis of the Ste20 sequence revealed a putative bipartite NLS between amino acids 272-288 with two clusters of positively charged amino acids separated by a spacer of 11 amino acids (Figure 3A). Notably, this putative nuclear localization signal (NLS) overlaps with the BR domain, which targets Ste20 to the plasma membrane (Takahashi and Pryciak, 2007). In fact, the first of three basic clusters within the BR domain (BR-1) is identical to the second cluster of the NLS (Figure 3A). To test whether the identified NLS is functional, it was fused with green fluorescent protein (GFP) and expressed in yeast cells. This GFP fusion also contained a homodimerizing GST moiety, which can help to reveal weak localization determinants by increasing binding avidity (Winters *et al.*, 2005). However, the GFP-NLS fusion protein was found in the cytoplasm (data not shown). We reasoned that the NLS might

**Figure 3.** Ste20 localizes to the nucleus. (A) Sequence of the NLS and BR domain. The NLS is composed of two clusters of basic residues, whereas the BR domain consists of three clusters (BR-1, -2, and -3) (Takahashi and Pryciak, 2007). The second cluster of the NLS is identical to BR-1. (B) The Ste20 NLS is functional. Exponentially growing wild-type cells carrying the indicated Ste20 fragments fused to GST-GFP were incubated with galactose for 1 h. Cells were subsequently fixed with formaldehyde and stained with DAPI. One asterisk denotes the fusion with the peptide SGAGAGAGAGAIL. Two asterisks indicate addition of the sequence LGYFLFFEGGPGTQFAL. (C) Ste20 lacking the NLS is excluded from the nucleus. Logarithmically growing wild-type strains carrying the indicated *GFP-STE20* alleles under control of a galactose-inducible promoter on a centromeric plasmid were cultivated in galactose-containing media for 1 h. Arrowheads indicate the nucleus. (D) Quantification of C. n > 100. (E) In few cells, Ste20 was enriched in the nucleus. Exponentially growing wild-type cells harboring GFP-Ste20 under control of a galactose-inducible promoter on a centromeric plasmid were incubated with galactose for 1 h.
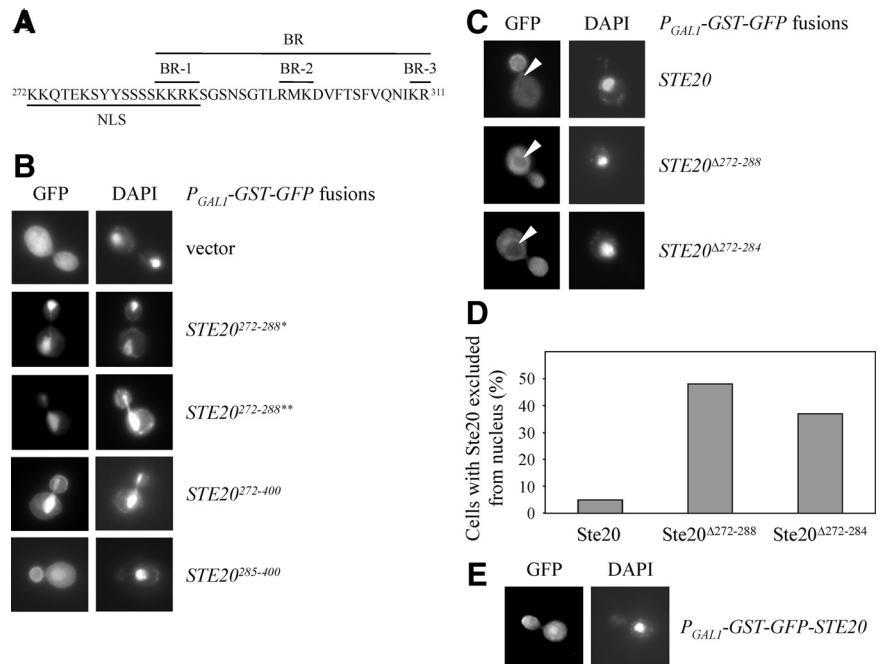


not be recognized by the nuclear import machinery, possibly due to steric hindrance. Therefore, we added a short stretch of amino acids (SGAGAGAGAGAIL), which is used as a spacer for GFP fused to a protein of interest (Miller and Lindow, 1997) at the C terminus of the NLS. This protein (termed GFP-Ste20$^{272-288*}$) exclusively localized to the nucleus as demonstrated by DAPI costaining (Figure 3B). To rule out the possibility that the attached spacer sequence was responsible for the nuclear localization, another unrelated random sequence (LGYFLFFEGGPGTQFAL) was C-terminally fused to the NLS. This fusion protein (termed GFP-Ste20$^{272-288**}$) was restricted to the nucleus as well (Figure 3B). From these data we conclude that the NLS is functional but requires a short nonspecific stretch of amino acids at the C terminus.

As mentioned above, the NLS overlaps with the BR domain (Figure 3A) (Takahashi and Pryciak, 2007). To find out whether the BR domain alone is sufficient for nuclear localization, we compared the localization of GFP fused with Ste20 residues 272-400 (comprising the entire NLS and BR domain plus some C-terminal amino acids) and Ste20 residues 285-400 (comprising only the second cluster of positively charged amino acids of the NLS, the complete BR domain and some C-terminal amino acids). Notably, GFP-Ste20$^{272-400}$ was strongly enriched in the nucleus and present in the cytoplasm and at the plasma membrane, whereas GFP-Ste20$^{285-400}$ was associated with the bud cortex and only a weak nuclear signal was observed (Figure 3B). Thus, the entire NLS is required for efficient nuclear targeting of Ste20, whereas the BR domain alone, which includes the second basic cluster of the NLS, plays only a minor role in this process. Importantly, in contrast to wild-type Ste20 fused to GFP, GFP-Ste20 without the complete NLS (GFP-Ste20$^{\Delta272-288}$) was excluded from the nucleus (Figure 3, C and D). Similarly, much of GFP-Ste20 lacking the first basic cluster but with an intact BR domain (GFP-Ste20$^{\Delta272-284}$) was no longer present in the nucleus (Figure 3, C and D). However, the effect was less pronounced compared with GFP-Ste20$^{\Delta272-288}$ (Figure 3D). Together, these data show that the BR-1 region contributes only to a minor extent to nuclear

targeting of Ste20. Furthermore, our observations suggest that during normal vegetative growth at least some Ste20 localizes to the nucleus. Consistently, in few cells we observed nuclear localization of wild-type Ste20 (Figure 3E). However, since Ste20 was only weakly enriched in the nucleus compared with the surrounding cytoplasm, the number of cells with a nuclear Ste20 signal was difficult to quantify.

Very little is known about Skm1, and its localization has not been reported previously (Martín *et al.*, 1997). Therefore, we first analyzed the localization of full-length Skm1 fused to GFP. *SKM1-GFP* under control of its endogenous promoter could be detected by immunoblotting but was to weak to be visualized by fluorescence microscopy (data not shown). Therefore, we overexpressed *GFP-SKM1* from the inducible *GAL1* promoter. Unexpectedly, full-length Skm1 was strongly enriched in the nucleus with only a faint signal in the cytoplasm and at the plasma membrane (Figure 4A). Between amino acids 213-230 we identified a putative bipartite NLS (*KR*TNSIKRSVSRTL*RKGK*; positively charged amino acids of the two basic clusters are in italics). Skm1 lacking this putative NLS was no longer enriched in the nucleus (Figure 4A). We also made point mutations in either the first or the second cluster of positively charged amino acids. The mutant protein Skm1$^{C1}$, in which Lys213 and Arg214 of the first basic cluster were changed to Ala, mostly localized to the cytoplasm (Figure 4A). In a second mutant, Arg227, Lys228, and Lys230 of the second positively charged cluster were changed to Ala. The corresponding protein, termed Skm$^{C2}$, was not or only faintly enriched in the nucleus (Figure 4A). By immunoblotting it was confirmed that Skm1 wild-type and mutant proteins were expressed at comparable levels (Figure 4B). Thus, both clusters play an important role in nuclear targeting of Skm1. We also fused Skm1 residues 213-230 to GFP. This fusion protein was present in the cytoplasm (data not shown), but a slightly larger Skm1 fragment (residues 201-230) exclusively localized to the nucleus (Figure 4C). Together, it seems that, similar to Ste20, the predicted Skm1 NLS is functional but
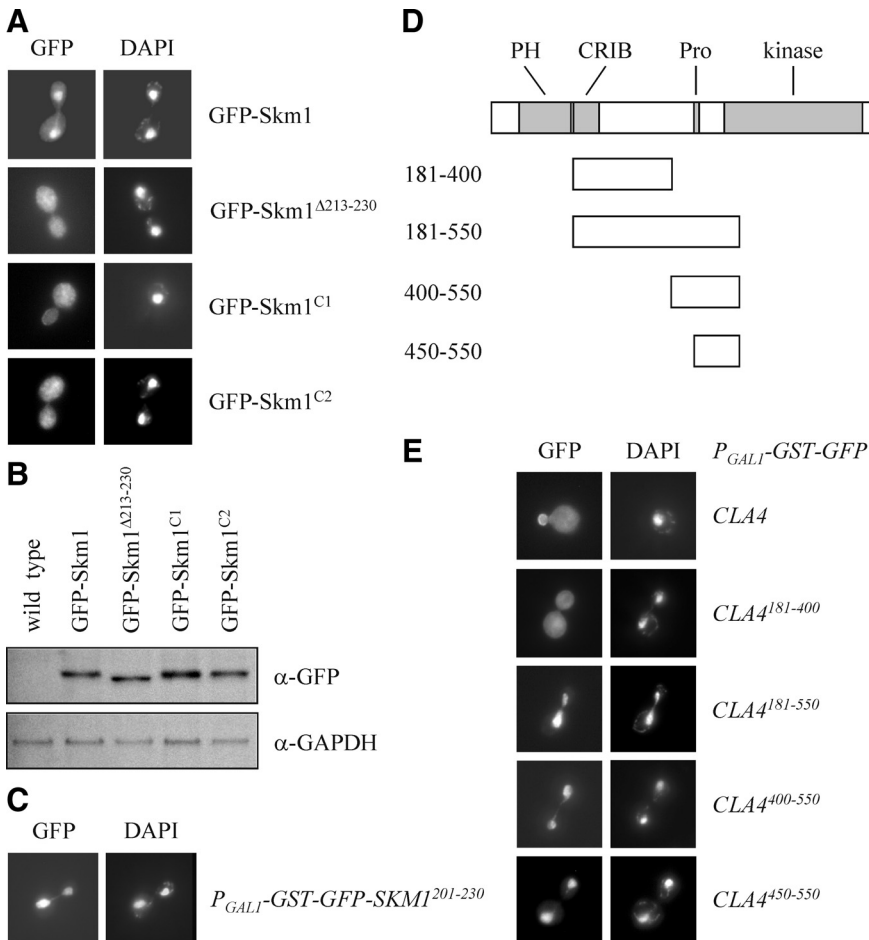
**A**

GFP     DAPI



GFP-Skm1

GFP-Skm1$^{\Delta213-230}$

GFP-Skm1$^{C1}$

GFP-Skm1$^{C2}$

**B**



wild type
GFP-Skm1
GFP-Skm1$^{\Delta213-230}$
GFP-Skm1$^{C1}$
GFP-Skm1$^{C2}$

α-GFP

α-GAPDH

**C**

GFP     DAPI



$P_{GAL1}$-GST-GFP-SKM1$^{201-230}$

**D**



PH    CRIB    Pro    kinase

181-400

181-550

400-550

450-550

**E**

GFP     DAPI     $P_{GAL1}$-GST-GFP



CLA4

CLA4$^{181-400}$

CLA4$^{181-550}$

CLA4$^{400-550}$

CLA4$^{450-550}$

**Figure 4.** Nuclear localization of Skm1 and Cla4. (A) Skm1 is strongly enriched in the nucleus. Exponentially growing cells carrying the indicated *GFP-SKM1* alleles were induced for 1 h by the addition of galactose. Cells were fixed with formaldehyde and stained with DAPI. (B) Wild-type *SKM1* and its derivatives are expressed at comparable levels. The indicated strains were incubated with galactose for 1 h. Cells were lysed and equal amounts of protein extract were analyzed by immunoblotting using antibodies against GFP and GAPDH (loading control). (C) Residues 201-230 are sufficient for nuclear targeting of Skm1. The Skm1 fragment was expressed as galactose-inducible GST-GFP fusion. (D) Fragments used to map the sequence that targets Cla4 to the nucleus. The kinase domain and regions that are required for membrane association are highlighted. The CRIB domain is the minimal Cdc42-binding motif (Cvrckova *et al.*, 1995), a proline-rich region (Pro) mediates binding to the scaffold protein Bem1 (Winters and Pryciak, 2005), and the PH domain is required for the association with membrane phosphoinositides (Wild *et al.*, 2004). (E) Cla4 fragments localize to the nucleus. Cla4 fragments were expressed as GST-GFP fusions from a galactose-inducible promoter in wild-type cells.

requires a few more amino acids to target GFP to the nucleus.

Like Ste20, Cla4 associates with the bud cortex (Holly and Blumer, 1999) (Figure 4E) and analysis of the Cla4 protein sequence did not reveal an NLS. To determine whether Cla4 could translocate into the nucleus, a series of Cla4 fragments fused to GFP were constructed (Figure 4D). In line with a previous report (Wild *et al.*, 2004), Cla4 fragments lacking the N-terminal 180 residues, which contain a PH domain that binds to membrane lipids, did not associate with the plasma membrane (Figure 4E). A fragment containing residues 181-550 exclusively localized to the nucleus, whereas a smaller portion of Cla4 (residues 181-400) was cytoplasmic (Figure 4E). Therefore, we reasoned that a nuclear targeting sequence may be present between residues 400-550. Indeed, a fragment comprising this sequence and also a slightly smaller fragment (amino acids 450-550) were only found in the nucleus (Figure 4E). Further truncations resulted in nuclear enrichment but in these cells we also observed cytoplasmic staining (data not shown). Thus, residues 450-550 are sufficient for proper nuclear targeting of Cla4. Together, we demonstrated that all three PAKs can localize to the nucleus, where they could interact with the transcriptional regulator Sut1.

## PAKs Specifically Down-Regulate the Expression of *AUS1*, *DAN1*, and *PDR11*

Sut1 promotes sterol uptake under anaerobic conditions by enhancing the transcription of *AUS1* and *DAN1* (Régnacq *et*

*al.*, 2001; Alimardani *et al.*, 2004). Therefore, we wanted to know whether the expression of these genes also depends on Ste20, Cla4, and Skm1. To this end, the promoter regions of *AUS1*, *DAN1*, and *PDR11*, were fused to the reporter gene *lacZ*. *PDR11* was included because it also mediates sterol influx but is not regulated by Sut1 (Wilcox *et al.*, 2002; Alimardani *et al.*, 2004). Using these constructs, we confirmed that *AUS1*, *DAN1*, and *PDR11* are not expressed in wild-type cells under aerobic conditions (Figure 4, A–C). In contrast, in a *hem1Δ* background mimicking anaerobic conditions these genes were strongly induced (Figure 4, A–C). Furthermore, *SUT1* overexpression under aerobic conditions led to expression of *AUS1* and *DAN1* but not of *PDR11* (Supplemental Figure S1). This is in line with previous reports (Régnacq *et al.*, 2001; Alimardani *et al.*, 2004) and indicates that *AUS1* and *DAN1* are both target genes of Sut1. Interestingly, *AUS1* expression levels were markedly reduced in *hem1Δ* cells overexpressing either *STE20*, *CLA4*, or *SKM1* (Figure 5A), whereas high levels of either *GIC2*, which, like the PAKs, is a Cdc42 effector (Brown *et al.*, 1997; Chen *et al.*, 1997), or *YCK1*, a kinase that is involved in cell polarity (Robinson *et al.*, 1993), did not affect *AUS1* expression (Figure 5A). Thus, the effects of PAKs are specific. Overexpression of *STE20* and *CLA4*, respectively, but not of *SKM1* lowered *DAN1* levels (Figure 5B). Finally, high levels of *CLA4* and *SKM1*, but not of *STE20* decreased *PDR11* expression (Figure 5C). In summary, all three PAKs down-regulate the expression of genes involved in sterol uptake, but with very different specificities. High levels of *GIC2* and
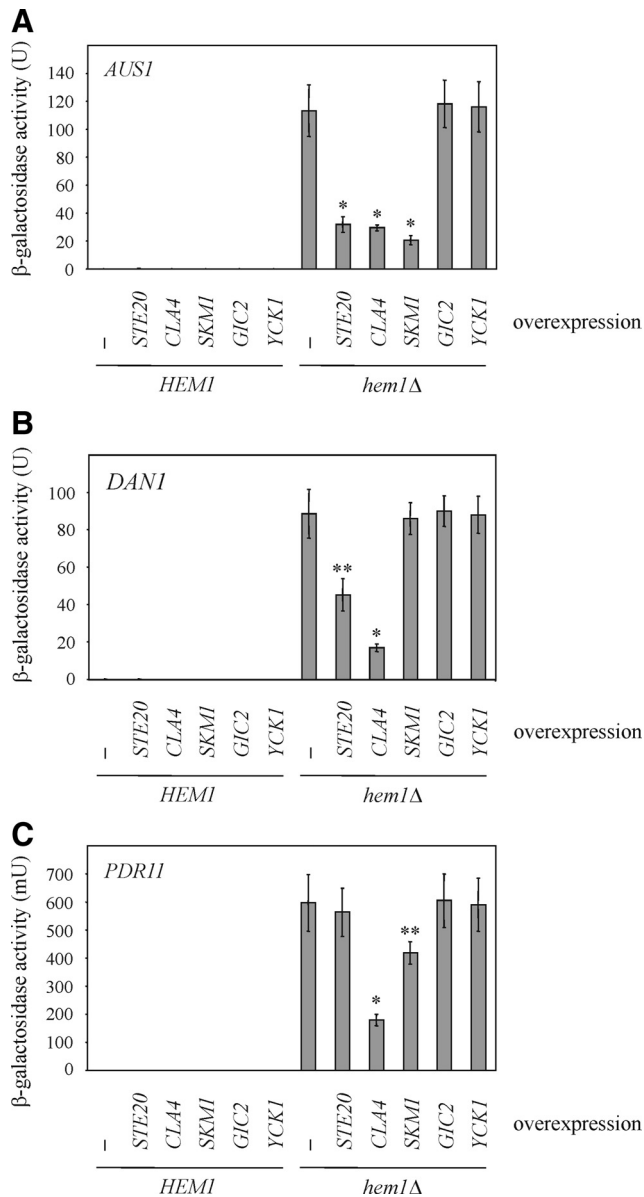
**Figure 5.** PAKs down-regulates the expression of *DAN1*, *AUS1*, and *PDR11*. (A) *AUS1* expression is regulated by Ste20, Cla4, and Skm1. The indicated strains harboring a plasmid on which the *lacZ* was fused to the promoter region of *AUS1* were grown in selective raffinose medium in the presence of ergosterol and Tween 80. The indicated genes were overexpressed for 18 h by the addition of galactose. Shown is the mean of β-galactosidase activity of at least six independent experiments with SD, *p < 0.0001, compared with *hem1*Δ cells without any overexpression. (B) Ste20 and Cla4 control the expression of *DAN1*. The indicated strains carrying a *DAN1-lacZ* plasmid were treated as described in A. Shown is the mean of β-galactosidase activity of at least six independent experiments. *p < 0.0001 and **p < 0.001, compared with *hem1*Δ cells without any overexpression. (C) *PDR11* expression is negatively regulated by Cla4 and Skm1. β-Galactosidase activity of the indicated strains harboring a *PDR11-lacZ* plasmid was determined in at least 6 independent experiments. *p < 0.0001 and **p < 0.005, compared with *hem1*Δ cells without any overexpression.

*YCK1* did not affect *DAN1* and *PDR11* expression levels (Figure 5, B and C).

Next, it was tested whether deletion of PAKs has an effect on the expression of *AUS1*, *DAN1* and *PDR11*. We observed
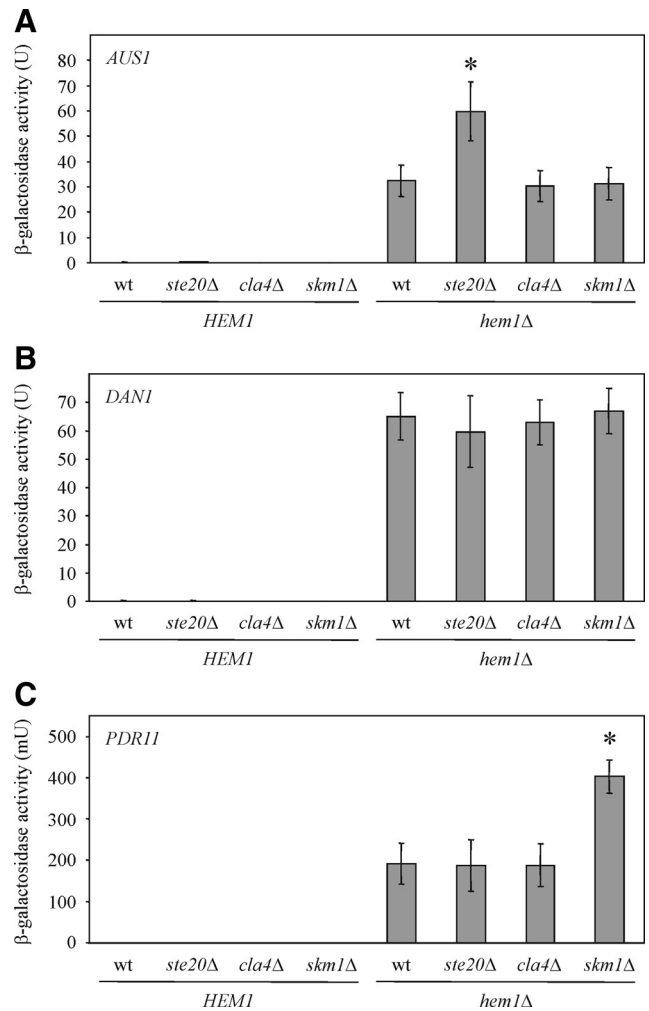


**Figure 6.** Expression of *AUS1*, *DAN1*, and *PDR11* in PAK deletion strains. (A) *STE20* deletion results in higher *AUS1* expression. The indicated strains carrying an *AUS1-lacZ* plasmid were grown in selective medium supplemented with ergosterol and Tween 80, and β-galactosidase activity was determined in at least six independent experiments. wt, wild type; *p < 0.001, compared with *hem1*Δ cells. (B) PAK deletion does not affect *DAN1* levels. Shown is the mean β-galactosidase activity of at least six independent experiments with SD of the indicated strains harboring *DAN1-lacZ* on a plasmid. (C) Cells lacking *SKM1* express higher *PDR11* levels. β-Galactosidase activity of the indicated strains harboring a *PDR11-lacZ* plasmid was determined in at least 6 independent experiments. *p < 0.0005, compared with *hem1*Δ cells.

higher *AUS1* levels in cells lacking *STE20* (Figure 6A), and *SKM1* deletion led to increased *PDR11* expression (Figure 6C). Notably, these results are consistent with the data obtained following PAK overexpression (Figure 5, A and C).

To test whether the observed effects of PAKs on gene expression are mediated by Sut1, expression studies were performed in cells lacking *SUT1* using *AUS1* as a reporter. The deletion of *SUT1* did not affect *AUS1* expression levels (Figure 7A), which is not surprising since *AUS1* expression is also under control of Upc2 and possibly other transcriptional regulators, which could compensate for the loss of *SUT1* (Wilcox *et al.*, 2002). High levels of either *STE20*, *CLA4*, or *SKM1* in the absence of *SUT1* led to no (Figure 7B) or only a minor reduction of *AUS1* levels (Figure 7, A and C). Thus, the PAK-mediated down-regulation of *AUS1* depends
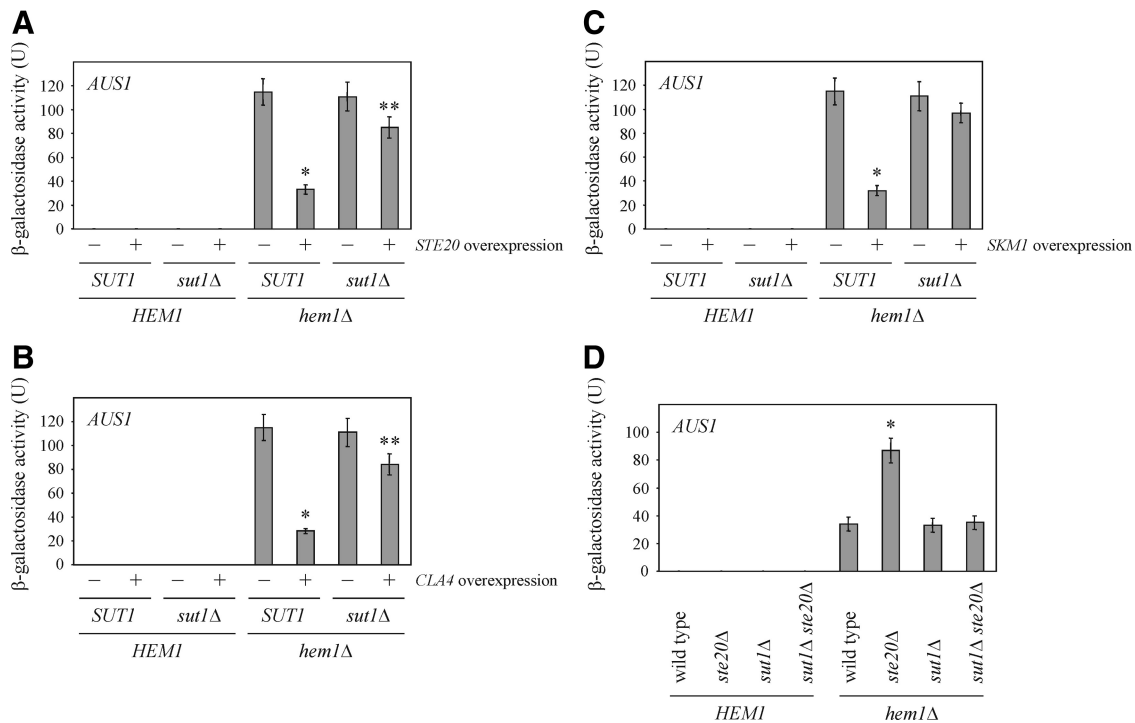
**Figure 7.** Down-regulation of *AUS1* expression by PAKs depends on *SUT1*. (A) Effect of *SUT1* deletion on *AUS1* levels following *STE20* overexpression. The β-galactosidase activity of the indicated strains was determined in six independent experiments as described in Figure 5A. *$p < 0.0001$ and **$p < 0.05$, compared with *hem1*Δ cells without *STE20* overexpression. (B) Regulation of *AUS1* expression by Cla4 depends on *SUT1*. Shown is the mean β-galactosidase activity of six independent experiments with SD of the indicated strains. *$p < 0.0001$ and **$p < 0.05$, compared with *hem1*Δ cells without *CLA4* overexpression. (C) The down-regulation of *AUS1* expression by Skm1 requires *SUT1*. β-Galactosidase activity of the indicated strains was determined in at least six independent experiments. *$p < 0.0001$, compared with *hem1*Δ cells without *SKM1* overexpression. (D) Effect of *SUT1* deletion on *AUS1* expression in cells lacking *STE20*. The β-galactosidase activity of the indicated strains was determined in at least six independent experiments as described in Figure 6A. *$p < 0.0005$, compared with *hem1*Δ cells.

mostly on *SUT1*. Possibly, PAKs to some extent also regulate Upc2 and maybe other transcriptional regulators of *AUS1*. This would explain the slight decrease of *AUS1* levels observed after overexpression of *STE20* and *CLA4* in cells lacking *SUT1*.

The role of Sut1 was also examined in cells deleted for *STE20*. The additional deletion of *STE20* in the absence of *SUT1* had no effect on *AUS1* expression levels (Figure 7D). This is consistent with the notion that specific inhibition of Sut1 by Ste20 results in a down-regulation of *AUS1* expression. In *ste20*Δ cells, Sut1 seems to have a higher activity, which results in stronger *AUS1* expression, possibly due to the missing inhibition by Ste20. As mentioned above, the *sut1*Δ strain probably expresses *AUS1* at levels comparable to the wild type because of proteins such as Upc2, which are functionally redundant with Sut1. The fact that *AUS1* expression is unaltered in *sut1*Δ *ste20*Δ cells suggests that Ste20 negatively regulates for the most part Sut1 and not or only to a minor extent other transcriptional regulators like Upc2. Whereas the experiment in Figure 7D suggests that Sut1 activates *AUS1* expression in a manner that is antagonized by Ste20, the experiments in Figure 7, A–C, shows that overexpression of PAKs inhibits *AUS1* expression in a manner that requires *SUT1*. It seems that Sut1 negatively regulates *AUS1* expression, a process that is promoted by high PAK levels. Importantly, in both models the effect on *AUS1* expression would be the same but it is not clear how these two different modes of action can be reconciled. Unfortunately, it is not known how Sut1 controls the expression of

genes such *AUS1*. Thus, it is even less clear how PAKs could regulate Sut1 activity.

Next, the mechanisms by which PAKs contribute to the regulation of gene expression were further characterized. For these experiments we focused on the regulation of *AUS1* by Ste20. Whereas overexpression of wild-type *STE20* decreased *AUS1* levels, overexpression of a mutated *STE20* lacking a major part of the NLS but leaving the BR domain intact (*STE20*$^{\Delta272-284}$) did not affect *AUS1* (Figure 8A). Thus, the nuclear localization of Ste20 is essential for its role in the control of gene expression. High levels of *STE20*$^{K649A}$, an allele that lacks kinase activity (Wu *et al.*, 1995), resulted in a minimal decrease of *AUS1* expression, but this effect, which is possibly due to minute residual kinase activity, was much weaker than after overexpression of wild-type *STE20* (Figure 8A). Thus, Ste20 kinase activity is required for the regulation of *AUS1* expression. Notably, wild-type *STE20* and the described mutant alleles were expressed at comparable levels as confirmed by immunoblotting (Figure 8B). Therefore, the observed effects are not due to different amounts of Ste20 protein. We also examined phenotypes of *STE20* mutant alleles when expressed from the native *STE20* promoter. Deletion of *STE20* led to an increased *AUS1* expression in the *hem1*Δ background (Figure 6A). Therefore, we brought back wild-type and mutated versions of *STE20* under control of its own promoter into the *ste20*Δ *hem1*Δ strain. As expected *STE20* reduced the expression of *AUS1* (Figure 8C). In contrast, the kinase dead derivative *STE20*$^{K649A}$ and the NLS mutant *STE20*$^{\Delta272-284}$ had no effect

## A



*AUS1*

β-galactosidase activity (U)

overexpression

*HEM1*    *hem1Δ*

## B

*HEM1*    *hem1Δ*

overexpression

α-HA

α-Cdc11

## C

*AUS1*

β-galactosidase activity (U)
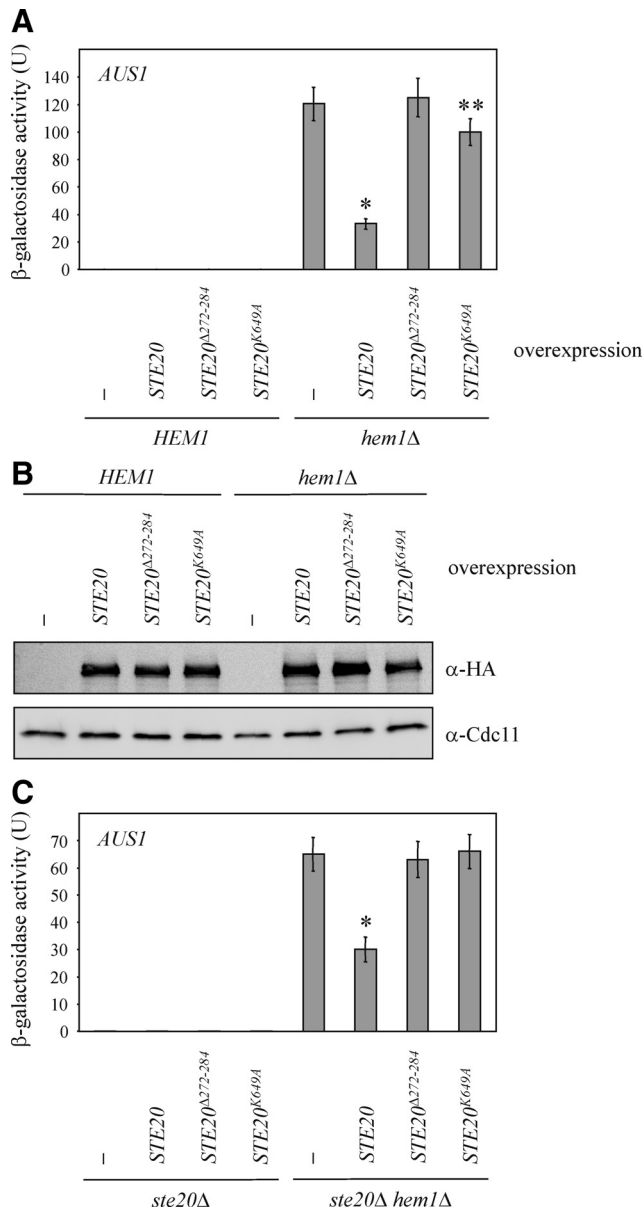
*ste20Δ*    *ste20Δ hem1Δ*

**Figure 8.** The regulation of *AUS1* expression requires kinase activity and nuclear localization of Ste20. (A) Wild-type *STE20* and mutant versions were overexpressed in the indicated strains as described in Figure 5A. Shown is the mean β-galactosidase activity of at least six independent experiments with SD, *$p < 0.0001$ and **$p < 0.05$, compared with *hem1Δ*cells without *STE20* overexpression. (B) Wild-type *STE20* and its mutant alleles are expressed at comparable levels. The indicated strains were incubated with galactose for 18 h. Cells were lysed and equal amounts of protein extract were analyzed by immunoblotting. An N-terminal 3HA tag allowed the detection of Ste20. Cdc11 was used as loading control. (C) Wild-type and mutant alleles of *STE20* expressed from the native *STE20* promoter were integrated into *ste20Δ* and *ste20Δ hem1Δ* cells. *AUS1* expression was determined in at least six independent experiments as described in Figure 6A. *$p < 0.0005$, compared with *ste20Δ hem1Δ* cells.

on *AUS1* levels (Figure 8C), suggesting that Ste20 kinase activity and nuclear localization are required for the inhibition of *AUS1* expression.

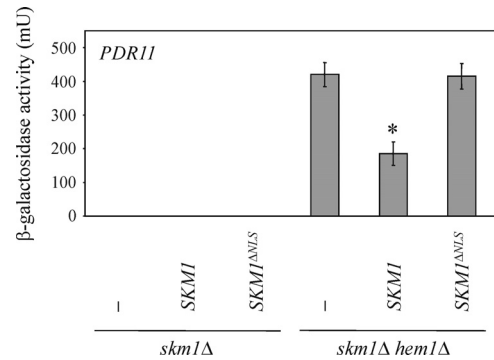In a similar experiment, we tested whether the NLS of Skm1 plays a role in the down-regulation of sterol uptake



*PDR11*

β-galactosidase activity (mU)

*skm1Δ*    *skm1Δ hem1Δ*

**Figure 9.** The down-regulation of *PDR11* expression requires nuclear localization of Skm1. *SKM1* and *SKM1*^ΔNLS, respectively, were integrated into *skm1Δ* and *skm1Δ hem1Δ* cells. *PDR11* expression was determined in at least six independent experiments as described in Figure 6C. *$p < 0.0005$, compared with *skm1Δ hem1Δ* cells.

genes. As shown in Figure 6C, *SKM1* deletion results in an up-regulation of *PDR11*. Integrating wild-type *SKM1* into the *skm1Δ hem1Δ* double mutant reduced *PDR11* expression to normal levels, whereas *SKM1*^ΔNLS had no effect (Figure 9). Thus, nuclear localization of Skm1 seems to be important for the down-regulation of *PDR11*.

### The regulation of AUS1 Expression Is Independent of Ste20 MAPK Signaling

Ste20 controls the expression of numerous genes by the activation of MAPK cascades regulating mating, filamentous growth, and the hyperosmotic response (Roberts and Fink, 1994; O'Rourke and Herskowitz, 1998; Raitt *et al.*, 2000). All three MAPK pathways share the MAPK kinase kinase Ste11, which is a target of Ste20 and essential for Ste20-dependent activation of MAPK cascades (Roberts and Fink, 1994; Wu *et al.*, 1995). To examine whether the Ste20-mediated transcriptional changes observed by us depend on MAPK signaling, we determined *AUS1* expression in cells lacking *STE11*. The decrease of *AUS1* levels after *STE20* overexpression was independent of *STE11* (Figure 10A). Thus, Ste20 seems to regulate gene expression by two distinct mechanisms: the activation of MAPK cascades and a Sut1-dependent pathway. Because the down-regulation of *AUS1* required nuclear Ste20 (Figure 8, A and C), we tested whether the nuclear localization of Ste20 was also necessary for its role in filamentous growth and mating. As described previously, Ste20 is essential for haploid filamentous growth and the formation of a mating projection in response to pheromone (Leberer *et al.*, 1992; Roberts and Fink, 1994) (Figure 10, B and C). Importantly, we observed normal filamentous growth for cells with Ste20 lacking the NLS (Figure 10B). These cells also responded normally to mating pheromone by arresting in G1 phase and formation of a mating projection (Figure 10C).

Together, Ste20 controls gene expression by two different mechanisms. MAPK cascades depend on Ste11, but do not require nuclear Ste20. In contrast, the Sut1-mediated pathway relies on nuclear Ste20, but is independent of Ste11.

### Ste20, Cla4, and Skm1 Negatively Regulate Sterol Uptake

Because Ste20, Cla4, and Skm1 down-regulate the expression of genes involved in sterol influx, it was tested whether individual deletion of either *STE20*, *CLA4*, or *SKM1* affects sterol uptake. To this end, heme-deficient wild-type and deletion strains were grown in the presence of [$^{14}$C]cholesterol, and uptake and esterification were quantified by TLC
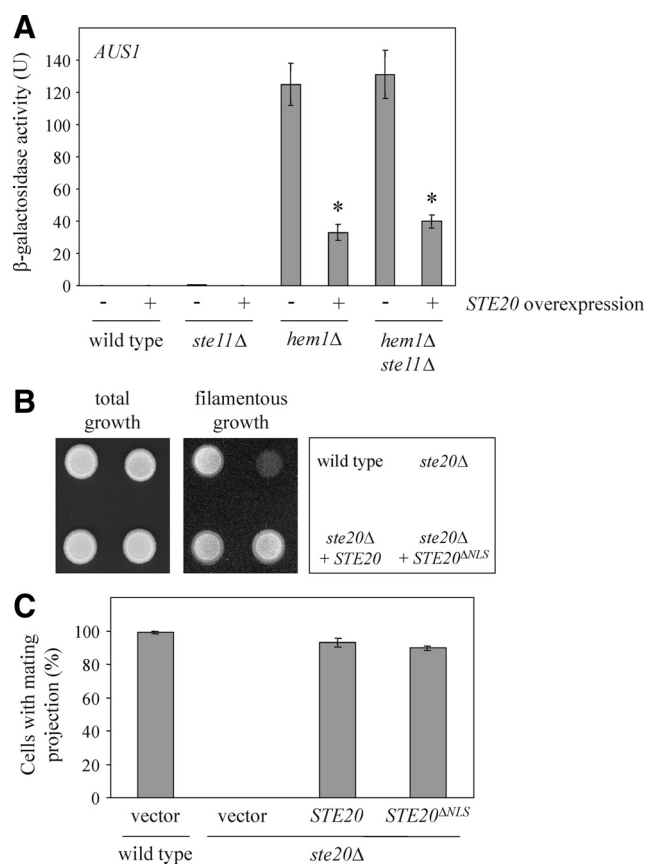
**Figure 10.** Ste20 regulates gene expression by two distinct mechanisms. (A) The down-regulation of *AUS1* expression is independent of the MAPK kinase kinase Ste11. The β-galactosidase activity was determined in eight independent experiments as described in Figure 5A. *$p < 0.0001$, compared with *hem1*Δ cells without *STE20* overexpression. (B) Haploid invasive growth does not require nuclear localization of Ste20. Cells ($10^5$) of the indicated strains were spotted on a YPD plate and incubated for 2 d at 30°C. Pictures were taken before and after gentle rinsing with water. (C) The NLS of Ste20 is not necessary for the formation of a mating projection. Exponentially growing cells of the indicated strains were incubated in selective medium with 1 μg/ml α-factor for 3 h. Cells were then fixed with formaldehyde and the percentage of cells with a mating projection was determined. Given is the mean of three independent experiments with SD bars (n > 100 for each experiment).

analysis of radiolabeled lipids. Consistent with the gene expression data, increased levels of free and esterified sterol were observed for *ste20*Δ, *cla4*Δ, and *skm1*Δ (Figure 11A). Sterol uptake was also analyzed in a strain that simultaneously overexpresses *STE20*, *CLA4*, and *SKM1*. In these cells, sterol import was markedly reduced compared with the wild type (Figure 11B). Thus, PAKs have a negative effect on sterol uptake. Finally, it was tested whether the inhibition of sterol import by Ste20 depends on nuclear localization of this PAK. Sterol uptake of cells expressing *STE20*ΔNLS from its own promoter was indistinguishable from the wild type (Supplemental Figure S2), suggesting that Ste20 may also have functions in sterol uptake that do not require nuclear translocation (see *Discussion*).

## DISCUSSION

Kinases of the PAK-family have a well established role in cell polarization (Hofmann *et al.*, 2004). Budding yeast ex-

presses three PAKs: Ste20, Cla4, and Skm1. To gain insight into molecular mechanisms of Ste20 action, we screened for interactors of Ste20 using the split-ubiquitin technique (Tiedje *et al.*, 2007). Here, we show that Sut1, a transcriptional regulator that controls sterol uptake (Bourot and Karst, 1995; Ness *et al.*, 2001), forms a complex with Ste20. By using pull-down assays, we demonstrate that not only Ste20 but also Cla4 and Skm1 bind to Sut1. Sut1 localizes exclusively to the nucleus, even after strong overexpression (data not shown) (Ness *et al.*, 2001). Thus far, Ste20 and Cla4 have been shown to localize mainly to the plasma membrane at sites of polarized growth and to the cytoplasm, whereas the localization of Skm1 has not been reported previously (Peter *et al.*, 1996; Leberer *et al.*, 1997; Holly and Blumer 1999). In this work, we show strong nuclear enrichment of Skm1 and we reveal an NLS. Furthermore, we identify an NLS for Ste20. The fact that a mutated Ste20, which lacks the entire NLS or a major part of it, is excluded from the nucleus under normal growth conditions, suggests that at least a small amount of Ste20 is present in the nucleus at any time. Consistently, in few cells, we observed a nuclear enrichment of wild-type Ste20. Cla4 does not contain an obvious NLS and the full-length protein is not enriched in the nucleus. Nevertheless, we observed that several Cla4 fragments that lacked the PH domain translocated into the nucleus. The PH domain is necessary for membrane association of Cla4 by binding to phosphoinositides (Wild *et al.*, 2004). We suggest that similar to Ste20, Cla4 that is not associated with membranes can localize to the cytoplasm and the nucleus. Therefore, it seems likely that the interaction between Sut1 and PAKs takes place in the nucleus. This notion is confirmed by our observation that the down-regulation of the Sut1 target *AUS1* by Ste20 and the inhibition of *PDR11* expression by Skm1 depends on the nuclear localization of these PAKs.

The expression of *AUS1*, *DAN1*, *PDR11*, and of other genes contributing to sterol uptake is repressed under aerobic conditions. In anaerobiosis, transcriptional regulators such as Sut1 and Upc2 allow expression of these genes, which is required for sterol uptake under these conditions (Régnacq *et al.*, 2001; Wilcox *et al.*, 2002; Alimardani *et al.*, 2004). Here, we demonstrate that PAKs negatively regulate the expression of genes involved in sterol influx. Interestingly, we observed very different expression profiles for Ste20, Cla4, and Skm1. Ste20 regulates the expression of *AUS1* and *DAN1*, Skm1 controls *AUS1* and *PDR11*, whereas Cla4 inhibits the expression of *AUS1*, *DAN1*, and *PDR11*. The reason for these different specificities is not clear.

Importantly, we demonstrate for all PAKs that the down-regulation of gene expression depends for the most part on *SUT1*. Because *AUS1* and *DAN1* are target genes of Sut1 and PAKs form a complex with Sut1, it seems very likely that PAKs control gene expression via Sut1. Nevertheless, it cannot be excluded that PAKs mediate their effects on transcription through other factors such as Upc2 or the less well characterized Ecm22 (Shianna *et al.*, 2001) as well. *PDR11* is also regulated by Cla4 and Skm1, but its transcription is controlled by Upc2 and there is no evidence that *PDR11* is a target of Sut1. Further, we observed a minor reduction of *AUS1* levels in the absence of *SUT1* after overexpression of either *STE20* or *CLA4*. Regulation of Upc2 or other factors by Ste20 and Cla4 could account for these weak effects on *AUS1* expression. We observed that sterol uptake in contrast to the regulation of *AUS1* expression is independent of the Ste20 NLS. The reason for this discrepancy is not clear. However, sterol uptake is a more complex process than the transcription of an individual gene. Possibly, Ste20 contributes also to sterol uptake by other mechanisms. Ste20
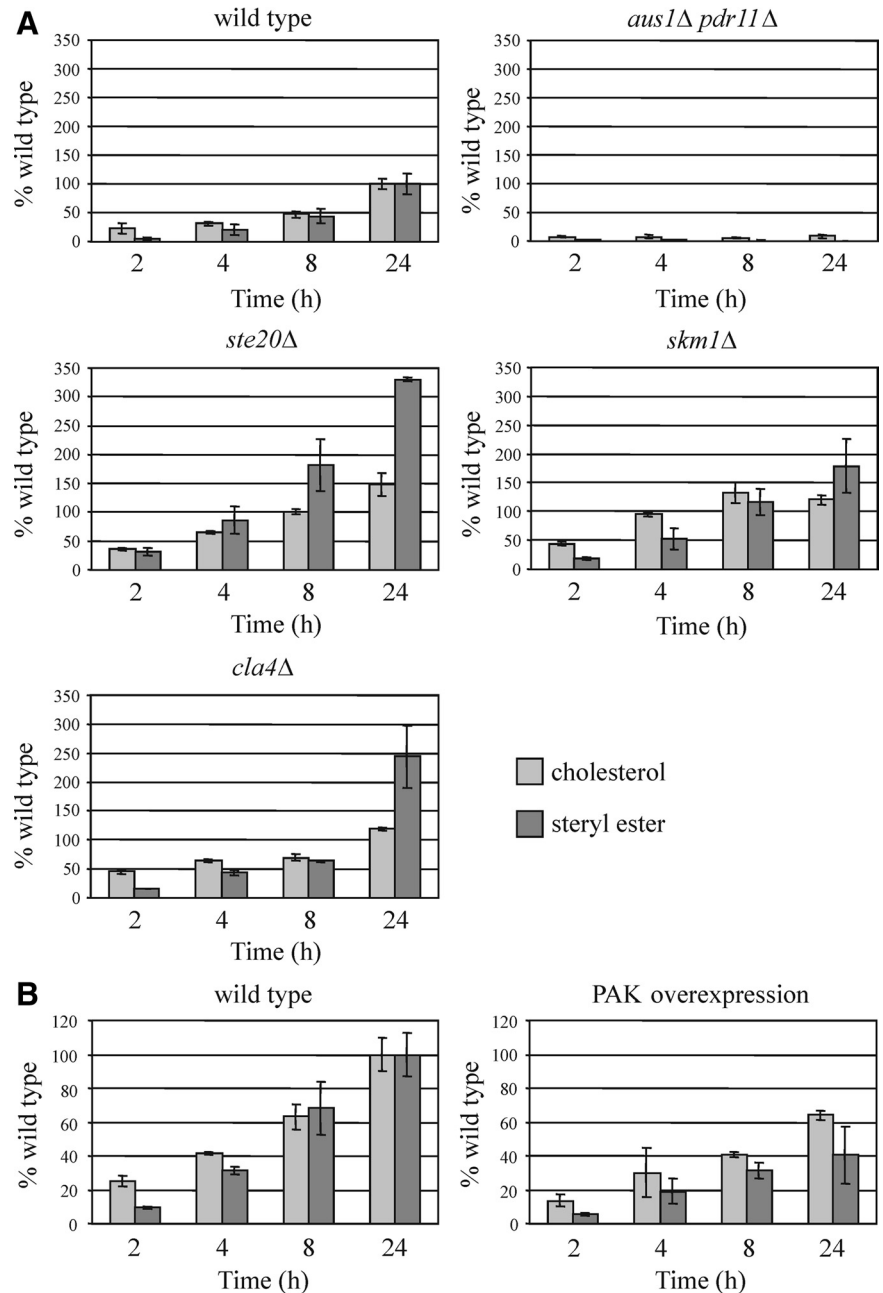
**Figure 11.** Ste20, Cla4, and Skm1 negatively regulate sterol uptake. (A) Ste20, Skm1, and Cla4 overaccumulate free sterols. Heme-deficient cells of the indicated genotype were cultured in media containing [$^{14}$C]cholesterol for the indicated period of time. Cells were collected, and lipids were isolated, separated by TLC, and quantified by radioscanning. Data are normalized to free sterols and steryl ester levels in wild-type cells after 24 h of uptake. Values are means and SD of two independent experiments. The *aus1Δ pdr11Δ* mutant was used as negative control. (B) Overexpression of PAKs results in a decreased sterol import. For PAK overexpression, *SKM1* and *CLA4* were placed under control of the *GAL1* promoter by genomic integration. These cells also harbored *STE20* expressed from the *GAL1* promoter on a plasmid. Wild-type cells carried the corresponding empty vector. Both strains were grown in SC-Ura medium supplemented with galactose and [$^{14}$C]cholesterol. Sterols and steryl ester were analyzed as described in A.

could control the activity of transcriptional regulators such as Upc2 and Ecm22, which localize to the cytoplasm as well (Marie *et al.*, 2008).

Thus far, the mechanisms of sterol uptake are only poorly understood. It seems that the transcriptional regulators involved in this process act redundantly, but very little is known about Ecm22 and it is not clear how Sut1 promotes the expression of *AUS1* and *DAN1*. Therefore, it is difficult to establish the mechanisms of inhibition of Sut1 and possibly other transcriptional regulators by PAKs. The down-regulation of *AUS1* expression requires Ste20 kinase activity. Nevertheless, we did not observe Sut1 phosphorylation by PAKs using in vivo and in vitro kinase assays (data not shown). Thus, it is conceivable that PAKs control transcriptional regulators via an unknown protein.

The control of gene expression by Ste20, Cla4, and Skm1 described here is clearly distinct from the regulation of transcription by Ste20 via MAPK modules as reported previously. During mating, filamentous growth and in response to hyperosmolarity Ste20 activates the MAPK kinase kinase Ste11 by phosphorylation, which eventually results in a change of the transcriptional pattern of numerous genes (Roberts and Fink, 1994; Wu *et al.*, 1995; O'Rourke and Herskowitz, 1998; Raitt *et al.*, 2000). As demonstrated for *AUS1*, the Sut1-mediated regulation of expression is independent of Ste11. Furthermore, it requires nuclear localization of Ste20. In contrast, the MAPK pathways promoting filamentation and the formation of a mating projection are independent of nuclear Ste20, but Ste11 is essential for these processes. Thus, the two mechanisms seem to be independent.

It has been reported that Ste20 translocates into the nucleus during hydrogen peroxide-induced cell death and directly phosphorylates histone H2B (Ahn *et al*., 2005). This presumably results in chromatin condensation. However, it has not been examined whether Ste20 alters gene expression under these conditions. Whether the inhibition of a transcriptional regulator involved in sterol uptake by Ste20 also involves chromatin modification as suggested for hydrogen peroxide-induced cell death remains to be tested.

Importantly, we demonstrated that the control of genes involved in sterol import mediated by Ste20, Cla4, and Skm1 affects sterol uptake. As expected from their role as negative regulators, the deletion of either *STE20*, *CLA4* or *SKM1* results in an increased sterol influx and subsequent esterification. Furthermore, sterol uptake was markedly reduced in cells overexpressing all three PAKs.

Our data raise the question why Ste20, Cla4, and Skm1 regulate sterol uptake. The fact that all three PAKs are involved in this process, suggests that the control of sterol influx is crucial for the cell and possibly for cell polarization. Previously, we could show that Ste20 binds to Erg4, Cbr1, and Ncp1, which all catalyze important steps in sterol biosynthesis (Tiedje *et al*., 2007). Interestingly, these proteins are also involved in bud site selection, apical bud growth, mating, filamentous growth, and exit from mitosis (Ni and Snyder, 2001; Keniry *et al*., 2004; Tiedje *et al*., 2007). These observations highlight the importance of sterol synthesis for cell polarization. Because Ste20 plays a crucial role in all these processes as well, it seems likely that Ste20 and sterol biosynthetic proteins act in the same pathway(s). In the absence of oxygen, sterols cannot be synthesized and cells completely depend on import of sterols from the extracellular medium. Under these conditions, control of sterol uptake may be as important for cell polarization as sterol biosynthesis in aerobiosis. Therefore, it is not surprising that PAKs regulate sterol uptake. Importantly, Cla4 not only has a negative effect on sterol uptake but also down-regulates sterol biosynthesis and storage (our unpublished data). Thus, it seems that Cla4 modulates sterol homeostasis under aerobic conditions and that all PAKs are involved in sterol import under anaerobiosis. In addition, homologues of oxysterol-binding proteins, a family of proteins, which regulate synthesis and transport of sterols, were found to participate in Cdc42-dependant cell polarity (Kozminski *et al*., 2006). How sterols contribute to cell polarization has been discussed controversially (Pichler and Riezman, 2004; Alvarez *et al*., 2007), and the elucidation of underlying molecular mechanisms will require further work.

In summary, we describe here a novel function for all three PAKs. They control transcription in the nucleus, which results in a change of sterol influx rate.

## ACKNOWLEDGMENTS

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