The zinc cluster proteins Upc2 and Ecm22 promote filamentation in Saccharomyces cerevisiae by sterol biosynthesis-dependent and -independent pathways

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Summary

The transition between a unicellular yeast form to multicellular filaments is crucial for budding yeast foraging and the pathogenesis of many fungal pathogens such as Candida albicans. Here, we examine the role of the related transcription factors Ecm22 and Upc2 in Saccharomyces cerevisiae filamentation. Overexpression of either ECM22 or UPC2 leads to increased filamentation, whereas cells lacking both ECM22 and UPC2 do not exhibit filamentous growth. Ecm22 and Upc2 positively control the expression of FHN1, NPR1, PRR2 and sterol biosynthesis genes. These genes all play a positive role in filamentous growth, and their expression is upregulated during filamentation in an Ecm22/Upc2-dependent manner. Furthermore, ergosterol content increases during filamentous growth. UPC2 expression also increases during filamentation and is inhibited by the transcription factors Sut1 and Sut2. The expression of SUT1 and SUT2 in turn is under negative control of the transcription factor Ste12. We suggest that during filamentation Ste12 becomes activated and reduces SUT1/SUT2 expression levels. This would result in increased UPC2 levels and as a consequence to transcriptional activation of FHN1, NPR1, PRR2 and sterol biosynthesis genes. Higher ergosterol levels in combination with the proteins Fhn1, Npr1 and Prr2 would then mediate the transition to filamentous growth.

Introduction

Many fungal species form filaments in response to extracellular stimuli such as nutrient deprivation (Cullen and Sprague, 2012). In the budding yeast Saccharomyces cerevisiae, filamentation can be observed when cells are grown on solid medium with limited nutrients (Cullen and Sprague, 2012). Filamentation in haploid cells is also termed invasive growth and is triggered by the lack of a fermentable carbon source such as glucose (Cullen and Sprague, 2000). In diploids, filamentous growth is also called pseudohyphal growth and can be induced by low nitrogen levels (Gimeno et al., 1992). Under these conditions, round yeast cells become more elongated and do not separate following cytokinesis. Cells also attach to and penetrate the substratum they grow on. Together, these mechanisms allow cells to forage for nutrients. Several signalling cascades are critical for filamentous growth including a mitogen-activated protein kinase (MAPK) pathway, the cAMP-dependent protein kinase A pathway and the target of rapamycin (TOR) pathway (Cullen and Sprague, 2012). These signalling pathways regulate a complex network of transcription factors that includes Flo8, Mga1, Phd1, Sok2, Ste12 and Tec1 (Borneman et al., 2006). These transcription factors alter the gene expression pattern which then drives the transition to filamentous growth.

Sut1, a transcription factor of the Zn(II)2Cys6 family, which is also known as zinc cluster proteins (Schjerling and Holmberg, 1996; Ness et al., 2001), plays an important role in filamentation of both haploids and diploids (Foster et al., 2013). During vegetative growth, Sut1 represses the expression of the genes GAT2, HAP4, MGA1, MSN4, NCE102, PRR2, RHO3 and RH05, which are involved in the switch to filamentous growth. During filamentation, a MAPK pathway activates the transcription factor Ste12 (Liu et al., 1993; Roberts and Fink, 1994), which lowers SUT1 expression (Foster et al., 2013). As a consequence, the repression of GAT2, HAP4, MGA1, MSN4, NCE102, PRR2, RH03 and RH05 is relieved, and the corresponding gene products induce filamentation. SUT1 has a paralogue, SUT2 (Ness et al., 2001; Byrne and Wolfe, 2005), which is not very well characterized. As for SUT1, overexpression of SUT2 leads to inhibition of haploid invasive growth (Rützler et al., 2004; Foster et al., 2013). However, the underlying molecular mechanisms are not known for Sut2.

Sut1 and Sut2 were originally identified as regulators of sterol uptake (Bourot and Karst, 1995; Ness et al., 2001). Under anaerobic conditions, ergosterol, the predominant sterol in yeast, cannot be synthesized because this...
process requires oxygen, and sterols are therefore imported from the extracellular medium (Jacquier and Schneiter, 2012). In the absence of oxygen, Sut1 upregulates the expression of Aus1 and Dan1, which mediate sterol uptake (Régnaqc et al., 2001; Alimardani et al., 2004).

Sterol uptake is also regulated by Upc2 and its paralogue Ecm22, which like Sut1 and Sut2, are members of the zinc cluster protein family (Schjerling and Holmberg, 1996; Crowley et al., 1998; Shianna et al., 2001). Like Sut1, Upc2 induces expression of AUS1 and DAN1, and another gene involved in sterol uptake, PDR11, under anaerobic conditions (Abramova et al., 2001; Wilcox et al., 2002). In addition, Upc2 seems to regulate the expression of nearly a third of anaerobically induced genes (Kwast et al., 2002). The role of Ecm22 under anaerobic conditions and sterol import is less clear. However, Ecm22 seems to induce DAN1 expression in the absence of oxygen (Davies and Rine, 2006).

Ecm22 and Upc2 also regulate sterol biosynthesis (Vik and Rine, 2001). Both proteins bind to sterol regulatory elements in the promoter of ergosterol biosynthesis (ERG) genes (Vik and Rine, 2001). Under normal laboratory growth conditions, Ecm22 seems to be the main activator, whereas when sterols are depleted, Ecm22 is replaced by Upc2 (Davies et al., 2005). It was shown that Upc2 acts as a sterol sensor (Marie et al., 2008; Yang et al., 2015). Under sterol-rich conditions, Upc2 is predominantly cytosolic and directly binds to ergosterol. When sterol levels drop, ergosterol dissociates from Upc2, which leads to the translocation of Upc2 to the nucleus where it induces expression of ERG genes.

In this study, we demonstrate that Ecm22 and Upc2 are important regulators of filamentation. In contrast to Sut1 and Sut2, which repress filamentous growth, Ecm22 and Upc2 are activators of filamentation. Ecm22 and Upc2 regulate the expression of PRR2, NPR1, FHNL1 and ERG genes, which are all involved in filamentous growth, and upregulated in an Ecm22/Upc2-dependent manner during filamentation. ERG11 expression is also under control of several transcription factors that play a crucial role in filamentation, suggesting that ergosterol biosynthesis is critical for filamentous growth. We further show that UPDC2 transcription is regulated by Sut1 and Sut2 and that UPC2 levels increase during filamentation. Thus, zinc cluster proteins not only have overlapping functions in filamentation, they also regulate each other.

Results

Sut2 regulates the expression of Sut1 target genes

We have previously shown that the zinc cluster protein Sut1 regulates filamentous growth (Foster et al., 2013). SUT1 overexpression using a multicopy plasmid and the strong constitutive PMA1 promoter leads to inhibition of haploid invasive growth and diploid pseudohyphal growth (Foster et al., 2013). We therefore tested whether overexpression of SUT2, a parologue of SUT1, has the same effect. Increased levels of SUT2 indeed led to the inhibition of haploid invasive growth (Fig. 1A), which is consistent with a previous observation (Rützler et al., 2004). Diploid cells overexpressing SUT2 also failed to undergo the transition to filamentous growth (Fig. 1B), suggesting that Sut2 is equally important for filamentation in both cell types. However, for this study we decided to focus on haploid cells.

The transcription factor Sut1 regulates filamentation through its targets GAT2, HAP4, MGA1, MSN4, NCE102, PRR2, RHO3 and RHO5 (Foster et al., 2013). Under optimal growth conditions, Sut1 represses the expression of these genes, whereas under filamentation-inducing conditions, this repression is lifted. Increased expression of the Sut1 targets then contributes to filamentation. Because of the similarity between Sut1 and Sut2, we tested whether Sut2 also acts as a repressor for Sut1 target genes. We have shown before that Sut2 negatively regulates the expression of NCE102, PRR2 and RHO5 (Blanda and Höfken, 2013). SUT2 overexpression also decreased the levels of GAT2, HAP4, MGA1, MSN4 and RHO3 (Fig. 1C). Increasing SUT2 levels did not affect the expression of other genes such as RHO4 (Fig. 1C), indicating that the observed downregulation is specific.

SUT1 expression is negatively regulated by Ste12 (Foster et al., 2013), a key transcription factor for the switch to filamentous growth (Liu et al., 1993; Roberts and Fink, 1994). As a consequence of Ste12 activation during filamentation, SUT1 levels decrease and expression of Sut1 targets increases. SUT2 levels are regulated in the same way. Overexpression of STE12 reduces SUT2 expression (Fig. 1D). Taken together, Sut1 and Sut2 seem to play the same role in filamentation. They are both negative regulators, they control expression of the same genes, and their expression is regulated by Ste12.

Ecm22 and Upc2 are positive regulators of filamentation

As overexpression of SUT1 and SUT2 leads to inhibition of filamentous growth, we asked whether Ecm22 and Upc2, which are like Sut1 and Sut2 zinc cluster proteins that regulate sterol import (Bourot and Karst, 1995; Schjerling and Holmberg, 1996; Crowley et al., 1998; Ness et al., 2001; Shianna et al., 2001), also control filamentous growth. Rather unexpectedly, overexpression of either ECM22 or UPC2 resulted in much stronger haploid invasive growth compared with the wild type (Fig. 2A). Thus, Ecm22 and Upc2 are activators of filamentation, unlike Sut1 and Sut2, which function as inhibitors. Expression levels of the filamentation marker FLO11 were also
considerably higher in cells overexpressing ECM22, and even more increased in the UPC2 overexpression strain (Fig. 2B). Higher levels of either ECM22 or UPC2 in diploid cells led to a marked increase in pseudohyphal growth (Fig. 2C), indicating that Ecm22 and Upc2 regulate filamentation in a positive manner in haploids and diploids. Nevertheless, for the further characterization of Ecm22 and Upc2, we focused on haploid cells.

Next, it was tested whether the deletion of ECM22 or UPC2 affects invasive growth. No phenotype was observed for single mutants (Fig. 2D). In contrast, simultaneous deletion of ECM22 and UPC2 resulted in a strong defect in invasive growth (Fig. 2D). In line with this observation, expression of the filamentation marker FLO11 was decreased in ECM22Δ upc2Δ cells but not in the corresponding single deletion strains (Fig. 2E). In summary, these data indicate that Ecm22 and Upc2 have an important and redundant role in filamentation.

Identification of target genes of Ecm22 and Upc2 that play a role in filamentation

Sut1, Ecm22, Upc2 and possibly Sut2 seem to control the expression of a similar set of genes for sterol uptake
under anaerobic conditions, including \textit{AUS1} and \textit{DAN1} (Régnacq \textit{et al}., 2001; Wilcox \textit{et al}., 2002; Alimardani \textit{et al}., 2004; Davies and Rine, 2006). It is therefore conceivable that Ecm22 and Upc2 also regulate the expression of Sut1/Sut2 target genes for filamentous growth.

However, levels of \textit{GAT2}, \textit{HAP4}, \textit{MGA1}, \textit{MSN4}, \textit{NCE102}, \textit{RHO3} and \textit{RHO5} in the \textit{ecm22Δ upc2Δ} double mutant were indistinguishable from the wild type (data not shown). As an example, \textit{NCE102} expression was also tested in cells overexpressing either \textit{ECM22} or \textit{UPC2}.

\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig2.png}
\caption{Ecm22 and Upc2 are positive regulators of filamentous growth.}
A. Overexpression of \textit{ECM22} and \textit{UPC2} leads to increased haploid invasive growth. The wild type (PPY966) and the sterol import mutant \textit{aus1Δ pdr11Δ} (SHY68) carrying the indicated plasmids (pNEV-N, pTH408, pMC8) were spotted onto selective medium plates and were grown for 3 days at 30°C. Pictures were taken before (total growth) and after (invasive growth) rinsing with water. This was done early when filamentation just started in the wild type to demonstrate the stronger invasive growth of strains overexpressing \textit{ECM22} and \textit{UPC2}.

B. Overexpression of \textit{ECM22} and \textit{UPC2} leads to increased \textit{FLO11} levels. Wild-type cells (PPY966) harboring a plasmid on which \textit{lacZ} was fused to the \textit{FLO11} promoter (pSH23), and carrying the indicated plasmids (pNEV-N, pTH408, pMC8) were grown in selective medium. Shown is the average \(\beta\)-galactosidase activity with standard deviation of four independent cultures. *, \(P < 0.01\) compared with the wild type carrying an empty plasmid.

C. Overexpression of \textit{ECM22} and \textit{UPC2} results in increased diploid pseudohyphal growth. Wild-type cells (PC344) carrying the indicated plasmids (pNEV-N, pTH408, pMC8) were grown on low-nitrogen SLAD medium for 4 days at 30°C. This was done early when filamentation just started in the wild type to demonstrate the stronger pseudohyphal growth of strains overexpressing \textit{ECM22} and \textit{UPC2}.

D. Simultaneous deletion of \textit{ECM22} and \textit{UPC2} results in a defect in haploid invasive growth. The indicated strains (PPY966, MCY19, MCY21, THY760) were spotted onto YPD plates and were grown for 2 days at 30°C. Pictures were taken before (total growth) and after (invasive growth) rinsing with water.

E. Deletion of both \textit{ECM22} and \textit{UPC2} results in decreased \textit{FLO11} expression. \(\beta\)-galactosidase activity was determined for the indicated strains (PPY966, MCY19, MCY21, THY760) all carrying a \textit{FLO11-lacZ} plasmid (pSH213). Bars indicate the average with standard deviation of four independent cultures. *, \(P < 0.01\) compared with the wild type.

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Fig. 3. PRR2 and NPR1 expression is regulated by Ecm22 and Upc2.

A. Deletion of ECM22 and UPC2 leads to decreased PRR2 expression. β-galactosidase activity was determined for the indicated strains (PPY966, MCY19, MCY21, THY760) carrying a PRR2-lacZ plasmid (pHU37). Shown is the average β-galactosidase activity with standard deviation of four independent cultures. *, P < 0.01 compared with the wild type.

B. Overexpression of ECM22 and UPC2 leads to increased PRR2 expression levels. Wild-type cells (PPY966) harboring a PRR2-lacZ plasmid (pHU37) in combination with the indicated plasmids (pNEV-N, pTH408, pMC8) were grown in selective medium, and β-galactosidase activity was determined for four independent cultures. *, P < 0.01 compared with the wild type carrying an empty plasmid.

C. Deletion of ECM22 and UPC2 results in decreased NPR1 expression. β-galactosidase activity was determined for the indicated strains (PPY966, MCY19, MCY21, THY760 carrying pTH421) (n = 4). *, P < 0.01 compared with the wild type.

D. NPR1 expression is regulated by Ecm22, Upc2, Sut1 and Sut2. Cells harbored a NPR1-lacZ plasmid (pTH421) in combination with the indicated vectors (pNEV-N, pTH408, pMC8, pNF1, pMC10). Shown is the average β-galactosidase activity with standard deviation of four independent cultures. *, P < 0.01 compared with the wild type carrying an empty plasmid.

Again no effect was observed (data not shown). Thus, the expression of GAT2, HAP4, MGA1, MSN4, NCE102, RHO3 and RHO5 is not under the control of Ecm22 and Upc2, and an altered expression of these genes is not the cause of the filamentation phenotypes of the ecΔΔ upcΔΔ mutant and the ECM22 and UPC2 overexpression strains. Interestingly, expression of the Sut1/Sut2 target PRR2 is lowered in ecΔΔ upcΔΔ cells but not in the corresponding single mutants (Fig. 3A). Furthermore, PRR2 expression is strongly increased in cells overexpressing UPC2 and to a lesser extent in cells overexpressing ECM22 (Fig. 3B), indicating that PRR2 is a target of Upc2 and Ecm22.

We also analyzed the expression of NPR1, a paralogue of PRR2 (Byrne and Wolfe, 2005). Interestingly, expression patterns of NPR1 and PRR2 are quite similar. Reduced NPR1 levels were observed in the ecΔΔ upcΔΔ double mutant but not in cells lacking only one gene (Fig. 3C). Furthermore, NPR1 expression is increased in cells overexpressing either ECM22 or UPC2 and reduced in strains overexpressing SUT1 or SUT2 (Fig. 3D). NPR1 and PRR2 are thus the only genes with a potential role in filamentation that are not only regulated by Sut1 and Sut2 but also by Ecm22 and Upc2.

As mentioned above, NCE102 expression is not affected by deletion or overexpression of ECM22 or UPC2 (data not shown). Nevertheless, we analyzed FHN1, the functional homologue of NCE102 (Byrne and Wolfe, 2005; Loibl et al., 2010). ECM22 deletion had no effect on FHN1 expression, whereas UPC2 deletion led to lower FHN1 levels (Fig. 4A). This was further reduced in a strain lacking both ECM22 and UPC2. FHN1 expression is strongly increased in cells overexpressing ECM22, and even stronger in cells overexpressing UPC2 (Fig. 4B). In contrast, overexpression of either SUT1 or SUT2 had no effect on the expression of FHN1 (Fig. 4B). Thus, FHN1 expression is regulated by Ecm22 and Upc2 but not by Sut1 or Sut2, whereas its paralogue NCE102 is under control of Sut1 and Sut2 but not of Ecm22 and Upc2.

Next, we examined whether the newly identified targets of Ecm22 and Upc2 (PRR2, NPR1 and FHN1) play a role in filamentation. As shown before, PRR2 expression is strongly upregulated during haploid and diploid filamentation (Foster et al., 2013). Furthermore, PRR2 expression...
correlates with filamentation phenotypes. PRR2 levels are reduced in strains that have a filamentation defect such as the ecm22Δ upc2Δ double mutant and strains that overexpress either SUT1 or SUT2 (Fig. 3A) (Blanda and Höfken, 2013; Foster et al., 2013). In strains that are hyperfilamentous due to overexpression of either UP2C or ECM22, PRR2 expression levels are increased (Fig. 3B). Together these data strongly suggest that Prr2 plays an important role in filamentation. However, a PRR2 deletion strain does not display a filamentation defect (Fig. 5A) (Foster et al., 2013). Because PRR2 has a paralogue, NPR1 (Byrne and Wolfe, 2005), it is conceivable that no defect was observed for the prr2Δ strain because both genes have overlapping functions in filamentation.

We therefore examined filamentous growth of the npr1Δ prr2Δ double mutant and the npr1Δ mutant. Both strains had an equally strong defect in invasive growth (Fig. 5A), establishing a role for NPR1 in filamentation but not for PRR2. However, cells overexpressing PRR2 exhibited increased invasive growth (Fig. 5B), suggesting that PRR2 like its paralogue NPR1 are involved in filamentous growth.

Deletion of either FHN1 or NCE102 or both genes did not affect filamentous growth (data not shown) (Foster et al., 2013). However, as for PRR2, overexpression of either FHN1 or NCE102 resulted in increased invasive growth (Fig. 5B), suggesting that the corresponding proteins play a positive role in filamentous growth.

ECm22 and Upc2 control the expression of genes that are involved in ergosterol biosynthesis in the presence of oxygen, and sterol import from the extracellular medium under anaerobic conditions (Crowley et al., 1998; Shianna et al., 2001; Vik and Rine, 2001). It is therefore conceivable that sterol biosynthesis and/or uptake contribute to filamentation. However, as no sterol was added to the medium the cells grow on and penetrate, it is unlikely that invasive growth requires sterol import. Furthermore, an aus1Δ pdr11Δ double mutant, which is unable to import sterols (Wilcox et al., 2002), displays normal invasive growth (Fig. 2A) (Foster et al., 2013).

Finally, the hyperfilamentation phenotype of strains overexpressing either ECM22 or UPC2 is not affected in the sterol uptake mutant aus1Δ pdr11Δ (Fig. 2A). Together, these data suggest that under the conditions examined here, invasive growth does not require sterol import.

ERG genes are also important targets of Ec22 and Upc2 (Vik and Rine, 2001; Wilcox et al., 2002). We chose ERG3, ERG11 and NCP1 to analyze the role of ERG genes in Ec22/Upc2-mediated filamentation. Erg3 and Erg11 directly catalyse steps in the biosynthetic pathway (Kalb et al., 1987; Arthington et al., 1991), whereas Ncp1 transfers electrons to several Erg enzymes (Yoshida, 1991).
1988; Aoyama et al., 1989; Kelly et al., 1995). The expression of ERG3, ERG11 and NCP1 is downregulated in ecm22Δ upc2Δ cells but not in the corresponding single mutants (Fig. 6A), which is consistent with published data (Vik and Rine, 2001). Notably, overexpression of either SUT1 or SUT2 does not affect levels of ERG3, ERG11 or NCP1 (data not shown), suggesting that the expression of these genes is specifically regulated by Ecm22 and Upc2, and not by Sut1 and Sut2. Importantly, ERG3, ERG11 and NCP1 are all required for invasive growth (Fig. 6B), suggesting that sterol biosynthesis plays an important role in filamentation.

Ecm22 and Upc2 are the main regulators of ERG gene expression (Vik and Rine, 2001), and little is known about other transcriptional regulators. However, a global screen for binding sites of the key transcription factors for filamentation Flo8, Mga1, Phd1, Sok2, Ste12 and Tec1 revealed that promoter regions of many ERG genes contain binding sites for these factors (Borneman et al., 2006). To our knowledge, it has not been examined whether these transcription factors actually regulate the expression of ERG genes. As all six transcription factors examined by Borneman et al. (2006) associate with the ERG11 promoter, we further analyzed this link. Using chromatin immunoprecipitation (ChIP), we found that Flo8-3HA expressed from its own promoter binds to the ERG11 promoter (Fig. 6C). Flo8-3HA overexpressed from the GAL1 promoter (THY841) and cells expressing untagged FLO8 from their own promoter (PPY966) were grown in galactose medium and subjected to ChIP. The immunoprecipitates (IP) were tested for the presence of the ERG11 promoter region. As a control for the PCR, cell lysates were tested without any anti-HA precipitation.

Fig. 6. Sterol biosynthesis enzymes play an important role in filamentation.
A. Deletion of ECM22 and UPC2 results in decreased expression of ERG genes. β-galactosidase activity was determined for the indicated strains (PPY966, MCY19, MCY21, THY760 carrying pTH376, pTH379 or pSH24). Given is the average β-galactosidase activity with standard deviation (n = 4). *, P < 0.01 compared with the wild type.
B. ERG genes are required for invasive growth. The indicated strains (PPY966, THY784, THY827, MBY16) were spotted onto YPD plates and grown for 2 days. Pictures were taken before (total growth) and after (invasive growth) rinsing with water.
C. Flo8 binds to the ERG11 promoter. Cells expressing FLO8-3HA from the endogenous promoter (THY839), cells expressing 3HA-tagged FLO8 from the GAL1 promoter (THY841), and cells expressing untagged FLO8 from their own promoter (PPY966) were grown in galactose medium and subjected to ChIP. The immunoprecipitates (IP) were tested for the presence of the ERG11 promoter region. As a control for the PCR, cell lysates were tested without any anti-HA precipitation.
D. Regulation of ERG11 expression by transcription factors that promote filamentous growth. ERG11-lacZ (pTH379) expression was determined for the wild-type strain (PPY966) and cells overexpressing the indicated transcriptional regulators from the GAL1 promoter (THY768, THY769, THY771, THY762, THY767). Bars indicate the average with standard deviation of four independent cultures. *, P < 0.01 compared with the wild type.
Notably, these strains have been shown to display strongly increased filamentous growth (Foster et al., 2013). Thus, there is a clear correlation between ERG11 expression and filamentation.

As increased levels of SOK2 and TEC1 did not affect ERG11 expression (Fig. 6D), we also analyzed SOK2 and TEC1 deletion strains. ERG11 levels in sok2Δ and tec1Δ mutants were comparable with the wild type (data not shown). Thus, there is no evidence that Sok2 and Tec1 control ERG11 expression, but Flo8, Mga1, Phd1 and Ste12 regulate ERG11 expression in a positive manner. The fact that ERG11 expression is regulated by so many transcription factors that promote filamentation is a further indication that ERG11 and probably other ERG genes play a crucial role in filamentous growth.

Fig. 7. Expression of Ecm22/Upc2 targets increases during filamentation.
A. Expression of Ecm22/Upc2 target genes during filamentous growth. β-galactosidase activity was determined for the indicated genes (pTH376, pTH379, pSH24, pTH407, pTH421, pMC7) in cells (PPY966, MCY19, MCY21, THY760) grown for 14 h at 30°C on minimal medium plates lacking glucose. Cells grown in liquid minimal medium containing glucose served as reference. Shown is the average increase of four independent replicates with standard deviation. *, P < 0.01 compared with the wild type.
B. ERG11 expression increases only under conditions that induce filamentation. Wild-type cells (PPY966) carrying an ERG11-lacZ plasmid (pTH379) were either grown in liquid minimal medium with or without glucose, or alternatively cells were grown for 14 h on minimal medium plates with or without glucose. Shown is the average β-galactosidase activity with standard deviation (n = 4). *, P < 0.01 compared with cells grown in high-glucose liquid medium.
C. Erg11 and Prr2 protein levels increase during filamentation. Cells expressing either Erg11-9Myc or Prr2-9Myc (THY837, SHY6) were grown in liquid high-glucose minimal medium or on plates lacking glucose. Cells were lysed and equal amounts were analyzed by immunoblotting using antibodies against the Myc epitope and Cdc11 (loading control).
D. Ergosterol levels increase during filamentation. Sterols were extracted from wild type cells (PPY966) grown in liquid minimal medium with 2% glucose or from plates lacking glucose. Ergosterol levels were determined from three independent cultures. *, P < 0.01 compared with cells grown in high-glucose liquid medium.

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Targets of Ecm22 and Upc2 are upregulated during filamentation

The Ecm22/Upc2 target genes examined here are either essential for filamentation (NPR1, ERG3, ERG11 and NCP1) (Figs 5A and 6B) or at least play a positive role in this process (Prr2 and FHN1) (Fig. 5B). It therefore seems likely that their expression increases during filamentous growth. We have previously shown a strong increase of PRR2 expression under filamentation-inducing conditions (Foster et al., 2013). The other Ecm22/Upc2 targets ERG3, ERG11, NCP1, FHN1 and NPR1 were also all upregulated during filamentation, in contrast to the control RHO4 (Fig. 7A). This induction is not affected by the deletion of either ECM22 or UPC2 but
reduced in strains lacking both genes (Fig. 7A). These data suggest that Ecm22 and Upc2 are partly responsible for the upregulation but that other transcription factors are involved as well. The expression of the Ecm22/Upc2 target genes only increased when cells were grown on plates with limited nutrients, as shown here for ERG11 (Fig. 7B). In liquid medium without glucose, and on glucose-rich plates ERG11 was expressed at levels comparable with liquid medium containing glucose (Fig. 7B). Thus, gene expression correlates with filamentous growth that only occurs when cells are grown on solid medium with limited nutrients (Gimeno et al., 1992; Cullen and Sprague, 2000). We next examined whether altered transcription observed here results in changes at protein level. Erg11 levels were significantly higher in cells grown under filamentation-inducing conditions (Fig. 7C). This correlates well with the five- to sixfold increase of glucose (Fig. 7C). This effect was even more pronounced for Prr2, which was barely or not detectable in liquid cultures with glucose but strongly expressed in cells grown on plates without glucose (Fig. 7C). This correlates well with the five- to sixfold increase of ERG11 expression during filamentation determined by β-galactosidase assays (Fig. 7A and B), and a 90-fold increase for PRR2 that we observed previously using quantitative real-time PCR (Foster et al., 2013). As a consequence of higher ERG gene expression during filamentous growth, the ergosterol content could also increase. In fact, we observed significantly higher ergosterol levels in cells grown on plates with limited nutrients (Fig. 7D). In summary, targets of Ecm22 and Upc2 are upregulated at transcriptional and protein level during filamentation. This probably results in physiological changes such as higher ergosterol levels.

Regulation of UPC2 expression

Upc2 has been shown to positively regulate its own expression (Abramova et al., 2001; Wilcox et al., 2002). We therefore tested the possibility that Ecm22, Sut1 and Sut2 are also involved in the regulation of UPC2 expression. Overexpression of UPC2 led to increased UPC2 levels (Fig. 8A), confirming UPC2 autoregulation that has been reported before (Abramova et al., 2001; Wilcox et al., 2002). Higher ECM22 levels had no effect on UPC2 expression, whereas overexpression of either SUT1 or SUT2 decreased UPC2 expression (Fig. 8A). Thus, UPC2 expression is positively regulated by Upc2, and in a negative way by Sut1 and Sut2. In contrast, ECM22 expression was not affected in cells overexpressing either ECM22, UPC2, SUT1 or SUT2 (data not shown).

As the expression of SUT1 and SUT2 is regulated by the transcription factor Ste12 (Foster et al., 2013) (Fig. 1D), it is tempting to speculate that Upc2 is indirectly regulated by Ste12. To test this hypothesis, we examined genetic interactions between STE12 and UPC2. Overexpression of UPC2 rescues the filamentation defect of the STE12 deletion strain (Fig. 8B). This is a highly specific interaction as increased ECM22 levels have no effect (Fig. 8B). This is consistent with the observation that the Ste12 targets Sut1 and Sut2 regulate the expression of UPC2 but not of ECM22 (Fig. 8A). We also found that STE12 overexpression suppresses the filamentation defect of the ecmm22Δ upc2Δ double mutant (Fig. 8C), which further strengthens the link between STE12 and UPC2.

Finally, we tested whether levels of ECM22 and UPC2 change during filamentation. The expression of ECM22 did not change under conditions that induce filamentous growth, whereas UPC2 levels increased during filamentation (Fig. 8D). Taken together, these data suggest that regulation of gene expression is an important control mechanism for Upc2 during filamentous growth. In contrast, Ecm22 seems to be regulated by a different unknown mechanism.

Discussion

Sut1, Sut2, Ecm22 and Upc2 are transcription factors of the zinc cluster protein family, and they all control sterol import under anaerobic conditions (Bourot and Karst, 1995; Schjerling and Holmberg, 1996; Crowley et al., 1998; Ness et al., 2001; Shianna et al., 2001). We have shown previously that Sut1 is also involved in filamentation (Foster et al., 2013). Here, we demonstrate that Ecm22, Upc2 and Sut2 play an important role in filamentation, too. Filamentation and sterol uptake seem to be regulated in a different manner. Overexpression or hyperactive alleles of SUT1, SUT2, ECM22 and UPC2 trigger sterol import, indicating a positive role for these factors in sterol uptake (Lewis et al., 1988; Bourot and Karst, 1995; Ness et al., 2001; Shianna et al., 2001). In contrast, Sut1 and Sut2 inhibit filamentation (Rützler et al., 2004; Foster et al., 2013), whereas Ecm22 and Upc2 play a positive role in filamentous growth. Furthermore, Sut1, Sut2, Ecm22 and Upc2 all seem to regulate the expression of similar genes for sterol uptake (Abramova et al., 2001; Régnacq et al., 2001; Wilcox et al., 2002; Alimardani et al., 2004), whereas Sut1/Sut2 and Ecm22/Upc2 largely regulate different sets of genes for filamentation (Fig. 9).

The expression of GAT2, HAP4, MGA1, MSN4, NCE102, RHO3 and RHO5 is under control of Sut1 and Sut2 (Blanda and Höfken, 2013; Foster et al., 2013) but not of Ecm22 and Upc2. Ecm22 and Upc2 specifically regulate the transcription of FHNI and the ERG genes. PRR2 and its parologue NPR1 are the only genes tested here that are regulated by all four transcription factors.

The Ecm22/Upc2 targets examined here are all either essential for filamentous growth or play at least an important role in this process. Furthermore, they are upregu-
lated during filamentation in an Ecm22/Upc2-dependent manner. Therefore, activation of Ecm22 and/or Upc2 during filamentous growth probably leads to increased expression of their targets\(\text{FHN1, NPR1, PRR2}\) and the\(\text{ERG}\) genes, which in turn promotes filamentation (Fig. 9). Other studies have shown that Upc2 is primarily activated through reduced sterol levels (Davies and Rine, 2006), which can be achieved through inhibition of sterol biosynthesis enzymes. As sterol synthesis requires oxygen, anaerobic conditions also lead to a reduction of sterol and therefore Upc2 activation. It was proposed that in sterol-rich conditions sterol directly binds to Upc2 that keeps it inactive in the cytoplasm (Marie et al., 2008; Yang et al., 2015). Dissociation of sterol from Upc2 leads to nuclear translocation of Upc2 and transcriptional activation. Starving conditions that trigger filamentation might also lead to reduced sterol levels. However, here we show that\(\text{UPC2}\) overexpression alone is sufficient to upregulate genes involved in filamentation. The observed increase of\(\text{UPC2}\) expression during filamentation might therefore also be sufficient for its role in filamentous growth.\(\text{UPC2}\) transcription is repressed by Sut1 and Sut2 and positively regulated by its own gene product. Furthermore, expression of\(\text{SUT1}\) and\(\text{SUT2}\) is inhibited by the transcription factor Ste12 (Foster et al., 2013), which is activated during filamentation (Liu et al., 1993; Roberts and Fink, 1993).
We propose a model in which Sut1 and Sut2 partially repress the expression of their targets \( \text{GAT2}, \text{HAP4}, \text{MGA1}, \text{MSN4}, \text{NCE102}, \text{RHO3}, \text{RHO5} \) and UPC2 under optimal growth conditions (Fig. 9A). When cells are grown on a solid medium with limited nutrients, Ste12 is activated, which results in reduced Sut1 and Sut2 levels, and as a consequence, in increased levels of Sut1/Sut2 targets. Together these targets mediate the transition to filamentous growth. UPC2 levels increase due to autoregulation and the reduced repression by Sut1 and Sut2. This then leads to transcriptional activation of Upc2 target genes. This model is supported by genetic interactions reported here. The filamentation defect of a \( \text{STE12} \) deletion strain is rescued by UPC2 overexpression. Increased levels of Upc2 targets, which are downstream of Ste12, are presumably sufficient for this effect. Interestingly, ECM22 overexpression had no effect on the filamentation defect of the \( \text{ste12} \Delta \) mutant that is consistent with other observations. In contrast to UPC2, ECM22 expression does not change during filamentation and is not regulated by the Ste12 targets Sut1 and Sut2. We also observed that \( \text{STE12} \) overexpression suppresses the fila-

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mentation defect of the \(\text{ecm}22\Delta\ \text{upc}2\Delta\) strain. This could be explained by the action of other Ste12 targets that function in parallel to the Upc2 pathway.

\(\text{GAT2},\ \text{HAP4},\ \text{MGA1},\ \text{MSN4},\ \text{NCE102},\ \text{PRR2},\ \text{RHO3}\) and \(\text{RH05}\) are not only regulated by \(\text{Sut1}\) and \(\text{Sut2}\). Their promoter regions also contain binding sites for the transcription factors \(\text{Flo8},\ \text{Mga1},\ \text{Phd1},\ \text{Sok2},\ \text{Ste12}\) and \(\text{Tec1}\), which promote filamentation (Borneman et al., 2006; Foster et al., 2013). Likewise, many \(\text{ERG}\) promoters have a binding site for at least one of these factors (Borneman et al., 2006). All six transcription factors bind to the \(\text{ERG11}\) promoter, and we show here that \(\text{Flo8},\ \text{Mga1},\ \text{Phd1}\) and \(\text{Ste12}\) actually control \(\text{ERG11}\) expression. It seems very likely that other \(\text{ERG}\) genes and therefore as a consequence ergosterol biosynthesis are regulated by these transcription factors. This would be a novel and interesting regulatory mechanism for this important metabolic pathway.

What is the function of the Ecm22/Upc2 targets in filamentation? \(\text{Fhn1}\), like \(\text{Nce102}\), is involved in the formation of a specialized plasma membrane domain termed eisosome (Loibl et al., 2010). This membrane domain could be important for polarized growth during filamentation. Pnt2 functions as a mating inhibitor (Burchett et al., 2001). It is not clear how this is relevant for filamentation. The kinase \(\text{Npr1}\) stabilizes and activates plasma membrane-bound nitrogen source transporters when nitrogen is limited (Schmidt et al., 1998; De Craene et al., 2001; Boeckstaens et al., 2014). This includes the ammonium permease \(\text{Mep2}\), which also functions as a nitrogen sensor for the transition to filamentous growth (Lorenz and Heitman, 1998; Van Nuland et al., 2006). \(\text{Npr1}\) activity is regulated by the TOR pathway. The increase of \(\text{NPR1}\) expression that we observed seems to be another regulatory mechanism to allow optimal ammonium transport and sensing during nitrogen limitation.

We not only observed transcriptional activation of \(\text{ERG}\) genes but also increased ergosterol levels during filamentation. It can only be speculated on the role of ergosterol in filamentation. However, eisosomes are rich in sterol (Grossmann et al., 2007). \(\text{Fhn1},\ \text{Nce102}\) and \(\text{Erg}\) enzymes might therefore act together to mediate filamentation. Interestingly, an Ecm22/Upc2-mediated change of sterol biosynthesis in response to an external signal has been reported before. \(\text{ECM22}\) is downregulated upon hyperosmotic stress (Montañés et al., 2011). This results in reduced \(\text{ERG}\) gene expression and lower sterol biosynthesis, which seems to be an important adaptation mechanism for hyperosmotic stress.

In \(\text{Candida albicans}\), the most common fungal pathogen in humans, filamentation plays important roles in host cell adherence, tissue invasion and virulence (Sudbery, 2011; Gow et al., 2012; Höfken, 2013). It would therefore be interesting to study the role of the \(\text{C. albicans}\) homo-
Table 1. Yeast strains used in this study.

| Name         | Genotype                                           | Source or reference |
|--------------|----------------------------------------------------|---------------------|
| MBY16        | PPY966 nap1::TRP1                                  | This study          |
| MCY19        | PPY966 ecm22::hphNT1                               | This study          |
| MCY21        | PPY966 upc21::hphNT1                               | This study          |
| PC344        | MATa/MATa ura3-52/ura3-52                          | Tiedje et al. (2008) |
| PPY966       | MATa his3::hisG leu2::hisG trp1::hisG ura3-52      | Tiedje et al. (2007) |
| SHY4         | PPY966 prr21::His3MX6                              | Foster et al. (2013) |
| SHY6         | PPy966 prr2::3Myc-kTRP1                            | This study          |
| SHY68        | PPy966 aux1::His3MX6 prr11::kTRP1                  | Foster et al. (2013) |
| THY760       | PPy966 upc21::hphNT1 ecms22::His3MX6               | This study          |
| THY762       | PPy966 KanMX6-pGAL1-3HA-STE12                      | Foster et al. (2013) |
| THY765       | PPy966 KanMX6-pGAL1-3HA-PHD1                       | Foster et al. (2013) |
| THY767       | PPy966 KanMX6-pGAL1-3HA-TEC1                       | Foster et al. (2013) |
| THY768       | PPy966 His3MX6-pGAL1-3HA-FLO8                      | Foster et al. (2013) |
| THY769       | PPy966 KanMX6-pGAL1-3HA-MGA1                       | Foster et al. (2013) |
| THY771       | PPy966 His3MX6-pGAL1-3HA-SOK2                      | This study          |
| THY784       | PPy966 erg3::His3MX6                               | This study          |
| THY808       | PPy966 npr1::KanMX6                                | This study          |
| THY809       | PPy966 prr21::His3MX6 npr1::hphNT1                 | This study          |
| THY826       | PPy966 upc21::hphNT1 ecms22::His3MX6 KanMX6-pGAL1-3HA-STE12 | This study          |
| THY827       | PPy966 erg11::His3MX6                              | This study          |
| THY837       | PPy966 ERG11-9Myc-His3MX6                          | This study          |
| THY839       | PPy966 FLO8-3HA-His3MX6                            | This study          |
| THY841       | PPy966 KanMX6-pGAL1-3HA-FLO8-His3MX6               | This study          |
| THY842       | PPy966 ste121::KanMX6                              | This study          |

For protein analysis, β-galactosidase assays and determination of ergosterol, cells were grown to exponential phase in SC medium. Cells were washed with water, and 10° cells were plated on SC medium lacking tetracycline and incubated for 14 h at 30°C. For protein analysis and β-galactosidase assays cells were scraped from one plate. Five plates were required for each measurement of the ergosterol content.

β-galactosidase assay

Densities of cell cultures were measured by optical density at 600 nm (A600). Cells were harvested by centrifugation and resuspended in 1 ml Z buffer (100 mM sodium phosphate [pH 7.0], 10 mM KCl, 1 mM MgSO4, 50 mM β-mercaptoethanol). Cells were permeabilized by addition of 20 μl chloroform and 20 μl 0.1% SDS. After 15 min incubation at 30°C, the reaction was started by addition of 140 μl n-o-nitrophenyl-β-D-galactopyranoside (4 mg ml-1 in 100 mM sodium phosphate, pH 7.0), incubated at 30°C until the solution became yellow, and the reaction was stopped by addition of 400 μl 1 M Na2CO3. Samples were centrifuged, and absorbance of the supernatant at 420 nm and 550 nm was determined. β-Galactosidase activity was calculated in Miller units as 1,000 × [A600 – (1.75 × A550)] / reaction time (min) × culture volume (ml) × A600.

Immunoblotting

One milliliter of cells was harvested by centrifugation and resuspended in 1 ml water. One hundred fifty microliters 1.85 M NaOH was added and incubated for 10 min on ice. After adding 150 μl 55% trichloroacetic acid, the samples were incubated for 10 min on ice. Following 20 min centrifugation 13 000 r.p.m. at 4°C, the supernatant was discarded. The pellet was resuspended in SDS sample buffer (150 mM Tris [pH 8.8], 2% SDS, 10% glycerol, 5% β-mercaptoethanol) and heated for 15 min at 65°C. The samples were then clarified by centrifugation at 13 000 r.p.m. for 1 min. Equal amounts were

Table 2. Plasmids used in this study.

| Name       | Genotype                        | Source or reference |
|------------|---------------------------------|---------------------|
| pHU36      | YEp367 carrying pMG1            | Foster et al. (2013) |
| pHU37      | YEp367 carrying pRPR2           | Foster et al. (2013) |
| pMC6       | YEp367 carrying pGA2            | Foster et al. (2013) |
| pMC7       | YEp367 carrying pRH04           | Foster et al. (2013) |
| pMC8       | pNEV-N, carrying UCP2           | This study          |
| pMC10      | pNEV-N carrying SUT2            | This study          |
| pNEV-N     | 2 μm, URA3, pMA1                | Sauer and Stolz (1994) |
| pNF1       | pNEV-N carrying SUT1            | Ness et al. (2001)  |
| pRS426     | 2 μm, URA3                      | Christianson et al. (1992) |
| pSH13      | YEp367 carrying pFLO1           | Foster et al. (2013) |
| pSH23      | YEp367 carrying pH4             | Foster et al. (2013) |
| pSH24      | YEp367 carrying pNCP1           | This study          |
| pTH376     | YEp367 carrying pERG3           | This study          |
| pTH379     | YEp367 carrying pERG11          | This study          |
| pTH387     | YEp367 carrying pH03            | Foster et al. (2013) |
| pTH391     | YEp367 carrying pMSN4           | Foster et al. (2013) |
| pTH401     | pRS426 carrying NECE102         | Blanda and Höfken (2013) |
| pTH402     | pRS426 carrying pRR2            | This study          |
| pTH407     | YEp367 carrying pFHN1           | This study          |
| pTH408     | pNEV-N carrying ECM22           | This study          |
| pTH412     | YEp367 carrying pECM22          | This study          |
| pTH414     | YEp367 carrying pUPC2           | This study          |
| pTH415     | YEp367 carrying pSUT2           | This study          |
| pTH421     | YEp367 carrying pNPR1           | This study          |
| pTH422     | pRS426 carrying FHN1            | This study          |
| YEp387     | 2 μm, LEU2, lacZ                | Myers et al. (1986) |

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separated by SDS-PAGE, transferred to nitrocellulose and incubated with mouse monoclonal anti-Myc (9E10) from Santa Cruz Biotechnology. To test whether equal amounts of protein were loaded, membranes were stripped after development by incubating membranes in stripping buffer (65 mM Tris [pH 6.8], 2% SDS, 20 mM β-mercaptoethanol) for 40 min at 50°C. After thorough washing with PBS, membranes were incubated with rabbit polyclonal anti-Cdc11 (Santa Cruz Biotechnology) as loading control. Secondary antibodies were from Jackson Research Laboratories.

**Ergosterol quantification**

Ergosterol levels were determined as described by Arthington-Skaggs et al. (1999), with minor modifications. Briefly, cells were harvested, washed with water and the wet weight was determined. Cells were resuspended in 1.5 ml 25% alcoholic potassium hydroxide solution (25 g KOH and 35 ml water were brought to 100 ml with ethanol) and vortexed for 1 min. Cells suspensions were transferred to borosilicate glass screw-cap tubes and incubated in an 85°C water bath for 1 h. The samples were then allowed to cool down to room temperature and sterols were extracted with a mixture of 500 μl of water and 1.5 ml of n-heptane followed by vortexing for 3 min. Ergosterol content was determined using a Hitachi U-1900 spectrophotometer and calculated as percentage of the wet weight as described by Arthington-Skaggs et al. (1999).

**ChIP**

ChIP was performed as described previously (Foster et al., 2013). The ERG11 promoter region was amplified using primers 5’TACTCTACTAAATCACAC3’ and 5’CATCCTTG-TATTTACTCGT3’.

**Quantitative real-time PCR**

ERG11 expression was determined by quantitative real-time PCR as described by Foster et al. (2013) using primers 5’TTCGGTGGTGGTAGACACAG3’ and 5’GGTGAACG-GTCTTACCCTC3’.

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The authors declare that there are no conflicts of interest.

**References**

Abramova, N.E., Cohen, B.D., Sertil, O., Kapoor, R., Davies, K.J., and Lowry, C.V. (2001) Regulatory mechanisms controlling expression of the DAN/TIR mannoprotein genes during anaerobic remodeling of the cell wall in Saccharomyces cerevisiae. *Genetics* **157**: 1169–1177.

Ailmardani, P., Régnacq, M., Moreau-Vauzelle, C., Ferreira, T., Rossignol, T, Blondin, B., and Bergès, T. (2004) *SUT1-* promoted sterol uptake involves the ABC transporter Aus1 and the mannoprotein Dan1 whose synergistic action is sufficient for this process. *Biochem J* **381**: 195–202.

Aoyama, Y., Yoshida, Y., Sonoda, Y., and Sato, Y. (1989) Deformylation of 32-oxo-24,25-dihydrolanosterol by the purified cytochrome P-4501A4DM (lanosterol 14 alpha-demethylase) from yeast e evidenced confirming the intermediate step of lanosterol 14 alpha-demethylase. *J Biol Chem* **264**: 18502–18505.

Arthington, B.A., Bennett, L.G., Skatrud, P.L., Guynn, C.J., Barbach, R.J., Ulbright, C.E., and Bard, M. (1991) Cloning, disruption and sequence of the gene encoding yeast C-5 sterol desaturase. *Gene* **102**: 39–44.

Arthington-Skaggs, B.A., Jradi, H., Desai, T., and Morrison, C.J. (1999) Quantitation of ergosterol content: novel method for determination of flucanazole susceptibility of *Candida albicans*. *J Clin Microbiol* **37**: 3332–3337.

Blanda, C., and Höfken, T. (2013) Regulation of mating in the budding yeast *Saccharomyces cerevisiae* by the zinc cluster proteins Sut1 and Sut2. *Biochem Biophys Res Commun* **438**: 66–70.

Boeckstaens, M., Linares, E., Van Vooren, P., and Marin, A.M. (2014) The TORC1 effector kinase Npr1 fine tunes the inherent activity of the Mep2 ammonium transport protein. *Nat Commun* **5**: 3101.

Borneman, A.R., Leigh-Bell, J.A., Yu, H., Bertone, P., Gerstein, M., and Snyder, M. (2006) Target hub proteins serve as master regulators of development in yeast. *Genes Dev* **20**: 435–448.

Bourot, S., and Karst, F. (1995) Isolation and characterization of the *Saccharomyces cerevisiae* *SUT1* gene involved in sterol uptake. *Gene* **165**: 97–102.

Burchett, S.A., Scott, A., Errede, B., and Dohman, H.G. (2001) Identification of novel pheromone-response regulators through systematic overexpression of 120 protein kinases in yeast. *J Biol Chem* **276**: 26472–26478.

Byrne, K.P., and Wolfe, K.H. (2005) The Yeast Gene Order Browser: combining curated homology and syntenic context reveals gene fate in polyploid species. *Genome Res* **15**: 1456–1461.

Christianson, T.W., Sikorski, R.S., Dante, M., Sher, J.H., and Hieter, P. (1992) Multifunctional yeast high-copy-number shuttle vectors. *Gene* **110**: 119–122.

Crowley, J.H., Leak, F.W., Jr, Shianna, K.V., Tove, S., and Parks, L.W. (1998) A mutation in a purported regulatory gene affects control of sterol uptake in *Saccharomyces cerevisiae*. *J Bacteriol* **180**: 4177–4183.

Cullen, P.J., and Sprague, G.F., Jr (2000) Glucose depletion causes haploid invasive growth in yeast. *Proc Natl Acad Sci USA* **97**: 13619–13624.

Cullen, P.J., and Sprague, G.F., Jr (2012) The regulation of filamentous growth in yeast. *Genetics* **190**: 23–49.

Davies, B.S., and Rine, J. (2006) A role for sterol levels in oxygen sensing in *Saccharomyces cerevisiae*. *Genetics* **174**: 191–201.

Davies, B.S., Wang, H.S., and Rine, J. (2005) Dual activators of the sterol biosynthetic pathway of *Saccharomyces cerevisiae*: similar activation/regulatory domains but different response mechanisms. *Mol Cell Biol* **25**: 7375–7385.

De Craene, J.O., Soetens, O., and Andre, B. (2001) The Npr1 kinase controls biosynthetic and endocytic sorting of the
yeast Gap1 permease. J Biol Chem 276: 43939–43948.

Douglas, L.M., Wang, H.X., and Konopka, J.B. (2013) The MARVEL domain protein Nce102 regulates actin organization and invasive growth of Candida albicans. MBio 4: e00723–13.

Dunkel, N., Liu, T.T., Barker, K.S., Homayouni, R., Morschhäuser, J., and Rogers, P.D. (2008) A gain-of-function mutation in the transcription factor Upc2p causes upregulation of ergosterol biosynthesis genes and increased fluconazole resistance in a clinical Candida albicans isolate. Eukaryot Cell 7: 1180–1190.

Flowers, S.A., Barker, K.S., Berkow, E.L., Toner, G., Chadwick, S.G., Gygax, S.E., et al. (2012) Gain-of-function mutations in UPC2 are a frequent cause of ERG11 upregulation in azole-resistant clinical isolates of Candida albicans. Eukaryot Cell 11: 1289–1299.

Foster, H.A., Cui, M., Naveenathayalan, A., Unden, H., Schwanbeck, R., and Höfken, T. (2013) The zinc cluster protein Sut1 contributes to filamentation in Saccharomyces cerevisiae. Eukaryot Cell 12: 244–253.

Gallo-Ebert, C., Donigan, M., Stroke, I.L., Swanson, R.N., Gow, N.A., van de Veerdonk, F.L., Brown, A.J., and Netea, Hoen, T. (2013) Candida albicans Candida cerevisiae, and candidiasis. In C. albicans affecting sterol uptake and metabolism. Yeast 4: 99–106.

Höfken, T., Liu, H., Styles, C.A., and Fink, G.R. (1993) Elements of the yeast pheromone response pathway required for filamentous growth of diploids. Science 262: 1741–1744.

Lohberger, A., Coste, A.T., and Sanglard, D. (2014) Distinct roles of Candida albicans drug resistance transcription factors TAC1, MRR1, and UPC2 in virulence. Eukaryot Cell 13: 127–142.

Longtine, M.S., McKenzie, A., Demarini, D.J., Shah, N.G., Wach, A., Brachat, A., et al. (1998) Additional modules for versatile and economical PCR-based gene deletion and modification in Saccharomyces cerevisiae. Yeast 14: 953–961.

Lorenz, M.C., and Heitman, J. (1998) The MEP2 ammonium permease regulates pseudohyphal differentiation in Saccharomyces cerevisiae. EMBO J 17: 1236–1247.

Lupetti, A., Danesi, R., Campa, M., Del Tacca, M., and Kelly, S. (2002) Molecular basis of resistance to azole antifungals. Trends Mol Med 8: 76–81.

MacPherson, S., Akache, B., Weber, S., De Deken, X., Raymond, M., and Turcotte, B. (2005) Candida albicans zinc cluster protein Upc2p confers resistance to antifungal drugs and is an activator of ergosterol biosynthetic genes. Antimicrob Agents Chemother 49: 1745–1752.

Marie, C., Leyde, S., and White, T.C. (2008) Cytoplasmic localization of sterol transcription factors Upc2p and Ecm22p in S. cerevisiae. Fungal Genet Biol 45: 1430–1438.

Montañés, F.M., Pascual-Ahuir, A., and Proft, M. (2011) Repression of ergosterol biosynthesis is essential for stress resistance and is mediated by the Hog1 MAP kinase and the Mot3 and Rox1 transcription factors. Mol Microbiol 79: 1008–1023.

Myers, A.M., Tzagoloff, A., Kinney, D.M., and Lustig, C.J. (1986) Yeast shuttle and integrative vectors with multiple cloning sites suitable for construction of lacZ fusions. Gene 45: 299–310.

Ness, F., Bourot, S., Régniac, M., Spagnoli, R., Bergès, T., and Karst, F. (2001) SUT1 is a putative Zn[II]Cys6 transcription factor whose upregulation enhances both sterol uptake and synthesis in aerobically growing Saccharomyces cerevisiae cells. Eur J Biochem 268: 1585–1595.

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Régnacq, M., Alimardani, P., El Moudni, B., and Bergès, T. (2001) SUT1p interaction with Cyc8p(Ssn6p) relieves hypoxic genes from Cyc8p-Tup1p repression in Saccharomyces cerevisiae. Mol Microbiol 40: 1085–1096.

Roberts, R.L., and Fink, G.R. (1994) Elements of a single MAP kinase cascade in Saccharomyces cerevisiae mediate two developmental programs in the same cell type: mating and invasive growth. Genes Dev 8: 2974–2985.

Rützler, M., Reissaus, A., Budzowska, M., and Bandlow, W. (2004) SUT2 is a novel multicopy suppressor of low activity of the cAMP/protein kinase A pathway in yeast. Eur J Biochem 271: 1284–1291.

Sauer, N., and Stolz, J. (1994) SUC1 and SUC2: two sucrose transporters from Arabidopsis thaliana; expression and characterization in baker’s yeast and identification of the histidine-tagged protein. Plant J 6: 67–77.

Schjerling, P., and Holmberg, S. (1996) Comparative amino acid sequence analysis of the C6 zinc cluster family of transcriptional regulators. Nucleic Acids Res 24: 4599–4607.

Schmidt, A., Beck, T., Koller, A., Kunz, J., and Hall, M.N. (1998) The TOR nutrient signalling pathway phosphorylates NPR1 and inhibits turnover of the tryptophan permease. EMBO J 17: 6924–6931.

Shianna, K.V., Dotson, W.D., Tove, S., and Parks, L.W. (2001) Identification of a UPC2 homolog in Saccharomyces cerevisiae and its involvement in aerobic sterol uptake. J Bacteriol 183: 830–834.

Silver, P.M., Oliver, B.G., and White, T.C. (2004) Role of Candida albicans transcription factor Upc2p in drug resistance and sterol metabolism. Eukaryot Cell 3: 1391–1397.

Sudbery, P.E. (2011) Growth of Candida albicans hyphae. Nat Rev Microbiol 9: 737–748.

Tiedje, C., Holland, D.G., Just, U., and Höfken, T. (2007) Proteins involved in sterol synthesis interact with Ste20 and regulate cell polarity. J Cell Sci 120: 3613–3624.

Tiedje, C., Sakwa, I., Just, U., and Höfken, T. (2008) The Rho GDI Rdi1 regulates Rho GTPases by distinct mechanisms. Mol Biol Cell 19: 2885–2896.

Van Nuland, A., Vandormael, P., Donaton, M., Alenquer, M., Lourenço, A., Quintino, E., et al. (2006) Ammonium permease-based sensing mechanism for rapid ammonium activation of the protein kinase A pathway in yeast. Mol Microbiol 59: 1485–1505.

Vik, A., and Rine, J. (2001) Upc2p and Ecm22p, dual regulators of sterol biosynthesis in Saccharomyces cerevisiae. Mol Cell Biol 21: 6395–6405.

Wach, A., Brachat, A., Alberti-Segui, C., Rebischung, C., and Philippson, P. (1997) Heterologous HIS3 marker and GFP reporter modules for PCR-targeting in Saccharomyces cerevisiae. Yeast 13: 1065–1075.

Wilcox, L.J., Balderes, D.A., Wharton, B., Tinkelenberg, A.H., Rao, G., and Sturley, S.L. (2002) Transcriptional profiling identifies two members of the ATP-binding cassette transporter superfamily required for sterol uptake in yeast. J Biol Chem 277: 32466–32472.

Yang, H., Tong, J., Lee, C.W., Ha, S., Eom, S.H., and Im, Y.J. (2015) Structural mechanism of ergosterol regulation by fungal sterol transcription factor Upc2. Nat Commun 6: 6129.

Yoshida, Y. (1988) Cytochrome P-450 of fungi: primary target for azole antifungal agents. In Current Topics in Medical Mycology, Vol. 2. McGinnis, M.R. (ed.). New York: Springer, pp. 388–418.