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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 $Zn(II)_2Cys_6$ 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 *RHO5*, 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*, *RHO3* and *RHO5* 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

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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 (Régnacq *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*, *FHN1* 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 *UPC2* 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 paralogue 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* is 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

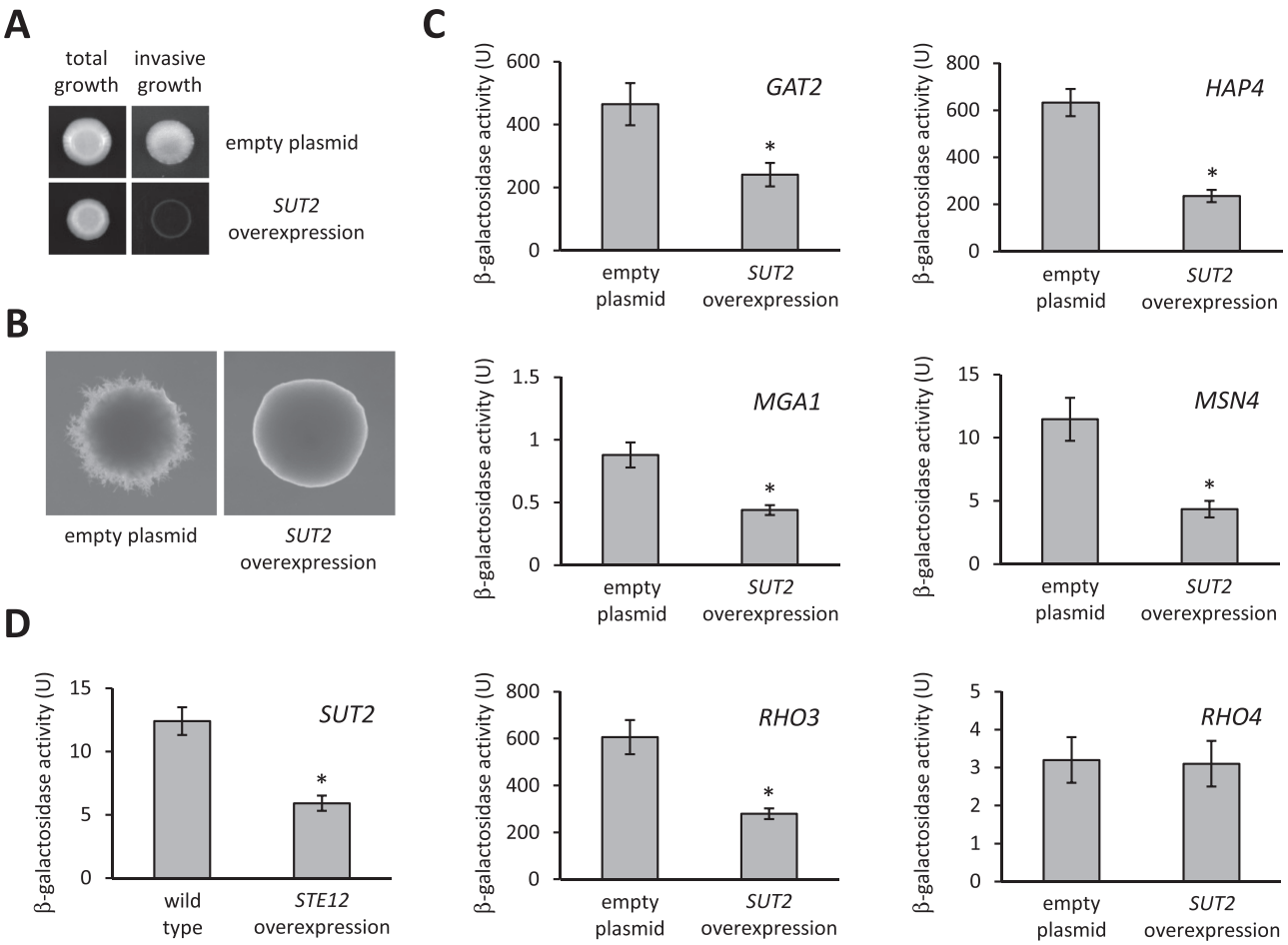

Fig. 1. Sut2 inhibits filamentous growth.
A. *SUT2* overexpression results in decreased haploid invasive growth. Haploid wild-type cells (PPY966) carrying either a *SUT2* overexpression plasmid (pMC10) or the corresponding empty vector (pNEV-N) were spotted onto a selective medium plate and were grown for 5 days at 30°C. Pictures were taken before (total growth) and after (invasive growth) rinsing with water.
B. Cells overexpressing *SUT2* have a defect in diploid pseudohyphal growth. Diploid cells (PC344) carrying either an empty vector (pNEV-N) or a *SUT2* overexpression plasmid (pMC10) were grown on low-nitrogen SLAD medium for 6 days at 30°C.
C. Sut2 negatively regulates the expression of Sut1 target genes. Cells harbored either a *SUT2* overexpression plasmid (pMC10) or the corresponding empty vector (pNEV-N) in combination with the *lacZ* reporter fused to the indicated promoter regions (pMC6, pSH23, pHU36, pTH391, pTH387, pMC7). 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.
D. Ste12 downregulates the expression of *SUT2*. *SUT2-lacZ* expression (pTH415) was determined for the wild-type strain (PPY966) and cells overexpressing *STE12* from the *GAL1* promoter (THY762). Bars indicate the average with standard deviation of four independent cultures. *, $P < 0.01$ compared with the wild type.

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 *ecm22*Δ *upc2*Δ 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

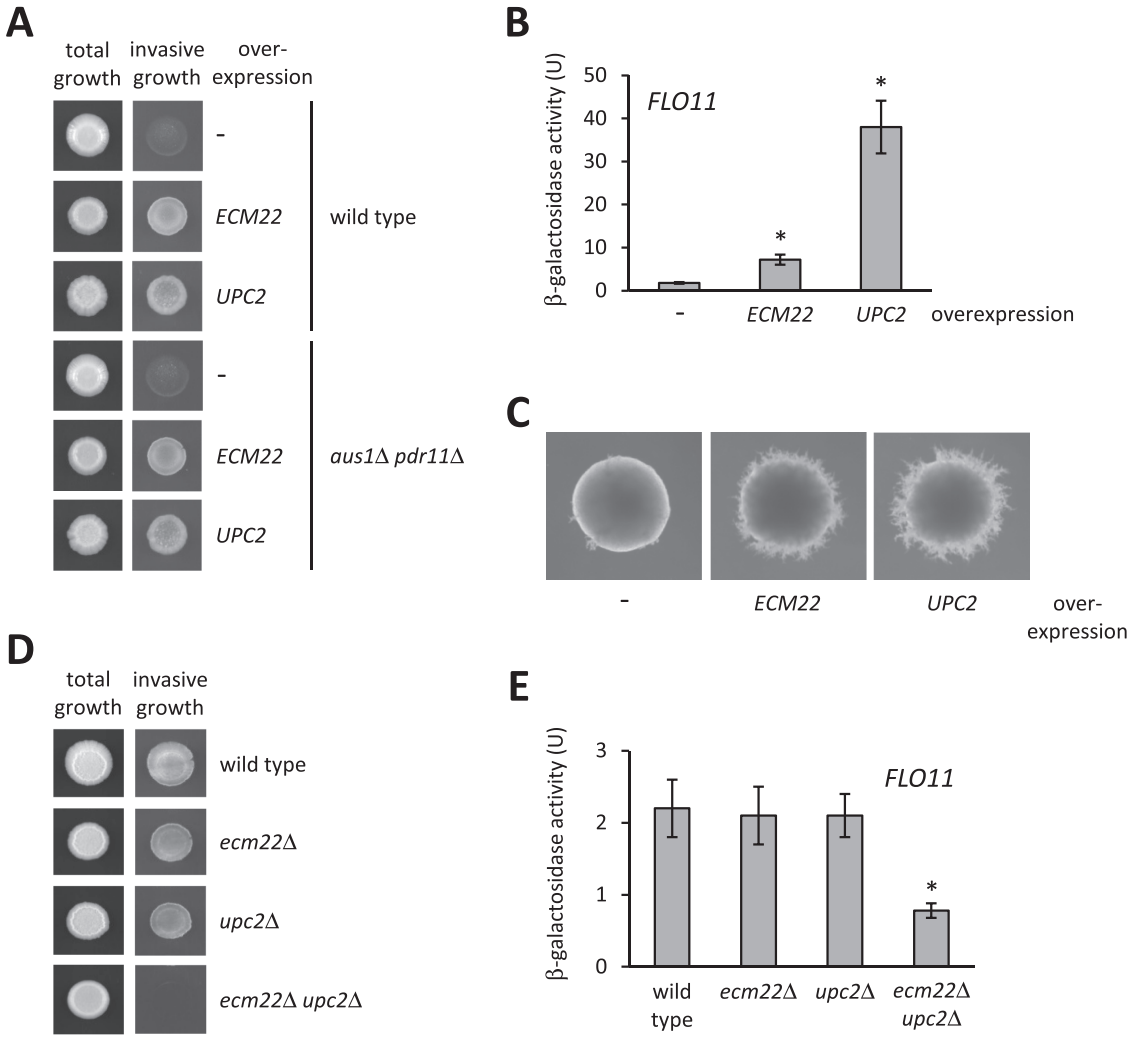

Fig. 2. Ecm22 and Upc2 are positive regulators of filamentous growth.
A. Overexpression of *ECM22* and *UPC2* leads to increased haploid invasive growth. The wild type (PPY966) and the sterol import mutant *aus1Δ pdr11Δ* (SHY68) carrying the indicated plasmids (pNEV-N, pTH408, pMC8) were spotted onto 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 *ECM22* and *UPC2*.
B. Overexpression of *ECM22* and *UPC2* leads to increased *FLO11* levels. Wild-type cells (PPY966) harboring a plasmid on which *lacZ* was fused to the *FLO11* promoter (pSH23), and carrying the indicated plasmids (pNEV-N, pTH408, pMC8) were grown in selective medium. 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.
C. Overexpression of *ECM22* and *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 *ECM22* and *UPC2*.
D. Simultaneous deletion of *ECM22* and *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 *ECM22* and *UPC2* results in decreased *FLO11* expression. β-galactosidase activity was determined for the indicated strains (PPY966, MCY19, MCY21, THY760) all carrying a *FLO11-lacZ* plasmid (pSH213). Bars indicate the average with standard deviation of four independent cultures. *, $P < 0.01$ compared with the wild type.

under anaerobic conditions, including *AUS1* and *DAN1* (Régnacq *et al.*, 2001; Wilcox *et al.*, 2002; Alimardani *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 filamentatous growth. However, levels of *GAT2*, *HAP4*, *MGA1*, *MSN4*, *NCE102*, *RHO3* and *RHO5* in the *ecm22Δ upc2Δ* double mutant were indistinguishable from the wild type (data not shown). As an example, *NCE102* expression was also tested in cells overexpressing either *ECM22* or *UPC2*.

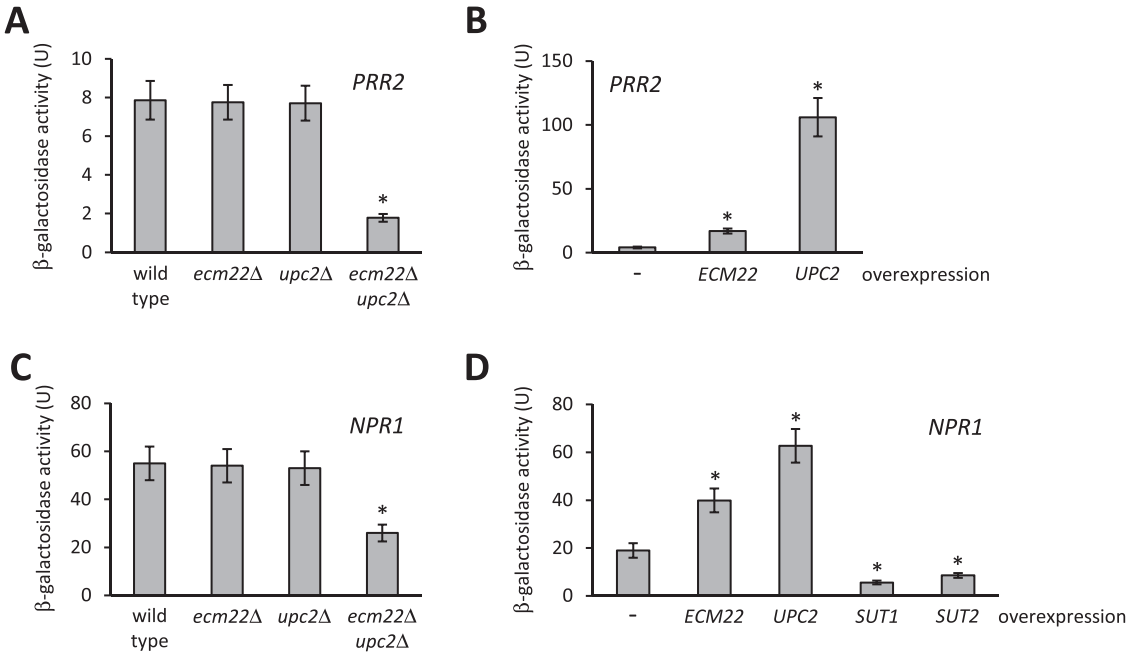

Fig. 3. *PRR2* and *NPR1* expression is regulated by Ecm22 and Upc2.
A. Deletion of *ECM22* and *UPC2* leads to decreased *PRR2* expression. β-galactosidase activity was determined for the indicated strains (PPY966, MCY19, MCY21, THY760) carrying a *PRR2-lacZ* plasmid (pHU37). Shown is the average β-galactosidase 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 *ecm22*Δ *upc2*Δ mutant and the *ECM22* and *UPC2* overexpression strains. Interestingly, expression of the Sut1/Sut2 target *PRR2* is lowered in *ecm22*Δ *upc2*Δ 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 *ecm22*Δ *upc2*Δ 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

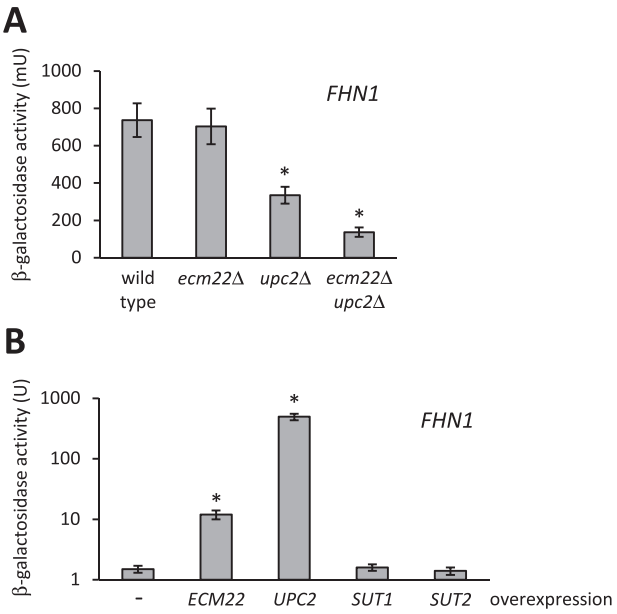

Fig. 4. *FHN1* expression is regulated by Ecm22 and Upc2.
A. Cells lacking *ECM22* and *UPC2* have reduced *FHN1* levels. Shown is the average β-galactosidase activity with standard deviation of four independent cultures of the indicated strains (PPY966, MCY19, MCY21, THY760 carrying pTH407). *, $P < 0.01$ compared with the wild type.
B. Overexpression of *ECM22* and *UPC2* leads to increased *FHN1* expression. β-galactosidase activity was determined from four independent cultures of the wild-type strain (PPY966) harboring an *FHN1-lacZ* plasmid (pTH407) and the indicated vectors (pNEV-N, pTH408, pMC8, pNF1, pMC10). *, $P < 0.01$ compared with the wild type carrying an empty plasmid.

correlates with filamentation phenotypes. *PRR2* levels are reduced in strains that have a filamentation defect such as the *ecm22*Δ *upc2*Δ 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 *UPC2* 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 *prr2*Δ strain because both genes have overlapping functions in filamentation. We therefore examined filamentous growth of the *npr1*Δ *prr2*Δ double mutant and the *npr1*Δ 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 *aus1*Δ *pdr11*Δ 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 *aus1*Δ *pdr11*Δ (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 Ecm22 and Upc2 (Vik and Rine, 2001; Wilcox *et al*., 2002). We chose *ERG3*, *ERG11* and *NCP1* to analyze the role of *ERG* genes in Ecm22/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,

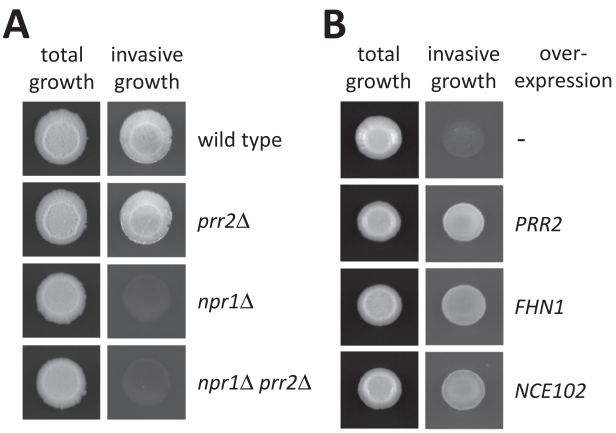

Fig. 5. Prr2, Npr1, Nce102 and Fhn1 have a role in filamentation.
A. *NPR1* deletion causes a defect in filamentation. The indicated strains (PPY966, SHY4, THY808, THY809) were spotted onto YPD medium and grown at 30°C. After 2 days, pictures were taken before (total growth) and after (invasive growth) rinsing with water.
B. Overexpression of *PRR2*, *NCE102* and *FHN1* results in stronger invasive growth. Wild-type cells (PPY966) carrying the indicated vectors (pRS426, pTH402, pTH422, pTH401) were spotted onto selective medium plates and incubated 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 *FHN1*, *NCE102* and *PRR2*.

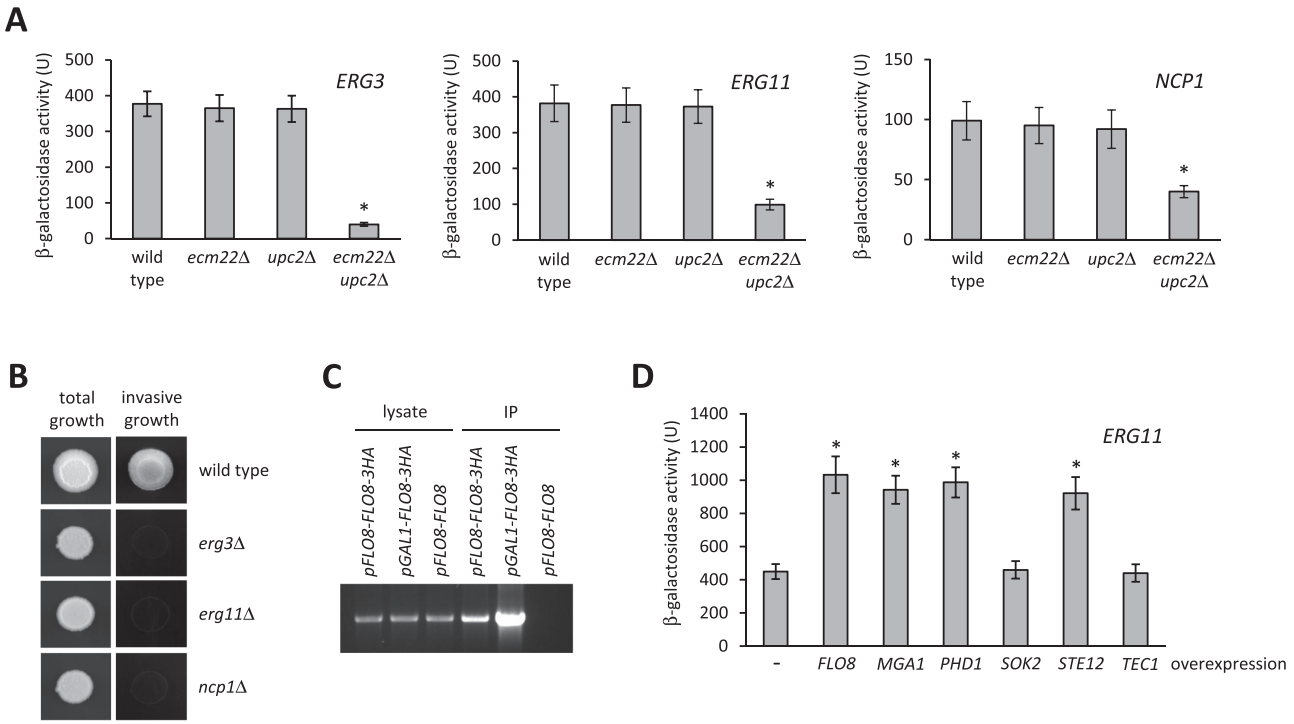

Fig. 6. Sterol biosynthesis enzymes play an important role in filamentation.
A. Deletion of *ECM22* and *UPC2* results in decreased expression of *ERG* genes. β-galactosidase activity was determined for the indicated strains (PPY966, MCY19, MCY21, THY760 carrying pTH376, pTH379 or pSH24). Given is the 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, THY765, THY771, THY762, THY767). Bars indicate the average with standard deviation of four independent cultures. *, $P < 0.01$ compared with the wild type.

1988; Aoyama *et al*., 1989; Kelly *et al*., 1995). The expression of *ERG3*, *ERG11* and *NCP1* is downregulated in *ecm22*Δ *upc2*Δ cells but not in the corresponding single mutants (Fig. 6A), which is consistent with published data (Vik and Rine, 2001; Wilcox *et al*., 2002). 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 associated more strongly with the promoter region of *ERG11*. The *ERG11* expression level was increased by 2.1 ± 0.15 in cells overexpressing Flo8-3HA as determined by quantitative real-time polymerase chain reaction (PCR). Thus, there is a clear correlation between Flo8-3HA levels, association of Flo8-3HA with the *ERG11* promoter and *ERG11* expression. As for *FLO8*, we also found that overexpression of *MGA1*, *PHD1* and *STE12* resulted in increased *ERG11* expression

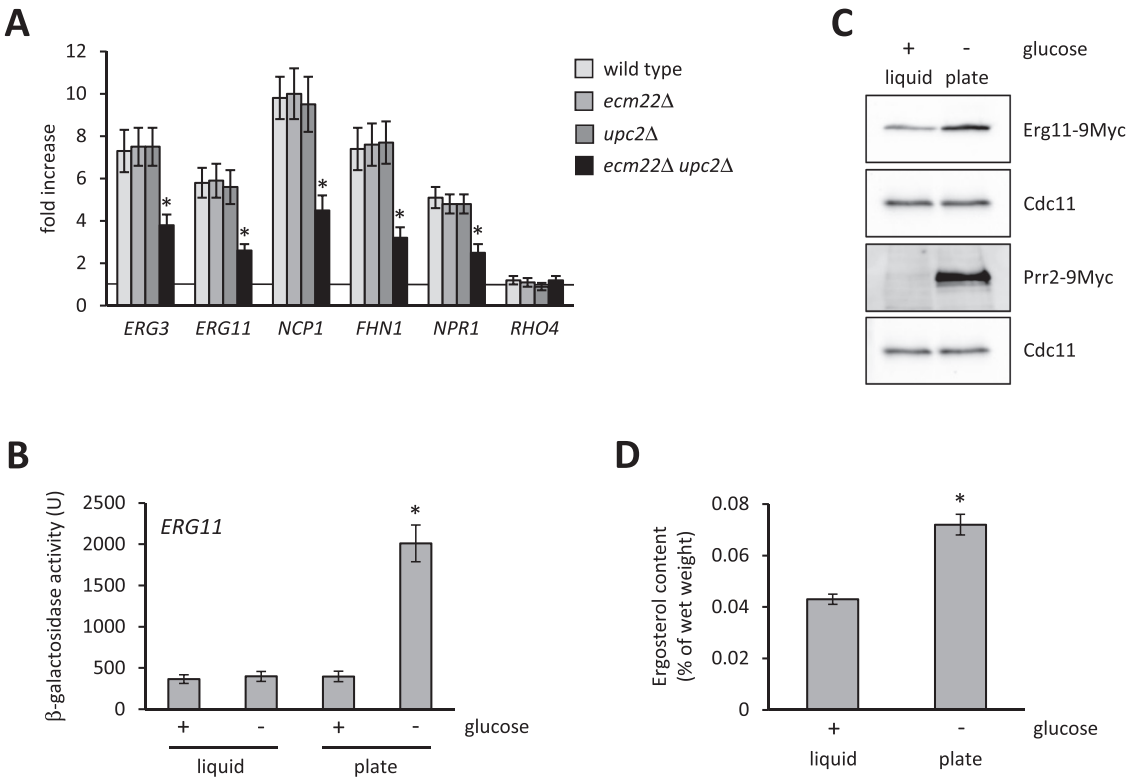

Fig. 7. Expression of Ecm22/Upc2 targets increases during filamentation.
A. Expression of Ecm22/Upc2 target genes during filamentous growth. β-galactosidase activity was determined for the indicated genes (pTH376, pTH379, pSH24, pTH407, pTH421, pMC7) in cells (PPY966, MCY19, MCY21, THY760) grown for 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 lyzed 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.

(Fig. 6D). 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 *sok2*Δ and *tec1*Δ 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.

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 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 *ecm22*Δ *upc2*Δ 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 *FHN1* and the *ERG* genes. *PRR2* and its paralogue *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-

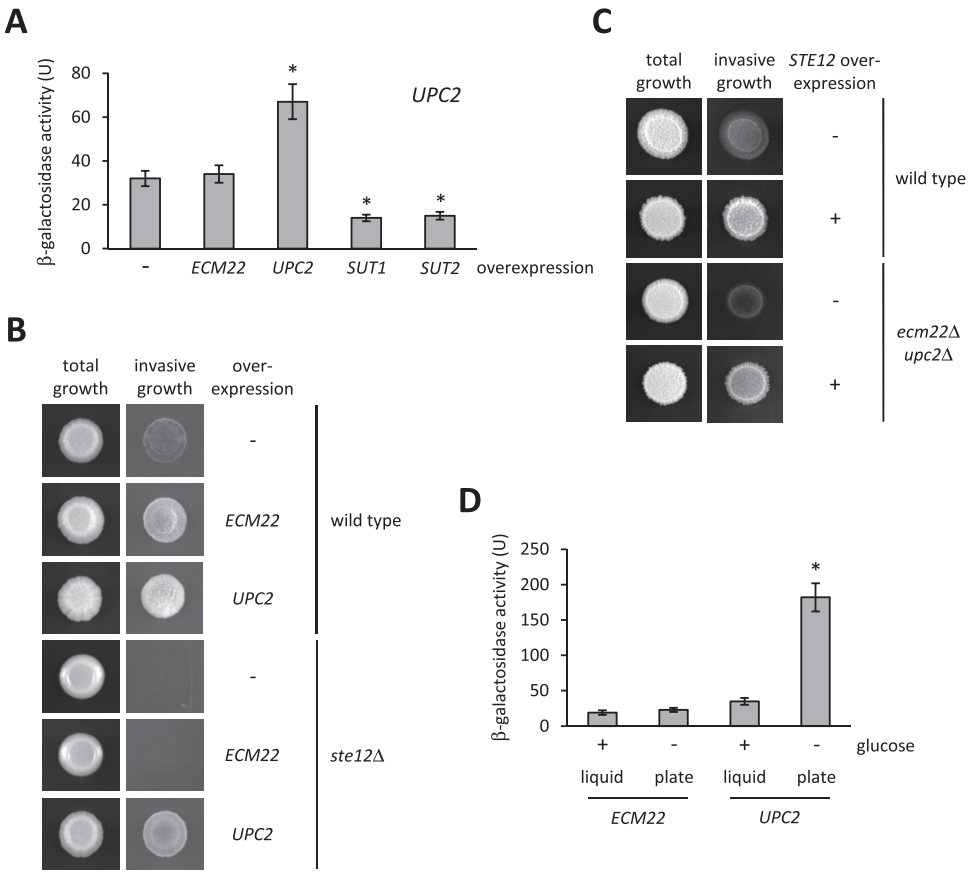

Fig. 8. Regulation of *UPC2* expression.
A. *UPC2* expression is under control of Sut1 and Sut2. The average β-galactosidase activity of wild-type cells (PPY966) carrying the indicated plasmids (pTH414 in combination with pNEV-N, pTH408, pMC8, pNF1, pMC10) is given with standard deviation ($n = 4$). *, $P < 0.01$ compared with the wild type carrying an empty plasmid.
B. Overexpression of *UPC2* rescues the filamentation defect of the *STE12* deletion strain. The wild type (PPY966) and the *ste12*Δ mutant (THY842) carrying the indicated vectors (pNEV-N, pTH408, pMC8) were spotted onto selective medium plates and incubated for 4 days at 30°C. Pictures were taken before (total growth) and after (invasive growth) rinsing with water.
C. *STE12* overexpression suppresses the filamentation defect of the *ecm22*Δ *upc2*Δ mutant. The indicated strains (PPY966, THY762, THY760, THY826) were spotted on minimal medium supplemented with galactose and raffinose for *STE12* overexpression and grown for 3 days at 30°C.
D. *UPC2* expression increases during filamentation. β-galactosidase activity was determined for the indicated genes (pTH412, pTH414) in wild-type cells (PPY966) grown either for 14 h on minimal medium plates lacking glucose or grown in liquid minimal medium containing glucose. Shown is the average activity with standard deviation ($n = 4$). *, $P < 0.01$ compared with the cells grown in liquid high-glucose medium.

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 *FHN1*, *NPR1*, *PRR2* and the *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 *UPC2* overexpression alone is sufficient to upregulate genes involved in filamentation. The observed increase of *UPC2* expression during filamentation might therefore also be sufficient for its role in filamentous growth. *UPC2* transcription is repressed by Sut1 and Sut2 and positively regulated by its own gene product. Furthermore, expression of *SUT1* and *SUT2* is inhibited by the transcription factor Ste12 (Foster *et al.*, 2013), which is activated during filamentation (Liu *et al.*, 1993; Roberts and Fink,

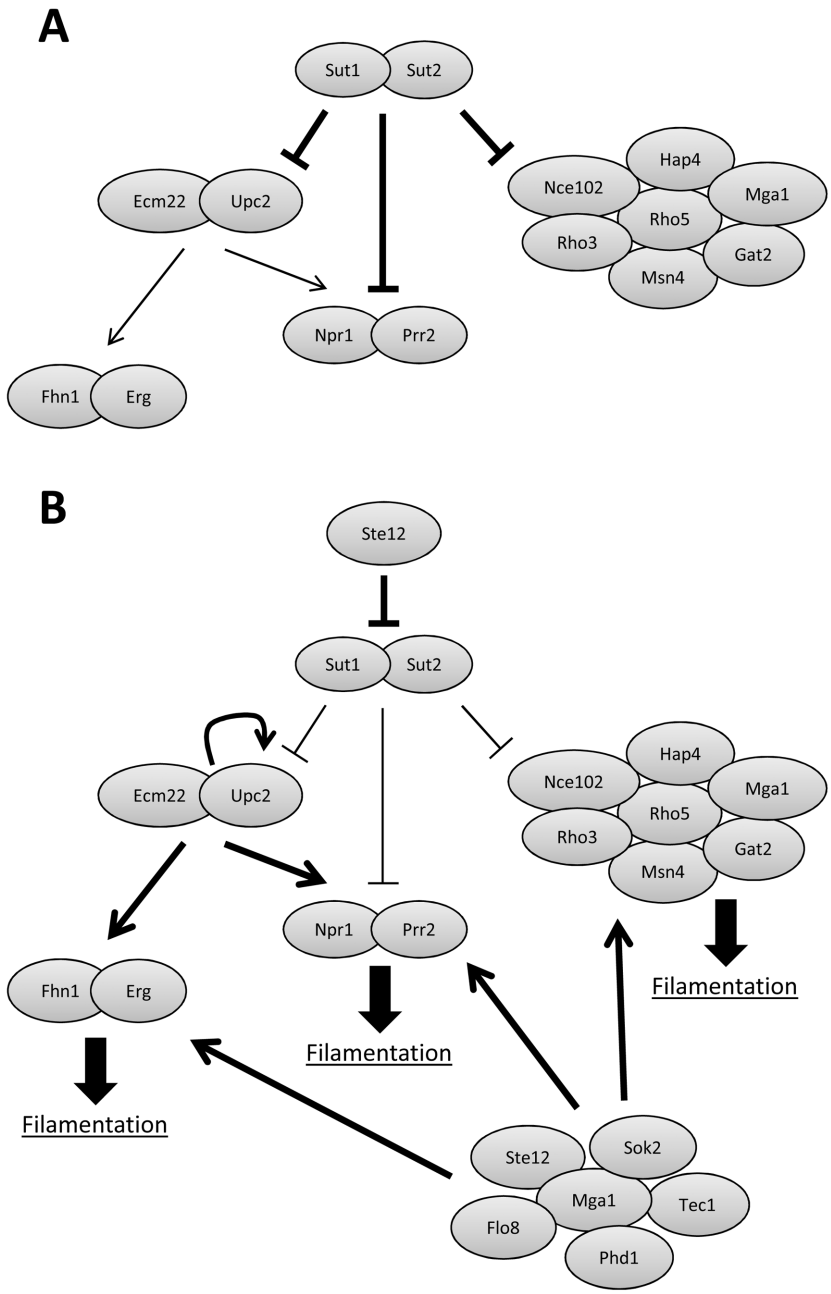

Fig. 9. Model for the regulation of filamentation by zinc cluster proteins. All factors shown represent proteins. Genes are not shown for the sake of simplicity. Activating and inhibitory arrows indicate regulation of expression of the corresponding genes.
A. When cells are grown in nutrient-rich liquid medium, Sut1 and Sut2 partially repress the expression of their targets. These include *GAT2*, *HAP4*, *MGA1*, *MSN4*, *NCE102*, *RHO3* and *RHO5*, which are only regulated by Sut1 (Foster *et al.*, 2013) and Sut2 (Blanda and Höfken, 2013; this study) but not by Ecm22 and Upc2 (this study). *NPR1* and *PRR2* are under control of all four transcription factors (this study). Importantly, *UPC2* expression is also repressed by Sut1 and Sut2 (this study).
B. When cells are grown on solid medium with limited nutrients, Ste12 becomes activated (Liu *et al.*, 1993; Roberts and Fink, 1994) and lowers *SUT1* and *SUT2* levels (Foster *et al.*, 2013; this study). As a consequence of the loss of repression, expression of the Sut1/Sut2 targets increases and the corresponding gene products contribute to filamentous growth (Foster *et al.*, 2013; this study). *UPC2* expression might also increase due to autoregulation (Abramova *et al.*, 2001; Wilcox *et al.*, 2002; this study). Higher Upc2 levels result in increased expression of its targets which include *FHN1*, *NPR1*, *PRR2* (this study) and *ERG* genes (Vik and Rine, 2001; this study). The corresponding proteins mediate the transition to filamentous growth (this study). Many targets of Ecm22, Upc2, Sut1 and Sut2 are probably also under control of other transcription factors that promote filamentation. Promoters of many *ERG* genes have binding sites for Flo8, Mga1, Phd1, Ste12, Sok2 and Tec1 (Borneman *et al.*, 2006; this study). All six transcription factors bind to the promoters of *GAT2*, *HAP4*, *MGA1*, *RHO3* and *RHO5*, and at least one of these transcription factors bind to the promoter regions of *PRR2*, *NCE102* and *MSN4* (Borneman *et al.*, 2006; Foster *et al.*, 2013). This suggests that Ecm22, Upc2, Sut1 and Sut1, and their targets are part of an important complex transcriptional network for the induction of filamentation.

1994). We propose a model in which Sut1 and Sut2 partially repress the expression of their targets *GAT2*, *HAP4*, *MGA1*, *MSN4*, *NCE102*, *NPR1*, *PRR2*, *RHO3*, *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 *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 *ste12*Δ 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 *STE12* overexpression suppresses the fila-

mentation defect of the *ecm22*Δ *upc2*Δ strain. This could be explained by the action of other Ste12 targets that function in parallel to the Upc2 pathway.

GAT2, *HAP4*, *MGA1*, *MSN4*, *NCE102*, *PRR2*, *RHO3* and *RHO5* are not only regulated by Sut1 and Sut2. Their promoter regions also contain binding sites for the transcription factors Flo8, Mga1, Phd1, Sok2, Ste12 and Tec1, which promote filamentation (Borneman *et al*., 2006; Foster *et al*., 2013). Likewise, many *ERG* promoters have a binding site for at least one of these factors (Borneman *et al*., 2006). All six transcription factors bind to the *ERG11* promoter, and we show here that Flo8, Mga1, Phd1 and Ste12 actually control *ERG11* expression. It seems very likely that other *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? Fhn1, like 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. Prr2 functions as a mating inhibitor (Burchett *et al*., 2001). It is not clear how this is relevant for filamentation. The kinase 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 Mep2, which also functions as a nitrogen sensor for the transition to filamentous growth (Lorenz and Heitman, 1998; Van Nuland *et al*., 2006). Npr1 activity is regulated by the TOR pathway. The increase of *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 *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). Fhn1, Nce102 and 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. *ECM22* is downregulated upon hyperosmotic stress (Montañés *et al*., 2011). This results in reduced *ERG* gene expression and lower sterol biosynthesis, which seems to be an important adaptation mechanism for hyperosmotic stress.

In *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 *C. albicans* homologues of *ECM22*, *UPC2*, *SUT1*, *SUT2* and their targets in filamentation and virulence. *UPC2*, the sole *C. albicans* orthologue of budding yeast *ECM22* and *UPC2*, is well studied because of its role in antifungal drug resistance (Silver *et al*., 2004; MacPherson *et al*., 2005). Many clinically important antifungals target ergosterol. Azoles inhibit Erg11, which results in ergosterol depletion and the accumulation of toxic sterols (Lupetti *et al*., 2002). Several gain-of-function mutants of *UPC2* have been identified from azole-resistant clinical isolates (Dunkel *et al*., 2008; Heilmann *et al*., 2010; Hoot *et al*., 2011; Flowers *et al*., 2012). Upc2 hyperactivation leads to *ERG11* overexpression, which contributes to azole resistance. Upc2 therefore represents a potential new target for antifungal drugs (Gallo-Ebert *et al*., 2014). It is not clear whether filamentation in *C. albicans* is regulated by Upc2 and its targets in a similar way as in budding yeast. Upc2 hyperactivation results in reduced filamentation and virulence (Lohberger *et al*., 2014), which is not consistent with our model. In contrast, deletion of *NCE102*, the only *C. albicans* orthologue of budding yeast *FHN1* and *NCE102*, leads to a defect in filamentation and reduced virulence (Douglas *et al*., 2013), which is in line with our observations in budding yeast. The role of zinc cluster proteins and their targets in *C. albicans* filamentation and virulence therefore certainly needs to be further examined.

Experimental procedures

Yeast strains, plasmids and growth conditions

All yeast strains used in this study are listed in Table 1. The strains are in the Σ1278b background. Yeast strains were constructed using PCR-amplified cassettes (Wach *et al*., 1997; Longtine *et al*., 1998; Janke *et al*., 2004). Yeast strains were grown in 1% yeast extract, 2% peptone, 2% dextrose (YPD) or synthetic complete (SC) medium. Synthetic low ammonium dextrose (SLAD) medium for induction of pseudohyphal growth contains 0.67% yeast nitrogen base without amino acids and without ammonium, 2% glucose and 50 μM $(NH_4)_2SO_4$. For induction of the *GAL1* promoter, yeast cells were grown in medium with 2% galactose and 3% raffinose instead of glucose. All constructs used in this work are listed in Table 2.

Filamentation assays

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

For pseudohyphal growth assays, cells were grown overnight, and 100 cells were spread on solid SLAD medium. Plates were incubated at 30°C. Colonies were examined with a Zeiss Axioskop 2 microscope equipped with a 5 × objective and images were captured using a ProgRes C12 camera (Jenoptik).

Table 1. Yeast strains used in this study.

Name	Genotype	Source or reference
MBY16	PPY966 *ncp1Δ::klTRP1*	This study
MCY19	PPY966 *ecm22Δ::hphNT1*	This study
MCY21	PPY966 *upc2Δ::hphNT1*	This study
PC344	*MAT***a**/*MATα ura3-52/ura3-52*	Tiedje *et al.* (2008)
PPY966	*MAT***a** *his3::hisG leu2::hisG trp1::hisG ura3-52*	Tiedje *et al.* (2007)
SHY4	PPY966 *prr2Δ::His3MX6*	Foster *et al.* (2013)
SHY6	PPY966 *PRR2-9Myc-klTRP1*	This study
SHY68	PPY966 *aus1Δ::His3MX6 pdr11Δ::klTRP1*	Foster *et al.* (2013)
THY760	PPY966 *upc2Δ::hphNT1 ecm22Δ::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 *erg3Δ::His3MX6*	This study
THY808	PPY966 *npr1Δ::KanMX6*	This study
THY809	PPY966 *prr2Δ::His3MX6 npr1Δ::hphNT1*	This study
THY826	PPY966 *upc2Δ::hphNT1 ecm22Δ::His3MX6 KanMX6-pGAL1-3HA-STE12*	This study
THY827	PPY966 *erg11Δ::His3MX6*	This study
THY837	PPY966 *ERG11-9Myc-His3MX6*	This study
THY839	PPY966 *FLO8-3HA-His3MX6*	This study
THY841	PPY966 *KanMX6-pGAL1-FLO8-3HA-His3MX6*	This study
THY842	PPY966 *ste12Δ::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 10^5 cells were plated on SC medium lacking glucose 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.

Table 2. Plasmids used in this study.

Name	Genotype	Source or reference
pHU36	YEp367 carrying *pMGA1*	Foster *et al.* (2013)
pHU37	YEp367 carrying *pPRR2*	Foster *et al.* (2013)
pMC6	YEp367 carrying *pGAT2*	Foster *et al.* (2013)
pMC7	YEp367 carrying *pRHO4*	Foster *et al.* (2013)
pMC8	pNEV-N carrying *UPC2*	This study
pMC10	pNEV-N carrying *SUT2*	This study
pNEV-N	2 μm, *URA3*, *pPMA1*	Sauer and Stolz (1994)
pNF1	pNEV-N carrying *SUT1*	Ness *et al.* (2001)
pRS426	2 μm, *URA3*	Christianson *et al.* (1992)
pSH13	YEP367 carrying *pFLO11*	Foster *et al.* (2013)
pSH23	YEp367 carrying *pHAP4*	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 *pRHO3*	Foster *et al.* (2013)
pTH391	YEp367 carrying *pMSN4*	Foster *et al.* (2013)
pTH401	pRS426 carrying *NCE102*	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
YEp367	2 μm, *LEU2*, *lacZ*	Myers *et al.* (1986)

β-galactosidase assay

Densities of cell cultures were measured by optical density at 600 nm (A_{600}). Cells were harvested by centrifugation and resuspended in 1 ml Z buffer (100 mM sodium phosphate [pH 7.0], 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol). Cells were permeabilized by 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 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 Na_2CO_3. 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 $1{,}000 \times [A_{420} - (1.75 \times A_{550})]$ / reaction time (min) × culture volume (ml) × A_{600}.

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

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′CATCCTTGTATTACTCGT3′.

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′GGTGGAACGGTCTTACCCTC3′.

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The authors declare that there are no conflicts of interest.

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