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Ecm22 and Upc2 regulate yeast mating through control of expression of the mating genes *PRM1* and *PRM4*

Thomas Höfken

Division of Biosciences, Brunel University London, Uxbridge, UB8 3PH, UK

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ABSTRACT

Budding yeast mating is an excellent model for receptor-activated cell differentiation. Here we identify the related transcription factors Ecm22 and Upc2 as novel regulators of mating. Cells lacking both *ECM22* and *UPC2* display strong mating defects whereas deletion of either gene has no effect. Ecm22 and Upc2 positively regulate basal expression of *PRM1* and *PRM4*. These genes are strongly induced in response to mating pheromone, which is also largely dependent on *ECM22* and *UPC2*. We further show that deletion of *PRM4* like *PRM1* results in markedly reduced mating efficiency. Expression of *PRM1* but not of *PRM4* is also regulated by Ste12, a key transcription factor for mating. *STE12* deletion lowers basal *PRM1* expression, whereas *STE12* overexpression strongly increases *PRM1* levels. This regulation of *PRM1* transcription is mediated through three Ste12-binding sites in the *PRM1* promoter. Simultaneous deletion of *ECM22* and *UPC2* as well as mutation of the three Ste12-binding sites in the *PRM1* promoter completely abolishes basal and pheromone-induced *PRM1* expression, indicating that Ste12 and Ecm22/Upc2 control *PRM1* transcription through distinct pathways. In summary, we propose a novel mechanism for budding yeast mating. We suggest that Ecm22 and Upc2 regulate mating through the induction of the mating genes *PRM1* and *PRM4*.

1. Introduction

The mating response of the unicellular budding yeast *Saccharomyces cerevisiae* is an ideal model for the study of receptor-activated cell differentiation [1–3]. Two different haploid cell types of budding yeast exist, termed **a** cells and α cells. When two cells of these opposite mating are in close proximity they can fuse to become diploid. The two haploid cell types secrete different peptide pheromones (**a**-factor and α-factor) which bind to a G protein-coupled receptor in the plasma membrane of the opposite cell type. This triggers the activation of a mitogen-activated protein kinase (MAPK) pathway which ultimately results in the induction of mating-specific genes, cell cycle arrest, polarized growth towards the mating partner and cell fusion. The transcription factor Ste12 plays a key role in the increased expression of mating-specific genes in response to pheromone [4–6].

In this study, we show that the transcription factors Ecm22 and Upc2 are novel regulators of mating. Ecm22 and the closely related Upc2 are both members of the zinc cluster protein family [7–10]. Initially, these transcription factors have been identified as key regulators of sterol import [8,9,11] and sterol biosynthesis [12,13]. Later it has been shown that Ecm22 and Upc2 also control filamentous growth [14], a response to nutrient limitation [15].

Sut1 and its paralog Sut2 are also transcription factors of the zinc cluster protein family [7,10,16]. Even though Sut1 and Sut2 are not related to Ecm22 and Upc2 they also control sterol uptake and filamentation [16–19]. Interestingly, Ecm22/Upc2 and Sut1/Sut2 regulate sterol import by induction of a very similar set of genes [13,20–24], and they share some target genes for the regulation of filamentation [14,19].

We have previously shown that Sut1/Sut2 also regulate mating [25]. Here, we show that Ecm22 and Upc2 control mating through the regulation of expression of the genes *PRM1* and *PRM4* which are strongly upregulated during mating and which are required for efficient mating.

2. Materials and methods

2.1. Yeast strains and growth conditions

All yeast strains used in this study are listed in Table 1.

E-mail address: thomas.hoefken@brunel.ac.uk.

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Simultaneous deletion of *ECM22* and *UPC2* is lethal in some yeast strains [9]. In this study, we used the Σ1278b background because here the lack of *ECM22* and *UPC2* has no effect on the growth rate [14]. Yeast strains were constructed using PCR-amplified cassettes [26–28]. Yeast strains were grown in 1% yeast extract, 2% peptone, 2% dextrose (YPD) or synthetic complete (SC) medium. For *STE12* overexpression, yeast cells were grown in medium with 2% galactose and 3% raffinose instead of glucose. To analyse gene expression in response to pheromone, cells were incubated with 10 μg/ml α-factor for 150 min.

2.2. Generation of plasmids

All plasmids used in this study are listed in Table 1. The promoter regions of *PRM1* (from −461 to +3) and *PRM4* (from −453 to +3) were amplified from chromosomal DNA using primers PRM1-1 (CGCGGATCCTACAAGGTCTATCTGATA), PRM1-2 (CCCAAGCTTCATATCATCAACGTTCAC), PRM4-1 (CGCGGATCCAATGATTAGGTGAGGGTC) and PRM4-2 (CCCAAGCTTCATCTTTAACTGTTATTT), digested with *Bam*HI and *Hin*dIII, and then cloned into the *Bam*HI and *Hin*dIII sites of YEp367 [29]. The three Ste12-binding sites in the *PRM1* promoter (from −181 to −175, from −170 to −164, and from −159 to −153) were changed from TGTTTCA to ATAAATT by a two-step site-directed mutagenesis. First, two PCR products were generated using primers PRM1-1 and PRM1-5 (ACGCACTTCAATTTATGTATAATTTATGTATAATTTATTATGTATTACCCGGACTC) and primers PRM1-2 and PRM1-6 (ACGCACTTCAATTTATGTATAATTTATGTATAATTTATTATGTATTACCCGGACTC) using chromosomal DNA as template. In a second PCR reaction the mutated *PRM1* promoter was amplified using these PCR products as templates and primers PRM1-1 and PRM1-2. The resulting PCR product was digested with *Bam*HI and *Hin*dIII, and then cloned into the *Bam*HI and *Hin*dIII sites of YEp367. The mutations were confirmed by DNA sequencing.

2.3. Quantitative mating assays

3×10^6 exponentially growing cells of each mating type were mixed and collected on nitrocellulose filters. The filters were placed on YPD plates for 5 h at 30 °C. Filters were then suspended in water and serial dilutions were plated on selective medium to determine the number of diploids. Mating efficiency was calculated as the percentage of input cells that formed diploids.

Table 1
Strains and plasmids.

Strain	Genotype	Source
MCY20	*MAT***a** *leu2::hisG ecm22Δ::kanMX6*	This study
MCY22	*MAT***a** *leu2::hisG upc2Δ::kanMX6*	This study
THY610	*MATα ura3-52*	This study
THY612	*MAT***a** *leu2::hisG*	This study
THY761	*MAT***a** *leu2::hisG ecm22Δ::kanMX6 upc2Δ::hphNT1*	This study
THY762	*MAT***a** *leu2::hisG kanMX6-pGAL1-3HA-STE12*	This study
THY862	*MAT***a** *leu2::hisG ste12Δ::hphNT1*	This study
THY866	*MAT***a** *leu2::hisG prm1Δ::kanMX6*	This study
THY868	*MATα ura3-52 prm1Δ::kanMX6*	This study
THY869	*MATα ura3-52 upc2Δ::kanMX6*	This study
THY870	*MATα ura3-52 ecm22Δ::kanMX6*	This study
THY871	*MAT***a** *leu2::hisG prm4Δ::kanMX6*	This study
THY872	*MATα ura3-52 prm4Δ::kanMX6*	This study
THY874	*MATα ura3-52 ecm22Δ::kanMX6 upc2Δ::hphNT1*	This study
Plasmid	**Genotype**	**Source**
pTH409	YEp367 carrying *pPRM1*	This study
pTH410	YEp367 carrying *pPRM4*	This study
pTH457	YEp367 carrying *pPRM1*^{Ste12a}	This study
YEp367	2 μm, *LEU2*, *lacZ*	[29]

[a] The three Ste12-binding sequences TGTTTCA in the *PRM1* promoter have each been mutated to ATAAATT.

2.4. β-Galactosidase assays

Densities of cell cultures were measured by optical density at 600 nm (A_{600}). 0.1–10 ml of 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 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 $1000 \times [A_{420} - (1.75 \times A_{550})]$/reaction time (min) × culture volume (ml) × A_{600}.

3. Results

The zinc cluster proteins Ecm22, Upc2, Sut1 and Sut2 all play important roles in sterol uptake and filamentation [10]. Since Sut1 and Sut2 also regulate mating [25], we tested whether this is the case for Ecm22 and Upc2, too. Deletion of either *ECM22* or *UPC2* had no effect on mating efficiency, whereas cells lacking both genes displayed a strong mating defect (Fig. 1A). Thus, Ecm22 and Upc2 combined have an important and hitherto unknown role in mating.

We next wanted to know how Ecm22 and Upc2 regulate mating. Both proteins control the expression of genes involved in sterol biosynthesis such as of *ERG3*, *ERG11* and *NCP1* [12–14], and sterol import including *AUS1*, *DAN1* and *PDR11* [13,20,23]. Ecm22/Upc2-dependent regulation of expression of these genes is not only important for sterol homeostasis but also for other processes such as filamentation and the hyperosmotic shock response [14,30]. However, *ERG3*, *ERG11*, *NCP1*, *AUS1*, *DAN1* and *PDR11* levels did not change in response to pheromone treatment (data not shown). It therefore seems that Ecm22 and Upc2 regulate mating through different target genes.

It has been reported that cells carrying the constitutively active *upc2-1* allele express *PRM1* and *PRM4* at increased levels [13]. Prm1 and Prm4 have been identified as transmembrane proteins that are strongly upregulated in response to pheromone [31,32]. A role in membrane fusion during mating has been well established for Prm1 [32–37]. In contrast, almost nothing is known about Prm4. We therefore examined whether Prm4 is actually involved in mating. Like *PRM1* [32], *PRM4* is required for efficient mating (Fig. 1B).

We next wanted to know whether Ecm22 and Upc2 mediate mating through regulation of *PRM1* and *PRM4* expression. *PRM1* and *PRM4* were both expressed at low levels in exponentially growing cells (Fig. 2A and B). This expression was not affected by deletion of either *ECM22* or *UPC2* but markedly reduced in the *ecm22 upc2* double mutant (Fig. 2A and B). These data suggest that both Ecm22 and Upc2 play an important role in the control of basal expression of *PRM1* and *PRM4*.

As mentioned above, *PRM1* and *PRM4* expression was strongly upregulated in response to pheromone (Fig. 3A and B) [31,32]. *PRM1* and *PRM4* induction in *ECM22* and *UPC2* single deletion strains was comparable to the wild type, in contrast to *ecm22*Δ *upc2*Δ cells in which *PRM1* and *PRM4* induction was considerably reduced (from 14.5- to 3.7-fold for *PRM1* and from 7.3- to 2-fold for *PRM4*) (Fig. 3A and B). Thus, Ecm22 and Upc2 are required for both basal and pheromone-induced transcription of *PRM1* and *PRM4*.

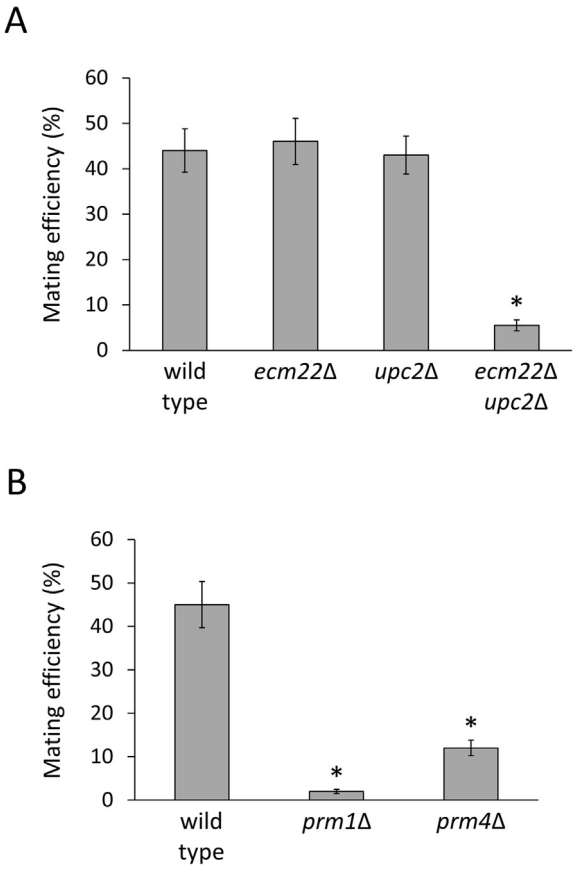

Fig. 1. *ECM22*, *UPC2* and their targets *PRM1* and *PRM4* are required for efficient mating. (A) Ecm22 and Upc2 play a role in mating. Shown is the average mating efficiency with standard deviation bars (n = 3). *, P < 0.01 compared with the wild type. (B) Deletion of either *PRM1* or *PRM4* results in reduced mating efficiency. Shown is the average with standard deviation (n = 3). *, P < 0.01 compared with the wild type.

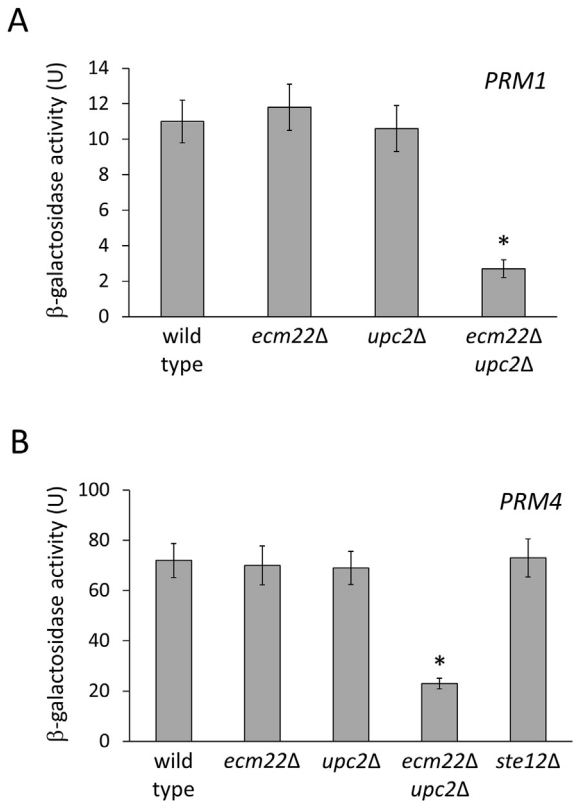

Fig. 2. Ecm22 and Upc2 regulate the expression of *PRM1* and *PRM4*. (A) Basal *PRM1* expression is strongly reduced in cells lacking both *ECM22* and *UPC2*. β-galactosidase activity was determined for the indicated strains all carrying a *PRM1-lacZ* plasmid. Bars indicate the average with standard deviation of 4 independent cultures. *, P < 0.01 compared with the wild type. (B) Deletion of both *ECM22* and *UPC2* results in decreased *PRM4* expression. Shown is the average β-galactosidase activity with standard deviation of 4 independent cultures. *, P < 0.01 compared with the wild type.

The fact that induced *PRM1* and *PRM4* expression was strongly reduced but not completely abolished in the *ecm22 upc2* double mutant (Fig. 3A and B) suggests that other factors also control the transcription of *PRM1* and *PRM4*. The transcription factor Ste12 plays a leading role in the regulation of mating gene expression [4–6]. It binds to pheromone-responsive elements (PREs) in the promoter region of its targets [38,39]. Three consensus Ste12-binding sites [38,39] can be found in the *PRM1* promoter (Fig. 4A) but to our knowledge it has not been tested whether Ste12 really regulates *PRM1* expression. *STE12* deletion resulted in a strong decrease of basal *PRM1* expression (Fig. 4B), whereas overexpression of *STE12* led to a massive increase of *PRM1* levels (Fig. 4C). In contrast, *STE12* deletion or overexpression had no effect on *PRM4* levels (Figs. 2B and 4C). Thus, Ste12 positively regulates the transcription of *PRM1* but not of *PRM4*.

We next wanted to know whether Ecm22 and Upc2 regulate *PRM1* expression independently of Ste12. For these experiments, a *STE12* deletion strain could not be used since *ste12* mutants are completely unresponsive to pheromone [40]. We therefore mutated the three Ste12-binding sites in the *PRM1* promoter (Fig. 4A) from TGTTTCA to ATAAATT. These binding sites have been shown to be sufficient for the recruitment of the *PRM1* gene to nuclear pores in response to pheromone in a Ste12-dependent manner [41] but to our knowledge it has not been demonstrated that Ste12 actually regulates *PRM1* transcription through these sites. Basal expression from the *PRM1* promoter with mutated Ste12-binding sites was strongly reduced and comparable to expression from the wild type *PRM1* promoter in the *STE12* deletion strain (Fig. 4B). *STE12* overexpression had no effect on expression from the *PRM1* promoter lacking the Ste12-binding sites (Fig. 4C). These observations indicate that Ste12 controls *PRM1* transcription through these three sites.

As mentioned above, basal *PRM1* levels were reduced in the *ecm22 upc2* double mutant and when expressed from the *PRM1* promoter lacking Ste12-binding sites but in both cases expression was readily detectable (Figs. 2A, 3A and 4B). In contrast, expression from the *PRM1* promoter without Ste12-binding sites in *ecm22*Δ *upc2*Δ cells was completely abolished (Fig. 3A), suggesting that Ecm22/Upc2 and Ste12 regulate *PRM1* expression independently. Using the *PRM1* promoter lacking Ste12-binding sites, pheromone induction was greatly reduced compared to the wild type (from 14.5-fold to 3-fold). In cells lacking both *ECM22* and *UPC2*, expression from the mutated *PRM1* promoter following pheromone treatment was not detectable (Fig. 3A). Taken together, these data suggest that Ecm22/Upc2 and Ste12 are equally important for basal and pheromone-induced *PRM1* expression, and that Ecm22/Upc2 and Ste12 act through separate pathways.

4. Discussion

The transcription factors Ecm22 and Upc2 have an important and well established role in sterol biosynthesis and sterol uptake [10]. Here, we show that Ecm22 and Upc2 also regulate mating.

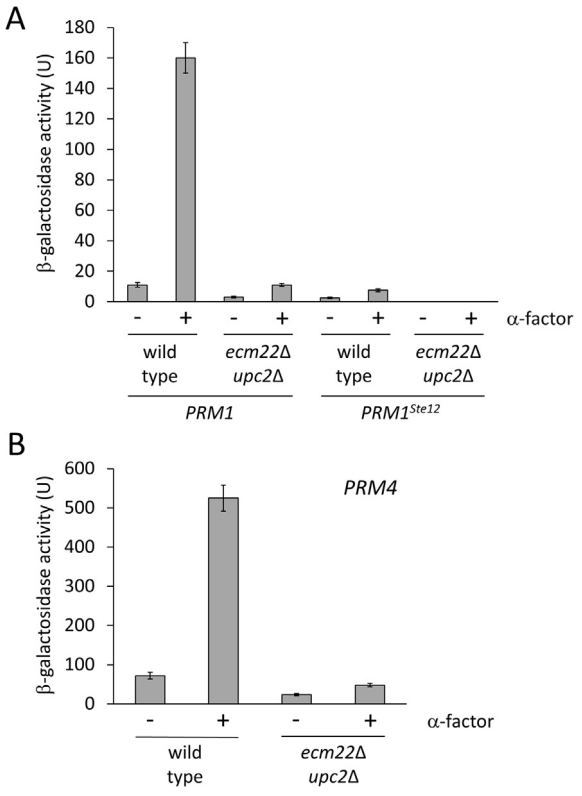

Fig. 3. Pheromone-induced transcription of *PRM1* and *PRM4* depends on *ECM22* and *UPC2*. (A) *PRM1* induction in response to pheromone requires Ste12 as well as Ecm22 and Upc2. β-galactosidase activity was determined for the indicated strains carrying either the wild type *PRM1* promoter (*PRM1*) or the *PRM1* promoter in which the three Ste12-binding sites have been mutated (*PRM1*^{Ste12}). Bars indicate the average with standard deviation of four independent cultures. (B) *PRM4* expression in the presence and absence of pheromone was quantified in the indicated strains using β-galactosidase assays. Shown is the average β-galactosidase activity with standard deviation of 4 independent cultures.

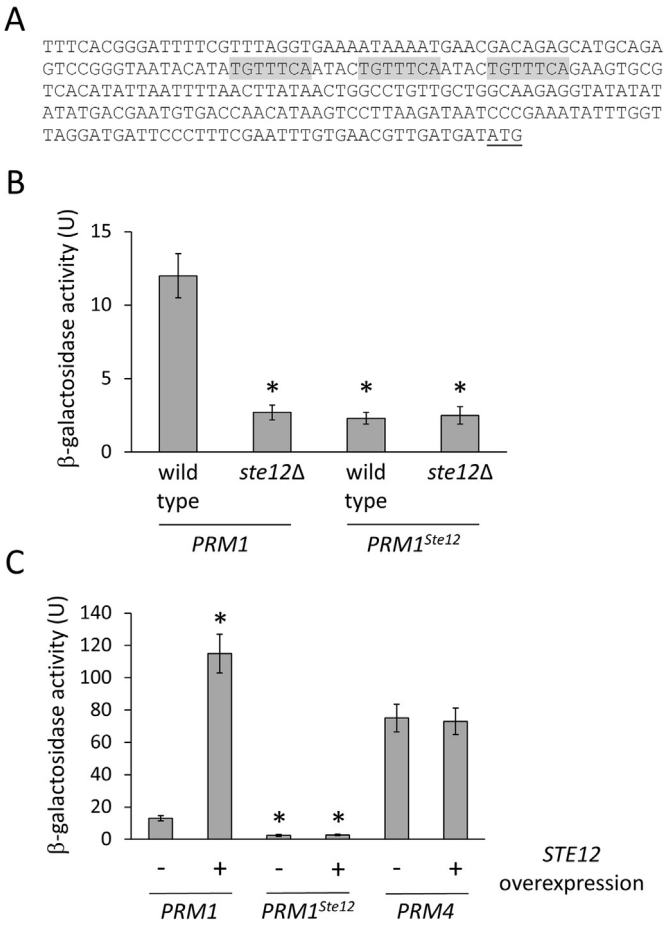

Fig. 4. Ste12 regulates *PRM1* transcription through three binding sites in the *PRM1* promoter. (A) Sequence of the *PRM1* promoter. Consensus Ste12-binding sites are highlighted in grey. The start codon is underlined. (B) Ste12 is required for basal *PRM1* expression. β-galactosidase activity was determined for the indicated strains either harboring the wild type *PRM1* promoter (*PRM1*) or the *PRM1* promoter lacking the three Ste12-binding sites (*PRM1*^{Ste12}). Shown is the average β-galactosidase activity with standard deviation of 4 independent cultures. *, $P < 0.01$ compared with the wild type *PRM1* promoter in the wild type strain. (C) *STE12* overexpression increases levels of *PRM1* but not of *PRM4*. β-galactosidase activity was measured for the wild type strain and cells overexpressing *STE12* from the *GAL1* promoter carrying either the wild type *PRM1* promoter (*PRM1*), the *PRM1* promoter lacking the three Ste12-binding sites (*PRM1*^{Ste12}) or the *PRM4* promoter (*PRM4*). Shown is the average β-galactosidase activity with standard deviation of 4 independent cultures. *, $P < 0.01$ compared with the wild type *PRM1* promoter in cells without *STE12* overexpression.

Deletion of either *ECM22* or *UPC2* alone has no effect on mating efficiency or expression of mating genes, whereas cells lacking both genes exhibit strong phenotypes. This suggests that the related proteins Ecm22 and Upc2 have overlapping functions in mating. Such a Ecm22-Upc2 redundancy has previously been shown for other processes such as filamentation [14].

Sterol biosynthesis genes are among the most important targets of Ecm22 and Upc2 [10]. However, expression of sterol biosynthesis genes does not change in response to pheromone (data not shown). This is in line with observations that while sterol composition is crucial for mating [42–45], sterol levels do not change in response to pheromone [46]. This is different from other processes regulated by Ecm22 and Upc2. During filamentation sterol biosynthesis genes and as a consequence sterol levels are upregulated [14], whereas the reverse can be observed in response to hyperosmotic stress [30].

We suggest that Ecm22 and Upc2 mediate mating through induction of *PRM1* and *PRM4*. In this study, we show that Ecm22 and Upc2 play an important role in basal and pheromone-induced transcription of *PRM1* and *PRM4*. The role of Prm1 in membrane fusion during mating has been well characterized [32–37]. Unfortunately, very little is known about Prm4. Since Prm4 is strongly upregulated in response to pheromone and because it contains a predicted transmembrane domain it is a good mating factor candidate [31,32]. Here, we show here that Prm4 like Prm1 is indeed required for efficient mating. Ecm22 and Upc2 also play a role in filamentation, another differentiation process [14]. However, deletion of *PRM1* or *PRM4* does not affect filamentous growth (data not shown). Furthermore, unlike target genes of Ecm22 and Upc2 that are involved in filamentation [14,19], *PRM1* and *PRM4* expression does not change during filamentation (data not shown). These observations suggest that Ecm22 and Upc2 regulate *PRM1* and *PRM4* specifically in mating and not during filamentation.

Around 200 genes are induced in response to pheromone, and almost all mating genes seem to be regulated through the transcription factor Ste12 [6]. However, here we show that *PRM4* expression is regulated by Ecm22 and Upc2, and *PRM4* levels are not affected by *STE12* deletion or overexpression. Furthermore, no Ste12-binding sites could be identified in the *PRM4* promoter (data not shown). Thus, it seems unlikely that Ste12 controls *PRM4* transcription. Instead, basal and pheromone-induced *PRM4* expression is regulated by Ecm22 and Upc2. For *PRM1* we could show that its basal expression and pheromone induction is under control of Ste12, Ecm22 and Upc2. Mutation of Ste12-binding sites

in the *PRM1* promoter or simultaneous deletion of *ECM22* and *UPC2* both markedly reduce *PRM1* expression. In the absence of *ECM22*, *UPC2* and Ste12 sites in the *PRM1* promoter, *PRM1* expression is no longer detectable. This additive effect suggests that Ecm22/Upc2 and Ste12 regulate *PRM1* through independent pathways.

It remains unknown how Ecm22 and Upc2 are activated in response to pheromone. Very little is known about Ecm22 regulation. Upc2 is regulated through sterol concentration and at the transcriptional level [10]. Upc2 acts as a sterol sensor [23,47,48]. When sterol levels drop, Upc2 translocates from the cytoplasm to the nucleus where it controls gene expression. However, it is unlikely that this is a mechanism for Upc2 activation during mating because sterol levels do not change following pheromone treatment [46]. Control of *UPC2* transcription is another important regulatory mechanism. Upc2 stimulates its own expression [13,20] and *UPC2* expression is also regulated by Sut1 and Sut2 [14]. However, transcriptional regulation can also be ruled out since expression of *UPC2* and *ECM22* is not affected by pheromone treatment (data not shown). During mating Ecm22 and Upc2 therefore seem to be regulated through a different yet unknown mechanism.

In conclusion, we have established that Ecm22 and Upc2 are novel mating factors that seem to act independently of Ste12 inducing expression of the mating genes *PRM1* and *PRM4*. Further research will show whether Ecm22 and Upc2 have other target genes, what functions these targets have, and how Ecm22 and Upc2 are regulated during mating.

Conflicts of interest

The author declares no conflicts of interest.

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Transparency document

Transparency document related to this article can be found online at https://doi.org/10.1016/j.bbrc.2017.10.005.

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