Differential requirement of the transcription factor Mcm1 for activation of the 
*Candida albicans* multidrug efflux pump *MDR1* by its regulators Mrr1 and Cap1

Running title: Role of Mcm1 in *MDR1* expression in *C. albicans*

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Abstract

Overexpression of the multidrug efflux pump Mdr1 causes increased fluconazole resistance in the pathogenic yeast *Candida albicans*. The transcription factors Mrr1 and Cap1 mediate *MDR1* upregulation in response to inducing stimuli, and gain-of-function mutations in Mrr1 or Cap1, which render the transcription factors hyperactive, result in constitutive *MDR1* overexpression. The essential MADS box transcription factor Mcm1 also binds to the *MDR1* promoter, but its role in inducible or constitutive *MDR1* upregulation is unknown. Using a conditional mutant in which Mcm1 can be depleted from the cells, we investigated the importance of Mcm1 for *MDR1* expression. We found that Mcm1 was dispensable for *MDR1* upregulation by H$_2$O$_2$, but required for full *MDR1* induction by benomyl. A C-terminally truncated, hyperactive Cap1 could upregulate *MDR1* expression both in the presence and absence of Mcm1. In contrast, a hyperactive Mrr1 containing a gain-of-function mutation depended on Mcm1 to cause *MDR1* overexpression. These results demonstrate a differential requirement of the coregulator Mcm1 for Cap1- and Mrr1-mediated *MDR1* upregulation. When activated by oxidative stress or a gain-of-function mutation, Cap1 can induce *MDR1* expression independently of Mcm1, whereas Mrr1 requires either Mcm1 or an active Cap1 to cause overexpression of the *MDR1* efflux pump. Our findings provide more detailed insight into the molecular mechanisms of drug resistance in this important human fungal pathogen.
Introduction

Overexpression of the multidrug efflux pump Mdr1 is one mechanism by which the pathogenic yeast *Candida albicans* can develop increased resistance to the antifungal drug fluconazole, which is widely used to treat *Candida* infections (reviewed in ref. (13)). The *MDR1* gene is not significantly expressed in drug-susceptible *C. albicans* strains under standard growth conditions, but its transcription can be induced by certain toxic compounds, like benomyl or H$_2$O$_2$ (6, 7, 9, 17). Many fluconazole-resistant, clinical *C. albicans* isolates constitutively upregulate *MDR1* under noninducing conditions, and all such isolates studied to date contain gain-of-function mutations in the zinc cluster transcription factor Mrr1 (3, 14).

*MRR1* plays a central role in *MDR1* expression, as its deletion abolishes both the inducible activation of the *MDR1* promoter in drug-susceptible strains and the constitutive *MDR1* overexpression in fluconazole-resistant, clinical isolates (14).

In addition to Mrr1, other transcription factors have also been implicated in the regulation of *MDR1* expression (1, 4, 16, 17, 20, 26, 27). The bZip transcription factor Cap1, which mediates oxidative stress responses in *C. albicans*, is required for the induction of *MDR1* transcription by H$_2$O$_2$ and also contributes to benomyl-induced *MDR1* expression (17, 26), (Schubert et al., in revision). Therefore, Mrr1 and Cap1 upregulate *MDR1* in response to inducing chemicals in a cooperative fashion. However, Cap1 is dispensable for the constitutive *MDR1* overexpression in strains containing gain-of-function mutations in Mrr1 (1, 17), (Schubert et al., in revision). Conversely, a C-terminally truncated, hyperactive Cap1 can also activate the *MDR1* promoter in the absence of *MRR1*, albeit less efficiently than in a wild-type background (Schubert et al., in revision). Hyperactive forms of either Mrr1 or Cap1 can therefore promote *MDR1* overexpression independently of each other.

Deletion analyses of the *MDR1* promoter identified a region that is important for benomyl-induced *MDR1* upregulation as well as for constitutive *MDR1* overexpression (16, 17). This
region, which was termed BRE (for benomyl response element) or MDRE (for MDR1 drug resistance element) contains a binding site for the MADS box transcription factor Mcm1. When inserted into a heterologous promoter, the BRE/MDRE rendered the promoter responsive to benomyl in a drug-susceptible strain and constitutively active in an MDRI overexpressing strain background (16, 17). Mcm1 is involved in a variety of cellular processes and mediates both repressing and activating functions, presumably by recruiting coregulatory proteins to the respective promoters (18, 23). Mcm1 has been shown to bind to the MDRI promoter in vivo (11, 23), where it may act together with Mrr1 and/or Cap1 to control expression of the efflux pump in response to inducing stimuli and enable constitutive MDRI overexpression in strains containing hyperactive MRR1 or CAP1 alleles.

As MCM1 is essential in C. albicans, the role of this transcription factor in MDRI regulation can not be addressed by deleting the gene. A conditional mcm1 mutant that contains a single copy of MCM1 under the control of a tetracycline-repressible promoter has therefore been used to analyze the function of Mcm1 in C. albicans. The addition of doxycycline to the conditional mutant results in depletion of Mcm1 within 3 h, but the cells remain viable for a prolonged period, allowing to study their behaviour in the absence of Mcm1 (18). It was shown that depletion of Mcm1 from the cells resulted in the loss of binding activity of protein extracts to an MDRI promoter fragment containing the MDRE (16). However, whether Mcm1 is indeed important for the regulation of MDRI expression and if it acts as an activator or a repressor of the MDRI promoter was not directly tested in that study. To understand in more detail how the expression of this multidrug efflux pump is controlled in C. albicans, we investigated if Mcm1 is necessary for the upregulation of MDRI in response to inducing chemicals and if hyperactive forms of Mrr1 and Cap1 require Mcm1 as a coregulator to mediate MDRI overexpression.
Materials and Methods

Strains and growth conditions

The *C. albicans* strains used in this study are listed in Table 1. All strains were stored as frozen stocks with 15% glycerol at -80°C and subcultured on YPD agar plates (10 g yeast extract, 20 g peptone, 20 g glucose, 15 g agar per liter) at 30°C. Strains were routinely grown in YPD liquid medium at 30°C in a shaking incubator. For selection of nourseothricin-resistant transformants, 200 µg/ml nourseothricin (Werner Bioagents, Jena, Germany) was added to YPD agar plates. To obtain nourseothricin-sensitive derivatives in which the SAT1 flipper cassette was excised by FLP-mediated recombination, transformants were grown overnight in YPM medium (10 g yeast extract, 20 g peptone, 20 g maltose per liter) without selective pressure to induce the MAL2 promoter that controls expression of the *caFLP* gene in the SAT1 flipper cassette. One hundred to 200 cells were then spread on YPD plates containing 10 µg/ml nourseothricin and grown for 2 days at 30°C. Nourseothricin-sensitive clones were identified by their small colony size and confirmed by restreaking on YPD plates containing 200 µg/ml nourseothricin.

Strain construction

*C. albicans* strains were transformed by electroporation (10) with the following gel-purified linear DNA fragments: A KpnI-SacII fragment from pMPG2S (14) was used to integrate a P<sub>MDR1</sub>-GFP reporter fusion at the *ACT1* locus of strains MRcan42 and MRcan43, resulting in strains can42MPG2A and -B and can43MPG2A and -B, respectively. A SacI-Apal fragment from pMRR1R3 (Schubert et al., in revision) was used to insert the hyperactive MRR1<sup>P683S</sup> allele in place of one of the MRR1 wild-type alleles of strains MRcan42 and MRcan43 with the help of the SAT1 flipper cassette, generating strains can42MRR1R31A and -B and can43MRR1R31A and -B, respectively. The SAT1 flipper cassette was then recycled to obtain
strains can42MRR1R32A and -B and can43MRR1R32A and -B. A second round of transformation and marker recycling resulted in strains can42MRR1R34A and -B and can43MRR1R34A and -B, in which both endogenous MRR1 alleles had been replaced by the hyperactive MRR1<sup>P683S</sup> allele. In an analogous fashion, a SacI-ApaI fragment from pCAP1R1 (Schubert et al., in revision) was used to substitute the wild-type CAP1 alleles of strains MRcan42 and MRcan43 for the hyperactive CAP1<sup>ΔC33</sup> allele in two rounds of transformation and recycling of the SAT1 flipper cassette, generating strains can42CAP1R14A and -B and can43CAP1R14A and -B, respectively. Nourseothricin-resistant transformants were selected as described previously (15) and correct integration of all constructs was confirmed by Southern hybridization with the upstream and downstream flanking sequences. The introduction of the P683S mutation into the first and second MRR1 allele of the transformants was confirmed by reamplification and direct sequencing of the PCR products.

**Isolation of genomic DNA and Southern hybridization**

Genomic DNA from *C. albicans* strains was isolated as described previously (15). The DNA was digested with appropriate restriction enzymes, separated on a 1% agarose gel, and, after ethidium bromide staining, transferred by vacuum blotting onto a nylon membrane and fixed by UV crosslinking. Southern hybridization with enhanced chemiluminescence-labeled probes was performed with the Amersham ECL<sup>TM</sup> Direct Nucleic Acid Labelling and Detection System (GE Healthcare, Braunschweig, Germany) according to the instructions of the manufacturer.

**Analysis of MDR1 promoter activity by FACS analysis**

YPD overnight cultures of GFP reporter and parental control strains were each diluted 10<sup>-2</sup> in six Erlenmeyer flasks containing 50 ml YPD medium (three without and three with 20 µg/ml doxycycline). After 3 h of growth at 30°C, 50 µg/ml benomyl or 0.005% H<sub>2</sub>O<sub>2</sub> was added to
two of the cultures (in both cases with and without doxycycline) to induce MDR1 expression; the third culture in each case was left untreated. After 60 min of further incubation the mean fluorescence of the cells was determined by flow cytometry. Fluorescence-activated cell sorter (FACS) analysis was performed with a FACSCalibur cytometry system equipped with an argon laser emitting at 488 nm (Becton Dickinson, Heidelberg, Germany). Fluorescence was measured on the FL1 fluorescence channel equipped with a 530-nm band-pass filter. Twenty thousand cells were analyzed per sample. Fluorescence data were collected by using logarithmic amplifiers. The mean fluorescence (arbitrary values) was determined with CellQuest Pro (Becton Dickinson) software.

Analysis of doxycycline-induced MCM1 repression by western immunoblotting

Strains can42MPG2A and -B were grown as described above for determining GFP expression. Whole cell protein extracts were prepared from two cultures grown for 3 h in the presence or absence of doxycycline and from the remaining cultures after further incubation for 60 min in the presence of benomyl or H2O2 (with and without doxycycline). Cells were collected by centrifugation, washed twice in water, and broken by vortexing for 10 min at 4°C with 300 µl 0.5-mm glass beads in 300 µl breaking buffer (100 mM Tris-Cl [pH 7.5], 200 mM NaCl, 20% glycerol, 5 mM EDTA, 4% of a Complete EDTA-free Protease Inhibitor Cocktail stock solution [Roche Diagnostics GmbH, Mannheim, Germany], 0.1% β-mercaptoethanol). Samples were centrifuged at 13,000 rpm for 5 min at 4°C, the supernatant collected, and the protein concentration quantified with a NanoDrop 1000 (Thermo Fisher Scientific, Wilmington, MA). Extracts were heated at 65°C for 10 min and 400 µg total protein of each sample was separated on an SDS-12% polyacrylamide gel. Proteins were transferred onto a nitrocellulose membrane with a Trans-Blot SD Semi-Dry transfer apparatus (Bio-Rad, Munich, Germany). For detection of Mcm1-Myc, a monoclonal anti-c-Myc antibody (purified mouse immunoglobulin, clone 9E10, Sigma-Aldrich Chemie GmbH, product number
M 4439) was used as primary antibody at a dilution of 1:6000, and goat anti-mouse IgG (Fab-specific) peroxidase conjugate (Sigma A 9917) was used as second antibody at a dilution of 1:6000. Blots were developed using a chemiluminescence detection system (GE Healthcare UK Limited, Chalfont, UK) under conditions recommended by the manufacturer.

**Analysis of MDR1 expression by quantitative real-time reverse transcription PCR**

YPD overnight cultures of strains SC5314, MRcan42, MRcan43, can42MRR1R34A and -B, can43MRR1R34A and -B, can42CAP1R14A and -B, and can43CAP1R14A and -B were diluted 10^{-2} in 50 ml fresh YPD medium without or with 20 µg/ml doxycycline and incubated for 4 h at 30°C. RNA was extracted by the hot phenol method (2) combined with a purification step with the RNeasy kit (Quiagen, Hilden, Germany). Contaminating DNA was removed by treatment with the Ambion Turbo DNA-free kit (Applied Biosystems, Darmstadt, Germany) and cDNA was prepared from the RNA with the SuperScript® III Reverse Transcriptase kit (Invitrogen, Karlsruhe, Germany). Generation of cDNA and absence of genomic DNA was controlled by a PCR reaction with the primers EFB1A (5’-ATTGAACGAATTCTTGGCTGAC-3’) and EFB1B (5’-CATCTTCTTCAACAGCAGCTTG-3’), which bind outside of the EFB1 intron and yield a PCR product of 0.55 kb from cDNA and a 0.8 kb PCR product from genomic DNA. qPCR was performed with the iQ™ SYBR® Green Supermix (Bio-Rad) and the primer pairs MDR5RT (5’-ATTTGTTTCAGATCAGTCATTGCTTGCTGACGTGT-3’) and MDR6RT (5’-GGTCCGTTCAAGTAAAACAAAACTGGAATA-3’) for MDR1 and ACT1RT (5’-AGTGTGACATGGATGTTAGAAAAGAATTATACGG-3’) and ACT2RT (5’-ACAGAGTATTTTCTTCTGGTGGAAG-3’) for ACT1, which served as the reference, using the following conditions: an initial denaturation for 3 min at 95°C, 40 cycles of 30 s 95°C, 40 s 50°C, 10 s 72°C, one cycle of 30 s 95°C, 30 s 57°C. Melt curves were generated...
and Ct values calculated by the Real Time program, Bio-Rad iQ5 V2.0 optical systems software. Ct values obtained by the software were then used to calculate the relative \textit{MDR1} mRNA levels adjusted to the \textit{ACT1} mRNA levels and using \textit{MDR1} expression in the wild-type strain SC5314 as a reference (set to 1). Two independent RNA extractions, each with two technical replicates, were used to calculate means and standard deviations of the final relative expression values.
Results

Mcm1 is required for benomyl-induced, but not H$_2$O$_2$-induced MDR1 expression

To monitor activation of the MDR1 promoter in response to inducing chemicals in the presence and absence of Mcm1, we introduced a $P_{MDR1}$-GFP reporter fusion into the conditional mcm1 mutant MRcan42, which contains a single, Myc-tagged MCM1 allele under the control of a tetracycline-repressible promoter. In addition, the reporter fusion was introduced into the control strain MRcan43, which contains both a tetracycline-repressible MCM1 copy and a Myc-tagged MCM1 allele with its own promoter. Two independent transformants of each parental strain were used for further analysis.

We first confirmed that Mcm1 could be efficiently depleted by addition of doxycycline to the conditional mutants carrying the reporter fusion and that the presence of the MDR1 inducers benomyl and H$_2$O$_2$ did not affect doxycycline-mediated repression. As can be seen in Fig. 1, Mcm1 was not detectably expressed after 3 h of growth in the presence of doxycycline, and the addition of benomyl or H$_2$O$_2$ did not alleviate this repression.

We then determined MDR1 promoter activity by quantifying the fluorescence of the reporter strains. As previously reported (14), MDR1 was not detectably expressed in the wild-type strain SC5314 in YPD medium, but could be induced by H$_2$O$_2$ and, even more efficiently, by benomyl (Fig. 2). The presence of doxycycline did not affect the activity of the MDR1 promoter under noninducing or inducing conditions in the wild-type background. H$_2$O$_2$ also induced the MDR1 promoter in the conditional mutant MRcan42, albeit somewhat less efficiently than in strain SC5314, and this induction was also observed after depletion of Mcm1. In contrast, Mcm1 depletion resulted in a reduced induction of the MDR1 promoter by benomyl, as the fluorescence of the conditional mutants was decreased in the presence of doxycycline. Doxycycline had little effect on benomyl-induced MDR1 expression in the
control strain MRcan43. These results demonstrate that Mcm1 is dispensable for the induction of *MDR1* expression by H$_2$O$_2$, but required for full *MDR1* induction by benomyl.

**Mcm1 is required for *MDR1* overexpression by hyperactive Mrr1, but not by hyperactive Cap1**

The induction of the *MDR1* promoter by H$_2$O$_2$ requires the bZip transcription factor Cap1, which is activated by oxidative stress (17, 26, Schubert et al., in revision). Cap1 also contributes to, but is not essential for benomyl-induced *MDR1* expression (17, 26, Schubert et al., in revision). In contrast, the zinc cluster transcription factor Mrr1 is indispensable for *MDR1* expression in the presence of either of these inducers (14). As Mcm1 was necessary for full benomyl-induced, but not for H$_2$O$_2$-induced *MDR1* expression, we investigated whether Mcm1 was required for the constitutive *MDR1* overexpression caused by hyperactive forms of Mrr1 and Cap1. To this aim, we introduced the P683S gain-of-function mutation (14) into both resident *MRR1* alleles of the conditional *mcm1* mutant MRcan42 and the control strain MRcan43. In addition, both wild-type *CAP1* alleles of these strains were replaced by the C-terminally truncated, hyperactive *CAP1*$_{∆C433}$ allele. Homozygous strains were generated because the activating mutations in *MRR1* and *CAP1* have a stronger effect on *MDR1* expression when they are present in both alleles (S. Schubert et al., in revision). Two independent series of mutants (A and B) were constructed in each case and used for further analysis.

As the *MDR1* promoter is constitutively activated in strains expressing hyperactive Mrr1 or Cap1, *GFP* was not useful as a reporter gene to measure the dependence of *MDR1* expression on Mcm1, because considerable amounts of the relatively stable GFP protein would remain in the cells after shutting off *MCM1* expression by the addition of doxycycline. We therefore measured *MDR1* mRNA levels in the presence and absence of doxycycline by quantitative RT-PCR. As can be seen in Fig. 3, *MDR1* was constitutively expressed at high levels in
strains carrying the \( \text{CAP1}^{\Delta C433} \) allele (between 250- and 400-fold higher than in the control strain SC5314) and Mcm1 depletion in the conditional \( \text{mcm1} \) mutants by doxycycline did not significantly reduce \( \text{MDR1} \) mRNA levels. \( \text{MDR1} \) expression was even higher in strains containing the hyperactive \( \text{MRR1}^{P683S} \) allele (between 1200- and 2200-fold higher than in the control strain SC5314). Mcm1 depletion by doxycycline resulted in a drastic reduction of \( \text{MDR1} \) mRNA levels in the conditional mutants (between 20- and 30-fold). A moderate reduction of \( \text{MDR1} \)-mRNA levels (ca. 2.5-fold) was also observed in the control strains, presumably because strain MRcan43 also strongly overexpresses \( \text{MCM1} \) from the Tet promoter and exhibits only low \( \text{MCM1} \) expression levels from the wild-type allele after the addition of doxycycline. These results indicate that a hyperactive Cap1 can mediate \( \text{MDR1} \) overexpression independently of Mcm1, whereas a hyperactive Mrr1 requires Mcm1 to promote \( \text{MDR1} \) expression.
The transcription factors Mrr1 and Cap1 are both involved in the induction of *MDR1* expression in response to inducing chemicals, and activating mutations in either of the two transcription factors result in constitutive overexpression of the efflux pump (1, 3, 14, 17, 26). However, little is known about how Mrr1 and Cap1 activate *MDR1* transcription and which additional regulatory factors may be required. In this study we have addressed the role of the MADS box transcription factor Mcm1, which has previously been implicated in the regulation of *MDR1* expression and was recently shown to bind to the *MDR1* promoter in vivo (11, 16, 17, 23).

Mcm1 can act as both a positive and a negative regulator of transcription (18, 23). We found that depletion of Mcm1 did not result in constitutive activity of the *MDR1* promoter, indicating that the role of Mcm1 is not that of a repressor of *MDR1* expression in the absence of inducing conditions (Fig. 2 and 3). Depletion of Mcm1 also did not affect the inducibility of the *MDR1* promoter by H$_2$O$_2$ or the constitutive *MDR1* overexpression caused by a hyperactive Cap1. On the other hand, benomyl-induced *MDR1* expression was reduced, but not abolished, after depletion of Mcm1, and a hyperactive Mrr1 could not upregulate *MDR1* in the absence of Mcm1. The *MDR1* transcripts that were still present after Mcm1 depletion in strains expressing the hyperactive Mrr1 may represent mRNAs that were produced before Mcm1 depletion and not yet degraded. Alternatively, the cells might have contained residual amounts of Mcm1 that were not detectable by western blot analysis (see Fig. 1). These results suggest that the induction of *MDR1* expression by H$_2$O$_2$ occurs mainly via activation of Cap1, which acts independently of Mcm1, and the induction by benomyl occurs partially via Mrr1, which depends on Mcm1 to activate the *MDR1* promoter, and partially via Cap1. Like H$_2$O$_2$, benomyl causes oxidative stress and activates Yap1, the homolog of Cap1, in *Saccharomyces cerevisiae* (12). In response to oxidative stress, conserved cysteine residues in the C-terminus
of Yap1 form disulfide bonds, resulting in conformational changes that interrupt the interaction of Yap1 with the nuclear export protein Crm1, so that Yap1 can accumulate in the nucleus and activate its target genes (24). In C. albicans, Cap1 activity is also regulated by nuclear-cytoplasmic shuttling and mutation of one of the conserved cysteines in Cap1 or removal of the C-terminus results in constitutive Cap1 activity (1, 25).

Based on the results of this and previous studies (1, 8, 11, 14, 16, 17, 23, 26, Schubert et al., in revision), the model shown in Fig. 4 depicts the involvement of Mrr1, Cap1, and Mcm1 in four scenarios in which *MDR1* expression is upregulated. In the presence of H$_2$O$_2$ (Fig. 4A), Cap1 is activated, accumulates in the nucleus, and induces *MDR1* expression. Under these conditions, Cap1 requires Mrr1 to activate the *MDR1* promoter, but Mcm1 is dispensable. In the presence of benomyl (Fig. 4B), Cap1 is also activated by oxidative stress and can induce *MDR1* expression together with Mrr1 independently of Mcm1 (a). However, *MDR1* induction by benomyl can also occur at a reduced level in the absence of Cap1 in an Mrr1-dependent fashion, and this requires the presence of Mcm1 (b). How benomyl activates Mrr1 is not known. It is possible that Mrr1 is activated by direct drug binding, similar to the zinc cluster transcription factors Pdr1 and Pdr3, which regulate efflux pump expression in *S. cerevisiae* and *Candida glabrata* (22), but benomyl may also activate Mrr1 in an indirect fashion. The C-terminally truncated, hyperactive Cap1 can upregulate *MDR1* expression even in the absence of Mrr1, but not as efficiently as in a wild-type background (Schubert et al., in revision) (Fig. 4C). In this respect, the hyperactive Cap1 differs from activated wild-type Cap1, which requires Mrr1 to induce the *MDR1* promoter in response to H$_2$O$_2$ or benomyl. Similar to oxidative stress-activated Cap1, however, the hyperactive Cap1 does not depend on Mcm1 to induce *MDR1* expression. Finally, a hyperactive Mrr1 can promote *MDR1* overexpression independently of Cap1, but in this case it requires Mcm1 (Fig. 4D). Altogether, these results indicate that Mrr1 can upregulate *MDR1* expression in cooperation with either Cap1 or
Mcm1. In response to oxidative stress, Mrr1 cooperates mainly with activated Cap1 to induce the \textit{MDR1} promoter and Mcm1 is largely dispensable. In the absence of inducers, Cap1 remains in the cytoplasm and a hyperactive Mrr1 depends on the presence of Mcm1 to cause \textit{MDR1} overexpression. These observations are in accordance with previous findings that deletion of the putative Mcm1 binding sequence (the BRE/MDRE, see introduction) from a truncated \textit{MDR1} promoter abolished benomyl-induced or constitutive \textit{MDR1} overexpression (16, 17). However, deletion of the BRE/MDRE from the full-length \textit{MDR1} promoter did not affect its constitutive activity in strains that contained gain-of-function mutations in Mrr1 (3, 8, 16). The result of our present study showing that a hyperactive Mrr1 requires Mcm1 to mediate \textit{MDR1} overexpression indicates that Mcm1 can contribute to \textit{MDR1} expression in a manner that is independent of its binding site in the BRE/MDRE. This may occur either by binding of Mcm1 to an additional region in the \textit{MDR1} promoter or by interacting with bound Mrr1 without itself binding to the DNA. Mcm1 has also been found to bind to the \textit{MRR1} promoter, indicating that Mcm1 can affect \textit{MDR1} expression also indirectly, by modulating the expression of \textit{MRR1} (23). In contrast, transcriptional profiling studies showed that the hyperactive Mrr1 did not affect expression of \textit{MCM1} or \textit{CAP1}, and the activated Cap1 also did not affect \textit{MCM1} or \textit{MRR1} expression levels (14, 26, Schubert et al., in revision).

An important goal for future research will be to unravel how Mrr1, Cap1, and Mcm1 activate \textit{MDR1} transcription. Depending on the conditions, Pdr1 and Pdr3 of \textit{S. cerevisiae} interact with different subunits of the mediator complex to recruit RNA polymerase II to the promoters of their target genes (21, 22). Mrr1 may act in a similar fashion, but it has also recently been shown that both Mrr1 and Cap1 recruit Ada2, a subunit of the SAGA/ADA coactivator complex, to induce transcription (19). It is likely that, depending on the inducing conditions and the combination of transcription factors involved, \textit{C. albicans} uses different mechanisms to upregulate expression of the \textit{MDR1} efflux pump.
Acknowledgments

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References


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Figure Legends

FIG. 1. Depletion of Mcm1 from reporter strains by treatment with doxycycline. Strains can42MPG2A and -B were grown in the absence (-) or presence (+) of doxycycline (Dox) and treated with benomyl or H$_2$O$_2$ as described in Materials and Methods. Whole cell protein extracts were prepared and analyzed by western immunoblotting with an anti-Myc antibody. The position of Myc-tagged Mcm1 is indicated; the lower band is an unspecific cross-reacting protein. The position of molecular weight markers is given on the right side of the blot. The figure shows the results for strain can42MPG2A; the same results were obtained with can42MPG2B.

FIG. 2. Activation of the MDR1 promoter by H$_2$O$_2$ and benomyl in the wild-type strain SC5314, the conditional mcm1 mutant MRcan42, and the control strain MRcan43. Parental strains and transformants carrying a P$_{MDR1}$-GFP reporter fusion were grown in the absence (-) or presence (+) of doxycycline (Dox) and treated with H$_2$O$_2$ or benomyl (Ben) as described in Materials and Methods. The mean fluorescence of the cells was determined by flow cytometry. The results obtained with two independently generated reporter strains (SCMPG2A and -B, can42MPG2A and -B, can43MPG2A and -B) are shown in each case. The background fluorescence of the parental strains, which do not contain the GFP gene, is indicated by the black part in each column.

FIG. 3. Effect of Mcm1 depletion on MDR1 overexpression mediated by hyperactive forms of Cap1 and Mrr1. The wild-type strain SC5314, the conditional mcm1 mutant MRcan42, control strain MRcan43, and independent derivatives (A and B) of MRcan42 and MRcan43 that were rendered homozygous for the CAP1$^{∆C333}$ or the MRR1$^{P683S}$ alleles were grown for 4 h in the
absence (light grey bars) or presence (dark grey bars) of doxycycline as described in Materials and Methods. \textit{MDR1} mRNA levels were determined by real time RT-PCR and are presented as relative expression levels compared to those of the reference strain SC5314 in the absence of doxycycline, which were set to 1. The graph shows the means and standard deviations of two independent experiments with duplicate measurements performed with each strain. *\textit{MDR1} expression levels in SC5314 and the parental strains MRcan42 and MRcan43 were too low to be visible in the graph.

FIG. 4. Model of the role of the transcription factors Cap1, Mrr1, and Mcm1 in \textit{MDR1} upregulation by inducing chemicals or gain-of-function mutations in Cap1 and Mrr1. Thinner bent arrows indicate reduced \textit{MDR1} promoter activity in the absence of the missing transcription factor. (A) H$_2$O$_2$ activates Cap1, resulting in accumulation of the transcription factor in the nucleus, where it can induce \textit{MDR1} expression together with Mrr1 in an Mcm1-independent fashion. (B) Benomyl activates Mrr1 in an unknown way and at least partially also Cap1. When Cap1 is available at the \textit{MDR1} promoter (a), Mrr1 and Cap1 can induce \textit{MDR1} expression independently of Mcm1. In the absence of Cap1 (b), Mrr1 requires Mcm1 to induce \textit{MDR1} expression. (C) A hyperactive form of Cap1 (labeled Cap1*) can induce the \textit{MDR1} promoter in the absence of inducing stimuli and independently of Mcm1 and Mrr1 (a), but full induction requires the presence of Mrr1 (b). (D) A hyperactive Mrr1 containing a gain-of-function mutation (labeled Mrr1*) can induce the \textit{MDR1} promoter independently of Cap1 (which is localized in the cytoplasm in the absence of inducing stimuli), but requires the coregulator Mcm1.
Table 1. *C. albicans* strains used in this study

<table>
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<tr>
<th>Strain</th>
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<td>SCMPG2A and -B</td>
<td>SC5314</td>
<td>ACT1:act1::P_{MRRI}::GFP-caSAT1</td>
<td>(14)</td>
</tr>
</tbody>
</table>
| MRcan42                 | SC5314      | ade2::hisG/ade2::hisG ura3::imm43/ura3::imm43
era1::EHO1/era1::EHO1-SS-HAP4AD-3xHA-ADE2 mcm1::URA3::97::MCM1::myc/mcm1::FRT | (18)      |
| MRcan43                 | SC5314      | ade2::hisG/ade2::hisG ura3::imm43/ura3::imm43
era1::EHO1/era1::EHO1-SS-HAP4AD-3xHA-ADE2 mcm1::URA3::97::MCM1::myc/mcm1::FRT | (18)      |
| can42MPG2A and -B       | MRcan42     | ACT1:act1::P_{MRRI}::GFP-caSAT1        | this study |
| can43MPG2A and -B       | MRcan43     | ACT1:act1::P_{MRRI}::GFP-caSAT1        | this study |
| can42MRR1R31A and -B    | MRcan42     | MRR1/MRR1P_{MRRI}::SAT1-FLIP           | this study |
| can42MRR1R32A           | can42MRR1R31A | MRRI1/MRRI1_{MRRI}::SAT1-FLIP         | this study |
| can42MRR1R32B           | can42MRR1R31B | MRR1P_{MRRI}::SAT1-FLIP/MRR1P_{MRRI}::FRT | this study |
| can42MRR1R33A           | can42MRR1R32A | MRR1P_{MRRI}::SAT1-FLIP/MRR1P_{MRRI}::FRT | this study |
| can42MRR1R33B           | can42MRR1R32B | MRR1P_{MRRI}::SAT1-FLIP/MRR1P_{MRRI}::FRT | this study |
| can42MRR1R34A           | can42MRR1R33A | MRR1P_{MRRI}::FRT/MRR1P_{MRRI}::FRT    | this study |
| can42MRR1R34B           | can42MRR1R33B | MRR1P_{MRRI}::FRT/MRR1P_{MRRI}::FRT    | this study |
| can43MRR1R31A and -B    | MRcan43     | MRR1/MRR1P_{MRRI}::SAT1-FLIP           | this study |
| can43MRR1R32A           | can43MRR1R31A | MRR1/MRR1P_{MRRI}::SAT1-FLIP           | this study |
| can43MRR1R32B           | can43MRR1R31B | MRR1P_{MRRI}::SAT1-FLIP/MRR1P_{MRRI}::FRT | this study |
| can43MRR1R33A           | can43MRR1R32A | MRR1P_{MRRI}::SAT1-FLIP/MRR1P_{MRRI}::FRT | this study |
| can43MRR1R33B           | can43MRR1R32B | MRR1P_{MRRI}::SAT1-FLIP/MRR1P_{MRRI}::FRT | this study |
| can43MRR1R34A           | can43MRR1R33A | MRR1P_{MRRI}::FRT/MRR1P_{MRRI}::FRT    | this study |
| can43MRR1R34B           | can43MRR1R33B | MRR1P_{MRRI}::FRT/MRR1P_{MRRI}::FRT    | this study |
| can42CAP1R11A           | MRcan42     | CAP1::AC33::SAT1-FLIP/CAP1::AC33::SAT1-FLIP | this study |
| can42CAP1R11B           | MRcan42     | CAP1::AC33::SAT1-FLIP/CAP1::AC33::SAT1-FLIP | this study |
| can42CAP1R12A           | can42CAP1R11A | CAP1::AC33::FRT/CAP1::AC33::FRT        | this study |
| can42CAP1R12B           | can42CAP1R11B | CAP1::AC33::FRT/CAP1::AC33::FRT        | this study |
| can42CAP1R13A           | can42CAP1R12A | CAP1::AC33::FRT/CAP1::AC33::SAT1-FLIP  | this study |
| can42CAP1R13B           | can42CAP1R12B | CAP1::AC33::FRT/CAP1::AC33::SAT1-FLIP  | this study |
| can42CAP1R14A           | can42CAP1R13A | CAP1::AC33::FRT/CAP1::AC33::FRT        | this study |
| can42CAP1R14B           | can42CAP1R13B | CAP1::AC33::FRT/CAP1::AC33::FRT        | this study |
| can43CAP1R11A and -B    | MRcan43     | CAP1::AC33::SAT1-FLIP                  | this study |
| can43CAP1R12A           | can43CAP1R11A | CAP1::AC33::SAT1-FLIP                  | this study |
| can43CAP1R12B           | can43CAP1R11B | CAP1::AC33::SAT1-FLIP                  | this study |
| can43CAP1R13A           | can43CAP1R12A | CAP1::AC33::SAT1-FLIP                  | this study |
| can43CAP1R13B           | can43CAP1R12B | CAP1::AC33::SAT1-FLIP                  | this study |
Apart from the indicated features all strains are identical to their parents. SAT1-FLIP denotes the SAT1 flipper cassette; FRT is the FLP recombination target sequence, one copy of which remains in the genome after recycling of the SAT1 flipper cassette. The CAP1 alleles in strain SC5314 were distinguished by a BglII restriction site polymorphism. The CAP1 allele containing the polymorphic BglII site in the upstream region was designated as CAP1-2.
Fig. 1

3 h YPD + 1 h Benomyl + 3 h YPD + 1 h H₂O₂

Dox - + - + - +

Mcm1-Myc

1 2 3 4 5 6

55 kDa 40 kDa 35 kDa
Fig. 2

![Bar graph showing mean fluorescence levels for different conditions: Dox, H$_2$O$_2$, and Ben with two different sets of strains: SC5314 and MRcan42, MRcan43. The x-axis represents different conditions, and the y-axis represents mean fluorescence levels. The graph indicates varying fluorescence intensities across the conditions and strains.]
Fig. 3

![Graph showing relative MDR1 expression with different conditions and treatments.

- SC3514
- MRcan42
- MRcan43
- MRcan42
- MRcan43
- CAP1 ∆C333
- MRR1 P683S

- Dox
+ Dox

Bars represent the relative expression levels with error bars indicating variability. The x-axis labels are SC3514, MRcan42, MRcan43, and CAP1 ∆C333, MRR1 P683S, while the y-axis represents the relative MDR1 expression levels ranging from 0 to 3000.
Fig. 4

A  Induction by H$_2$O$_2$

B  Induction by benomyl

C  Induction by hyperactive Cap1

D  Induction by hyperactive Mrr1