Regulation of efflux pump expression and drug resistance by the transcription factors Mrr1, Upc2, and Cap1 in Candida albicans

Running title: Transcriptional control of drug resistance in C. albicans

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Constitutive overexpression of the Mdr1 efflux pump is an important mechanism of acquired drug resistance in the yeast *Candida albicans*. The zinc cluster transcription factor Mrr1 is a central regulator of *MDR1* expression, but other transcription factors have also been implicated in *MDR1* regulation. To better understand how *MDR1*-mediated drug resistance is achieved in this fungal pathogen, we studied the interdependence of Mrr1 and two other *MDR1* regulators, Upc2 and Cap1, in the control of *MDR1* expression. A mutated, constitutively active Mrr1 could upregulate *MDR1* and confer drug resistance in the absence of Upc2 or Cap1. On the other hand, Upc2 containing a gain-of-function mutation only slightly activated the *MDR1* promoter, and this activation depended on the presence of a functional *MRR1* gene. In contrast, a C-terminally truncated, activated form of Cap1 could upregulate *MDR1* in a partially Mrr1-independent fashion. The induction of *MDR1* expression by toxic chemicals occurred independently of Upc2, but required the presence of Mrr1 and also partially depended on Cap1. Transcriptional profiling and *in vivo* DNA binding studies showed that a constitutively active Mrr1 binds to and upregulates most of its direct target genes in the presence or absence of Cap1. Therefore, Mrr1 and Cap1 cooperate in the environmental induction of *MDR1* expression in wild-type *C. albicans*, but gain-of-function mutations in either of the two transcription factors can independently mediate efflux pump overexpression and drug resistance.
Introduction

The overexpression of efflux pumps that transport endogenous metabolites as well as xenobiotics out of the cell is a common mechanism of resistance to drugs and other toxic compounds in organisms from bacteria to humans. Fungi possess two types of efflux pumps, ABC transporters and major facilitators, which use ATP or the proton gradient across the cytoplasmic membrane, respectively, to drive active transport of their substrates (9). In the pathogenic yeast *Candida albicans*, multidrug resistance is mediated mainly by the ABC transporters Cdr1 and Cdr2 and the major facilitator Mdr1 (22). These efflux pumps are usually expressed at low or nondetectable levels and are upregulated in the presence of certain chemicals. Constitutive overexpression of Cdr1 and Cdr2 or Mdr1 is frequently observed in *C. albicans* strains that have become resistant to the antifungal drug fluconazole, which inhibits ergosterol biosynthesis, especially after long-term therapy of oropharyngeal candidiasis in AIDS patients (35). The analysis of deletion mutants lacking these transporters has confirmed that their overexpression contributes to the multidrug-resistant phenotype of such strains (34, 36).

The transcription factors controlling the expression of multidrug efflux pumps in *C. albicans* have recently been identified. The zinc cluster transcription factor Tac1 mediates the upregulation of the *CDR1* and *CDR2* genes in response to inducing chemicals, and the constitutive overexpression of these efflux pumps in drug-resistant *C. albicans* strains is caused by gain-of-function mutations in Tac1 (5-8, 40). Another zinc cluster transcription factor, Mrr1, controls *MDR1* expression, and gain-of-function mutations in Mrr1 that render the transcription factor constitutively active are responsible for *MDR1* overexpression in all fluconazole-resistant strains investigated so far (10, 23, 30). Most drug-resistant strains that overexpress *CDR1* and *CDR2* or *MDR1* have become homozygous for hyperactive *TAC1* and *MRR1* alleles, respectively, because the loss of heterozygosity further increases drug
resistance once a gain-of-function mutation in these transcription factors has been acquired (5-6, 10, 23).

Mrr1 is essential for both the environmentally inducible expression of MDR1 and the constitutive overexpression of the efflux pump in drug-resistant strains. However, other transcription factors have also been implicated in the regulation of MDR1 expression. Upc2, another member of the zinc cluster transcription factor family, controls the expression of ergosterol biosynthesis (ERG) genes and mediates their upregulation in response to ergosterol depletion (20, 32). Upc2 also binds to the MDR1 promoter and, depending on the growth conditions, activates or represses MDR1 expression (41). A gain-of-function mutation in Upc2 that was found in a fluconazole-resistant, clinical C. albicans isolate and caused constitutive upregulation of ERG genes also resulted in moderately elevated MDR1 mRNA levels (11). The bZip transcription factor Cap1, which mediates the oxidative stress response in C. albicans (1, 37), was recently shown to bind to the MDR1 promoter in vivo (39). Although deletion of CAP1 in an MDR1 overexpressing strain did not reduce MDR1 transcript levels, expression of a C-terminally truncated, hyperactive CAP1 allele resulted in MDR1 upregulation and multidrug resistance (1). These findings suggest that, in addition to Mrr1, Upc2 and Cap1 might also be important regulators of MDR1 expression and activating mutations in these transcription factors could be a cause of Mdr1-mediated drug resistance in clinical C. albicans strains. To gain insight into the regulatory network controlling MDR1 expression and drug resistance in C. albicans, we investigated if Mrr1 requires Upc2 and Cap1 for upregulation of MDR1 and other Mrr1 target genes and if Upc2 and Cap1 can mediate MDR1 overexpression and drug resistance independently from Mrr1.
Materials and methods

Strains and growth conditions

The C. albicans strains used in this study are listed in Table S1. 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 20 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 100 µg/ml nourseothricin as described previously (25).

Plasmid constructions

To obtain a CAP1 deletion cassette, the CAP1 upstream region was amplified from genomic DNA of strain SC5314 by polymerase chain reaction (PCR) with the primers CAP1-5 (5'-TATTGAGCTCAGGATTGTAAACGGTGTGTC-3') and CAP1-6 (5'-AGTTCCGCGGTATCATCTAGATTTGCTGGTG-3'). The PCR product was digested at the introduced SacI and SacII sites (underlined) and cloned into plasmid pSFS4, a derivative of pSFS2 (25) in which a SalI site in the caSAT1 marker had been destroyed, to generate pCAP1M1. A fragment containing the CAP1 downstream region was then amplified with the primers CAP1-7 (5'-TTCTCTCGAGGAGGATAGTGATAAACTGC-3') and CAP1-8 (5'-
CATTTGGGCCC TGAAGACAAGAGGGAAGGG-3’), digested at the introduced XhoI and ApaI sites, and cloned between the same sites in pCAP1M1 to produce pCAP1M2.

An \textit{MDR1} deletion construct was obtained by amplifying the \textit{MDR1} upstream and downstream regions with the primer pair MDR1M1 (5’-GCTCGTTTAGTTGGGCCC ATTCGCATC-3’) and MDR1M2 (5’-GCATTCTCGAG TTCTATGTAAGTAGATGTATTGC-3’), and the primer pair MDR1M3 (5’-GATCTAAGTATGCTACCGCGG AGGTGTCATTG-3’) and MDR1M4 (5’-ATGAGAGCTC TACACCGTCTACGCGTAAAGCC-3’), respectively. The PCR products were digested with ApaI/XhoI and SacII/SacI, respectively, and cloned on both sides of the \textit{SAT1} flipper cassette in plasmid pCZF1M2 (24) to generate pMDR1M2.

Plasmid pUPC2R1, which was used to sequentially replace the \textit{UPC2} wild-type alleles by the \textit{UPC2}G648D allele with the help of the \textit{SAT1} flipper cassette, was described previously (12). Apart from the G648D gain-of-function mutation, which was originally identified in a fluconazole-resistant, clinical \textit{C. albicans} isolate, the \textit{UPC2} allele contained in pUPC2R1 is identical to one of the resident \textit{UPC2} alleles (\textit{UPC2}-I) of strain SC5314 (11). To obtain an analogous cassette for introduction of the \textit{MRR1}P683S allele, an XhoI-ApaI fragment from pZCF36M2 (23) containing the \textit{MRR1} downstream region was first substituted for the \textit{UPC2} downstream region in pUPC2R1, yielding pMRR1R1. A SacII-BglII fragment from pZCF36K3 (23) containing the \textit{MRR1}P683S allele was then inserted instead of the \textit{UPC2}G648D allele to obtain pMRR1R3. Apart from the P683S mutation, which was originally identified in a fluconazole-resistant, clinical \textit{C. albicans} isolate, the \textit{MRR1} allele contained in pMRR1R3 is identical to the \textit{MRR1} alleles of strain SC5314 (23). Similarly, for introduction of the \textit{CAP1}∆C333 allele, a C-terminally truncated \textit{CAP1} gene was first amplified from genomic DNA of strain SC5314 with the primers CAP1-1 (5’-ATATGTCGACAATGACAGATATTAAAAAGAAATTTTTTC-3’) and CAP1-4 (5’-ATATAGATCTTAAATGGAATGGAACCATTCTTGC-3’), digested at the introduced
SalI and BglII sites, and cloned into pNIM6 (24). The stop codon (reverse sequence) introduced at codon 334 of \textit{CAP1} is highlighted in bold. A SacI-BglII fragment containing the C-terminal part of the \textit{CAP1}^{\Delta333} allele was then cloned together with a BglII-XhoI fragment from pMRR1R3 containing the \textit{ACT1} transcription termination sequence (T_{\text{ACT1}}) and the \textit{SAT1}-flipper cassette into the SacI/XhoI-digested pCAP1M2 to generate pCAP1R1.

For C-terminal tagging of Mrr1 with a 3xHA epitope, the \textit{ACT1} transcription termination sequence from plasmid pMPG2 (13) was amplified with the primers HAT6 (5'-TGCTAGGATCC TACCCATACGATGTTCCGGATTACGCTTACCCATACGATGTTCCG GATTACGCTTACCCATACGATGTTCCGGATTACGCTTAAAGAGTGAAAATCTGGA-3') and URA16 (5'-TGTTTCCGCGGATACCATCCAAATCAATTCC-3'). The 3xHA epitope encoded in primer HAT6 is highlighted in italics and the stop codon in bold; the BamHI site, which creates a Gly-Ser linker, is underlined. The PCR product was digested with BamHI/SalI and cloned together with a Sacl-BamHI \textit{MRR1} fragment from pZCF36TF7-1 (23) and a SalI-ClaI \textit{caSAT1} fragment from pSAT1 (25) in the Sacl/ClaI-digested pZCF36K2 (23) to obtain pZCF36H2, in which the 3xHA-tagged \textit{MRR1} gene is placed under control of its own promoter. An EcoRI-ClaI fragment from this plasmid was then substituted for the corresponding fragment in pZCF36E2 (30) to produce pZCF36EH2, which allows expression of the 3xHA-tagged \textit{MRR1} gene from the \textit{ADH1} promoter. Substitution of the \textit{MRR1}^{P683S} allele for the wild-type \textit{MRR1} allele in pZCF36H2 and pZCF36EH2 generated plasmids pZCF36H3 and pZCF36EH3, respectively.

\textit{C. albicans} transformation

\textit{C. albicans} strains were transformed by electroporation (17) with the following gel-purified linear DNA fragments: the Sacl-ApaI fragments from pCAP1M2 and pMDR1M2 were used to delete the \textit{CAP1} and \textit{MDR1} genes, respectively, in strain SC5314. The Sacl-ApaI fragments from pMRR1R3, pUPC2R1, and pCAP1R1 were used to substitute the hyperactive
MRR1\textsuperscript{P683S}, UPC2\textsuperscript{G648D}, and CAP1\textsuperscript{\Delta C333} alleles, respectively, for the corresponding wild-type alleles in various strain backgrounds. The KpnI-SacII fragment from pMPG2S (23) was used to integrate a P\textsuperscript{MDR1}\textsuperscript{-GFP} reporter fusion at the ACT1 locus of different strains (see Table S1). The SacI-ApaI fragments from pZCF36H2 and pZCF36H3 were used for expression of 3xHA-tagged wild-type MRR1 and MRR1\textsuperscript{P683S} alleles, respectively, from the MRR1 promoter in mrr1\textsuperscript{\Delta} mutants of strain SC5314 and in the reporter strain CAG48MRR1M4B. The ApaI-SacII fragments from pZCF36EH2 and pZCF36EH3 served to express these HA-tagged MRR1 alleles from the ADH1 promoter in the same strains. The ApaI-SacII fragment from pZCF36EH3 was also used to express the 3xHA-tagged MRR1\textsuperscript{P683S} allele under control of the ADH1 promoter in mrr1\textsuperscript{\Delta} cap1\textsuperscript{\Delta} mutants of strain SC5314. Selection of nourseothricin-resistant transformants was performed as described previously (25) and the correct integration of each construct was confirmed by Southern hybridization with gene-specific probes.

Isolation of genomic DNA and Southern hybridization

Genomic DNA from C. albicans was isolated as described previously (25), 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 cross-linking. Southern hybridization with enhanced chemiluminescence-labeled probes was performed with the Amersham ECL\textsuperscript{TM} Direct Nucleic Acid Labelling and Detection System (GE Healthcare, Braunschweig, Germany) according to the instructions of the manufacturer.

FACS analysis

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 and were counted at a flow rate of 500 cells per second. Fluorescence data were collected by
using logarithmic amplifiers. The mean fluorescence values were determined with CellQuest Pro (Becton Dickinson) software.

Drug susceptibility tests

Stock solutions of the drugs were prepared as follows. Fluconazole (5 mg/ml) was dissolved in water and cerulenin (5 mg/ml) was dissolved in dimethylsulfoxide (DMSO). In the assays, serial twofold dilutions in the assay medium were prepared from an initial concentration of 100 µg/ml of each drug. Susceptibility tests were carried out in high resolution medium (14.67 g HR-Medium [Oxoid GmbH, Wesel, Germany], 1 g NaHCO₃, 0.2 M phosphate buffer [pH 7.2]), using a previously described microdilution method (28).

Transcription profiling

Gene expression profiles were obtained by hybridizing labeled cRNAs generated from C. albicans total RNA onto Affymetrix C. albicans custom expression arrays (CAN07, 49-5241 array format) described elsewhere (11). Total RNA was isolated using the hot sodium dodecyl sulfate-phenol method and subsequent cRNA synthesis and labeling was performed as previously described (11).

The cRNA pellet was suspended in 10 µl of RNase-free water, and 10 µg was fragmented by ion-mediated hydrolysis at 95°C for 35 min in 200 mM Tris-acetate (pH 8.1), 500 mM potassium acetate, 150 mM magnesium acetate. The fragmented cRNA was hybridized for 16 h at 45°C to the C. albicans Affymetrix expression arrays. Arrays were washed at 25°C with 6x SSPE (1x SSPE is 0.18 M NaCl, 10 mM NaH₂PO₄, and 1 mM EDTA [pH 7.7]), 0.01% Tween 20, followed by a stringent wash at 50°C with 100 mM MES [2-(N-morpholino)ethanesulfonic acid], 0.1 M NaCl, 0.01% Tween 20. Hybridizations and washes employed the Affymetrix Fluidics station 450 using their standard EukGE-WS2v5 protocol. The arrays were then stained with phycoerythrin-conjugated streptavidin (Molecular Probes), and the fluorescence intensities were determined using the GCS 3000 high-resolution...
confocal laser scanner (Affymetrix). The scanned images were analyzed using software resident in GeneChip operating system v2.0 (Affymetrix). Sample loading and variations in staining were standardized by scaling the average of the fluorescent intensities of all genes on an array to a constant target intensity (250). The signal intensity for each gene was calculated as the average intensity difference, represented by \[ \frac{\sum(PM - MM)}{\text{(number of probe pairs)}} \], where PM and MM denote perfect-match and mismatch probes.

The scaled gene expression values from GeneChip operating system v2.0 software were generated using the MAS5.0 algorithm. The value of each probe set is generated using Tukey’s biweight computation. The algorithm considers the contribution of each match and mismatch probe corrected for the background of the average of the probe set. A weighted mean is then calculated for each probe pair. The raw signal values are then log2 transformed, and the array is normalized to this value. Probe sets were deleted from subsequent analysis if they were determined to be absent by the Affymetrix criterion. Pairwise comparison of gene expression level was performed for each matched experiment. Among direct comparisons between strains, genes were considered to be differentially expressed if their change in expression was 2-fold or greater (for up-regulated genes) or 0.5-fold or less (for down-regulated genes) in both independent experiments of each comparison. For the purpose of direct comparison, the SCMRR1R34A vs. SC5314 and SCMRR1R34B vs. SC5314 comparisons are displayed in the same table (Table S2) as the \( \Delta cap/MRR1R34A \) vs. SCCAP1M4A and \( \Delta cap/MRR1R34B \) vs. SCCAP1M4B comparisons. Cells highlighted in green are genes that were differentially expressed by the above criteria for the corresponding comparison/experiment. Cells that are not highlighted but for which there is a value are genes whose values did not meet the differential expression criterion for the corresponding comparison/experiment. Cells that are empty are genes which were called “absent” by the Affymetrix criteria for the corresponding comparison/experiment and thus its expression value was not valid. In addition, genes whose promoter regions were bound by Mrr1 as
measured by ChIP-chip are indicated by yellow highlighting. Data files for each scanned chip were submitted to the Gene Expression Omnibus database (www.ncbi.nlm.nih.gov/geo/). The accession number for the series is GSE23532.

Western immunoblot analysis

YPD overnight cultures of the strains were diluted to an OD$_{600}$ of 0.2 and grown at 30°C to an OD$_{600}$ of 1.0. 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 [pH7.5], 200 mM NaCl, 20% glycerol, 5 mM EDTA, 4% Complete, EDTA-free Protease Inhibitor Cocktail [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 boiled for 5 min and 400 µg 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). Mrr1$^{P683S}$-HA was detected with a rat anti-HA monoclonal antibody (Anti-HA-Peroxidase, high affinity [3F10], Roche Diagnostics GmbH, Mannheim, Germany) at a dilution of 1:500 using a chemiluminescence detection system (GE Healthcare UK Limited, Chalfont, UK) under conditions recommended by the manufacturer.

ChIP-chip experiments

Three independent cultures of strains SCMRR1M4E3A (untagged control strain: mrr1Δ, $P_{ADH1-MRR1}^{P683S}$), SCMRR1M4EH3A (tagged strain: mrr1Δ, $P_{ADH1-MRR1}^{P683S}$-HA), and SCΔmrr1Δcap1MEH3A (tagged strain: mrr1Δ cap1Δ, $P_{ADH1-MRR1}^{P683S}$-HA) were grown in YPD at 30°C to an OD$_{600}$ of 1.0. The subsequent steps of DNA cross-linking, DNA shearing, chromatin-immunoprecipitation (ChIP), DNA labeling with Cy dyes, hybridization to DNA microarrays, and data analysis were performed as described previously (18). Both pools of
labeled DNA from the tagged strain (SCMRR1M4EH3A or SC\textit{∆}mrr1\textit{∆}cap1MEH3A, Cy5-labeled) and the untagged control strain (SCMRR1M4E3A, Cy3-labeled) were mixed and hybridized to a \textit{C. albicans} whole-genome tiled oligonucleotide DNA microarray (NimbleGen Systems, Inc.) (33) according to the manufacturer’s instructions. Scanning of the slides (\( n = 3 \)) was performed used a GenePix 4000B scanner (Molecular Devices). Scanned images were preprocessed using NimbleScan software (version 2.4; NimbleGen Systems, Inc.). General feature format reports were created for the Cy5 (tagged strain) and Cy3 (untagged control strain) intensity signals from each independent replicate and were then imported into the Tilescope program (http://tilescope.gersteinlab.org:8080/mosaic/pipeline.html) (38). Quantile normalization was applied to the data. The parameters used were as follows: a window size of 400 bp, a maximum genomic distance of 60 bp, and a minimum length of 120 bp. The replicate data were combined, and peak finding (i.e., determining the Mrr1 binding sites) was done using a pseudomedian signal threshold of at least twofold and p-value cut-off of 0.01.
The role of Upc2 in MDR1 expression and Mdr1-mediated drug resistance

To investigate the capacity of a constitutively active Upc2 to induce MDR1 expression, we integrated a $P_{MDR1}$-GFP reporter fusion into derivatives of strain SC5314 in which one or both endogenous UPC2 alleles had been replaced by the hyperactive $UPC2^{G648D}$ allele. The fluorescence of the cells was quantified by FACS analysis. MDR1 is not detectably expressed in the wild-type strain SC5314 under standard growth conditions and transformants of this strain exhibited only background fluorescence (Fig. 1, bar pair 1). The fluorescence of reporter strains that were homozygous for the $UPC2^{G648D}$ allele was increased about 2.5-fold over background, confirming that this hyperactive $UPC2$ allele can activate MDR1 expression (Fig. 1, bar pair 5). However, the Upc2-mediated MDR1 induction was negligible compared to the strong activation of MDR1 expression by the hyperactive $MRR1^{P683S}$ allele, especially when the P683S gain-of-function mutation was present in both $MRR1$ alleles (Fig. 1, bar pairs 8 and 9). In addition, while induction of the MDR1 promoter by the hyperactive $UPC2$ allele required the presence of $MRR1$ (Fig. 1, bar pairs 4-7), the $MRR1^{P683S}$ allele activated the MDR1 promoter with equal efficiency in the presence or absence of a functional $UPC2$ gene (Fig. 1, bar pairs 8-11). These results demonstrate that Upc2 plays only a minor role in MDR1 expression and Mrr1 is a more important regulator of this efflux pump.

To evaluate the effect of the hyperactive $UPC2^{G648D}$ allele on drug resistance in the presence and absence of $MRR1$, we tested the susceptibilities of the strains to fluconazole and cerulenin (Table 1). As previously reported (12), strains that carried the G648D mutation in both UPC2 alleles exhibited an 8-fold increased resistance to fluconazole compared to the wild-type parental strain (MIC increased from 0.2 µg/ml to 1.56 µg/ml), and the same increase was also observed in an mrr1Δ background. In contrast, the $UPC2^{G648D}$ allele did not confer increased resistance to cerulenin, which is a substrate of the Mdr1 efflux pump, even in the presence of
These results indicate that the increased fluconazole resistance of strains containing the \( \text{UPC2}^{G648D} \) allele was caused by the upregulation of \( \text{ERG11} \) and other \( \text{Upc2} \) target genes, but not by increased \( \text{MDR1} \) expression. On the other hand, the hyperactive \( \text{MRR1}^{P683S} \) allele caused a 16-fold increased resistance to cerulenin (MIC increased from 0.39 \( \mu \text{g/ml} \) to 6.25 \( \mu \text{g/ml} \)) in the presence or absence of \( \text{UPC2} \), in line with its ability to activate the \( \text{MDR1} \) promoter in both wild-type and \( \text{upc2} \Delta \) strains. As previously reported (11), the \( \text{upc2} \Delta \) mutants were hypersusceptible to fluconazole. Nevertheless, the P683S mutation in \( \text{MRR1} \) caused a 16-fold increase in fluconazole resistance also in the \( \text{upc2} \Delta \) mutants when present in both \( \text{MRR1} \) alleles (MIC increased from 0.05 \( \mu \text{g/ml} \) to 0.78 \( \mu \text{g/ml} \)), similar to its effect in a wild-type background. These results demonstrate that a hyperactive \( \text{Mrr1} \) confers increased drug resistance independently of \( \text{Upc2} \).

The role of \( \text{Cap1} \) in \( \text{MDR1} \) expression and \( \text{Mdr1} \)-mediated drug resistance

A C-terminally truncated \( \text{CAP1} \) allele has been shown to cause constitutive \( \text{MDR1} \) upregulation and increased fluconazole resistance (1). To investigate if \( \text{MDR1} \) activation by a hyperactive \( \text{CAP1} \) allele requires \( \text{MRR1} \), we replaced one or both wild-type \( \text{CAP1} \) alleles with a C-terminally truncated \( \text{CAP1} \) allele (\( \text{CAP1}^{\Delta \text{C333}} \)) in the wild-type strain SC5314 as well as in mutants lacking \( \text{MRR1} \). The activity of the \( \text{MDR1} \) promoter was monitored using a \( \text{P}_{\text{MDR1}}-\text{GFP} \) reporter fusion. \( \text{MDR1} \) expression was especially pronounced when the C-terminal truncation was present in both \( \text{CAP1} \) alleles (Fig. 2, bar pairs 5). \( \text{MDR1} \) activation by the \( \text{CAP1}^{\Delta \text{C333}} \) allele was also observed in the \( \text{mrr1} \Delta \) mutants, albeit at a reduced level compared to that in the wild-type background, demonstrating that the induction of the \( \text{MDR1} \) promoter by the hyperactive \( \text{CAP1}^{\Delta \text{C333}} \) allele was partially \( \text{Mrr1} \)-independent (Fig. 2, bar pairs 4-7). However, the \( \text{MRR1}^{P683S} \) allele caused a stronger \( \text{MDR1} \) activation than the \( \text{CAP1}^{\Delta \text{C333}} \) allele. This activation was largely \( \text{CAP1} \)-independent, because similar \( \text{MDR1} \) expression levels were
achieved by the hyperactive Mrr1 in the wild-type strain SC5314 and two independently constructed cap1Δ derivatives (Fig. 2, bar pairs 8-11). We also compared the effect of hyperactive CAP1 and MRR1 alleles on drug resistance in the different strain backgrounds (Table 2). An increased resistance to the Mdr1 substrate cerulenin was only observed when the hyperactive CAP1AC333 allele was substituted for both endogenous CAP1 wild-type alleles. In contrast, a reproducibly increased resistance to fluconazole was seen already in the heterozygous strains. Fluconazole resistance was further elevated in strains that were homozygous for the CAP1AC333 allele, but only in the presence of MRR1. Similar to its ability to activate the MDR1 promoter in a CAP1-independent fashion, the hyperactive MRR1P683S allele did not require the presence of an intact CAP1 gene to mediate resistance to fluconazole and cerulenin. These data show that hyperactive CAP1 and MRR1 alleles can independently confer increased drug resistance.

Cooperation between Mrr1 and Cap1 in MDR1 expression

As hyperactive MRR1 and CAP1 alleles are each able to activate the MDR1 promoter, we investigated whether the presence of both types of activated transcription factors would have an additive or even synergistic effect on the expression of the efflux pump. For this purpose, the two wild-type CAP1 alleles were replaced by the CAP1AC333 allele in strains that were homozygous for the MRR1P683S allele. The activity of the MDR1 promoter in these strains was compared with that in strains containing only the MRR1P683S or CAP1AC333 alleles using the Pmdr1-GFP reporter fusion. As can be seen in Fig. 3A, strains carrying activated forms of both transcription factors displayed higher MDR1 expression levels than strains expressing only hyperactive Mrr1 or Cap1. Similarly, the resistance to fluconazole and cerulenin was further increased when both Mrr1 and Cap1 were constitutively active (Fig. 5B). These results demonstrate that activating mutations in Mrr1 and Cap1 have an additive effect on MDR1 expression and drug resistance.
Inducibility of the MDR1 promoter in mrr1Δ, upc2Δ, and cap1Δ mutants

MDR1 expression can be induced in C. albicans wild-type strains by certain toxic compounds, like benomyl and H2O2 (23, 27). To compare the requirement of MRR1, UPC2, and CAP1 for MDR1 upregulation in response to these inducers, we quantified the activity of the MDR1 promoter in the presence or absence of benomyl and H2O2 in wild-type and mutant strains carrying a P<sub>MDR1</sub>-GFP reporter fusion (Fig. 4). As reported previously (23), the induction of the MDR1 promoter was abolished in mrr1Δ mutants. Deletion of UPC2 had no effect on the inducibility of the MDR1 promoter, whereas MDR1 expression in response to benomyl was reduced and no MDR1 induction by H2O2 was observed in the cap1Δ mutants under the experimental conditions used. These results demonstrate that Upc2 is not required for MDR1 induction in response to these chemicals, whereas Cap1 is required for H2O2-induced MDR1 expression and contributes to its induction by benomyl, and Mrr1 is essential for MDR1 upregulation by both inducers.

Contribution of the Mdr1 efflux pump to Mrr1-, Upc2-, and Cap1-mediated drug resistance

In order to evaluate the importance of the Mdr1 efflux pump for drug resistance conferred by hyperactive Mrr1, Upc2, and Cap1, we introduced the MRR1<sup>P683S</sup>, UPC2<sup>G648D</sup>, and CAP1<sup>ΔC333</sup> alleles into mdr1Δ mutants generated from strain SC5314 (see Experimental procedures and Table S1). As can be seen in Table 3, the increased fluconazole resistance conferred by the hyperactive Mrr1 was reduced, but not abolished in cells lacking the Mdr1 efflux pump (from 16-fold in the wild type to four- to eightfold in mdr1Δ mutants), demonstrating that other Mrr1 target genes contribute to the fluconazole-resistant phenotype of strains containing gain-of-function mutations in this transcription factor. On the other hand, the Mrr1-mediated cerulenin resistance seen in a wild-type background was largely abrogated in mdr1Δ mutants (from 16-fold to twofold), indicating that cerulenin resistance is mediated mostly, but not
exclusively, by the Mdr1 efflux pump. The increased fluconazole resistance caused by the
hyperactive \textit{CAP1}^{\Delta C333} allele was reduced from fourfold to twofold in the absence of \textit{MDR1},
whereas the moderate cerulenin resistance conferred by this allele depended on a functional
Mdr1 efflux pump. In contrast, the increased fluconazole resistance conferred by the
hyperactive \textit{Upc2} did not depend on the presence of a functional \textit{MDR1} gene, confirming that
Upc2-mediated azole resistance is caused by upregulation of the \textit{ERG} and possibly other
Upc2 target genes.

Comparison of the gene expression profiles of wild type and \textit{cap1}\textDelta mutants
expressing a hyperactive \textit{MRR1} allele

The results shown above demonstrate that overexpression of the Mdr1 efflux pump
contributes only partially to the fluconazole resistance conferred by activated \textit{MRR1} and
\textit{CAP1} alleles. Mrr1 and Cap1 share some of their target genes (23, 39). In order to evaluate
whether a hyperactive Mrr1 can activate all its target genes independently of Cap1, we
compared the global gene expression patterns of strains containing the P683S gain-of-
function mutation in both \textit{MRR1} alleles in wild-type and \textit{cap1}\textDelta backgrounds. In a previous
study, we found 19 genes to be significantly upregulated in strains expressing one copy of the
\textit{MRR1}^{P683S} allele (23). In line with the stronger effect of two copies of the \textit{MRR1}^{P683S} allele on
\textit{MDR1} expression (see Figs. 1 and 2), a higher number of genes (83) were reproducibly
upregulated in two independently constructed strains that were homozygous for the P683S
mutation (Table S2). For 67 of these 83 genes we obtained reliable hybridization signals in
both experiments with the \textit{cap1}\textDelta mutants. Twenty five of the 67 genes were also reproducibly
upregulated, while 27 were not significantly (< 2-fold) upregulated in the \textit{cap1}\textDelta mutants. For
15 genes the results of the two experiments were inconsistent (>2-fold upregulation in one
experiment, <2-fold in the other experiment). However, almost all genes that were
upregulated <2-fold in the \textit{cap1}\textDelta mutants (21/27) were also not strongly upregulated (<3-fold)
In the wild type. In contrast, among the 31 genes that were upregulated >4-fold in the wild type, only one gene \(OYE23\) was reproducibly not upregulated in the \(cap1\Delta\) mutants.

Identification of the \textit{in vivo} DNA binding sites of Mrr1

To find out whether genes that are differentially expressed in strains carrying a hyperactive \(MRR1\) allele are direct targets of Mrr1, we determined the \textit{in vivo} DNA binding pattern of Mrr1 by chromatin immunoprecipitation followed by hybridization on whole-genome oligonucleotide tiling microarrays (ChIP-chip). Mrr1 was tagged at its C-terminus with a 3xHA epitope to enable immunoprecipitation with an anti-HA antibody, an approach that has been previously used to identify the binding sites of the transcription factors Cap1, Upc2, and Tac1 in the \textit{C. albicans} genome (18, 39, 41). In pilot experiments, we evaluated the effect of the HA tag on the activity of wild-type and hyperactive Mrr1 proteins. Untagged and HA-tagged \(MRR1\) and \(MRR1^{P683S}\) alleles were first expressed from the endogenous \(MRR1\) promoter in \(mrr1\Delta\) mutants carrying a \(P_{MDR1}\)-GFP reporter fusion. Fig. 5A shows that the presence of the HA tag resulted in activation of Mrr1, as the \(MDR1\) promoter was constitutively upregulated in strains expressing HA-tagged wild-type \(MRR1\), similar to strains expressing the hyperactive \(MRR1^{P683S}\) allele. Such an activating effect of a C-terminal HA tag has also been observed for Upc2 (41) and is likely to be caused by unmasking of an activation domain. When the same proteins were expressed in \(mrr1\Delta\) mutants of strain SC5314, the presence of the HA tag also resulted in increased fluconazole resistance, confirming the activation of wild-type Mrr1 by the HA tag (Table 4).

In order to achieve a similar Mrr1 activity as in strains that are homozygous for a hyperactive \(MRR1\) allele (see Figs. 1 and 2 and Tables 1 and 2), we also expressed the same set of tagged and untagged \(MRR1\) alleles from the strong \(ADH1\) promoter in the \(mrr1\Delta\) mutants. While overexpression of the untagged wild-type \(MRR1\) gene had no detectable effect on \(MDR1\) promoter activity and drug susceptibility, expression of the HA-tagged \(MRR1\) alleles from the...
ADH1 promoter resulted in a further increase in MDR1 expression and fluconazole resistance compared to strains expressing the same alleles from the endogenous MRR1 promoter (Fig. 5A and Table 4). We chose to use the strains overexpressing the HA-tagged MRR1P683S allele for the ChIP-chip experiments. To compare binding of Mrr1 in the presence and absence of Cap1, the HA-tagged MRR1P683S allele was also expressed from the ADH1 promoter in mrr1Δ cap1Δ double mutants. Western blot analysis showed that the HA-tagged Mrr1P683S protein was expressed at comparable levels in independent transformants of the mrr1Δ single and mrr1Δ cap1Δ double mutants (Fig. 5B). Therefore, one clone was used for the ChIP-chip experiments in each case, and a strain that expressed the untagged MRR1P683S allele in the mrr1Δ background served as the reference.

Using the criteria described in the experimental procedures, we identified 710 Mrr1 binding sites in the wild-type strain, 608 of which were also found in the cap1Δ mutant (Table S3). Therefore, Mrr1 binds to most of its target sequences independently of Cap1. One hundred forty-nine of the Mrr1 binding sites could not be assigned to an open reading frame (ORF) and 71 binding sites could be associated with more than one ORF. The remaining 490 binding sites were assigned to specific ORFs, 453 of which had one Mrr1 binding peak and 31 of which had more than one peak, i.e., 484 defined ORFs (and additional ORFs that shared a binding site) were bound by Mrr1. Of the Mrr1-bound genes, 40 were upregulated in cells expressing the hyperactive Mrr1P683S and nine were downregulated (Table S3). Therefore, 40 of the 83 upregulated genes seem to be direct targets of Mrr1, including the MDR1 efflux pump and MRR1 itself, whereas the downregulation of genes seems to be an indirect effect (Table S2). Table 5 lists all genes that were both upregulated and bound by the hyperactive Mrr1P683S. Almost all of these (37/40) were also bound by Mrr1 in the absence of Cap1 and the majority of them (22/40) were upregulated in a Cap1-independent fashion, while only seven of these 40 genes were reproducibly not upregulated (<2-fold in both experiments) in the cap1Δ mutants, despite being bound by Mrr1.
Identification of potential Mrr1 binding motifs

To search for a putative Mrr1 DNA binding site, a set of DNA sequences corresponding to 710 hits identified in the HA-tagged MRR1<sup>P683S</sup> binding data was generated. It has been shown that DNA motifs bound by transcription factors in ChIP-chip fragments are distributed close to the center of the peaks (4). Therefore, for each of the 710 hits, the position with the highest signal intensity in the tiling arrays was identified and a region of 1 kb centered around the maximum peak (± 500 bp) was retrieved from the *C. albicans* Assembly 19 genomic sequences. The top score 100 sequences (displaying a fold enrichment ≥ 4) were analysed using the Suite for Computational identification Of Promoter Elements (SCOPE) program that allows determination of potential transcription factor binding sites in a set of sequences using three different motif discovery algorithms (http://genie.dartmouth.edu/scope/) (38). The following three highest scoring motifs were obtained: DCSGH (where D designates A, T, or G, S designates C or G, and H designates A, T or C; significance value of 668, 100% coverage) (Fig. 6A), AAAN<sub>4-5</sub>AAT (significance value of 208, 99-100% coverage), and TCCGA (significance value of 201, 84% coverage; a variant of the DCSGH motif). When compared to sets of random sequences, the DCSGH motif was significantly enriched in the center of the 710 sequences (Fig. 6B) as well as in the sequences corresponding to the 40 genes bound and upregulated by Mrr1 (Fig. 6C and Table 5). Unlike the two other motifs, the AAAN<sub>4-5</sub>AAT sequence was found to be randomly distributed (data not shown). Many zinc cluster factors have been shown to bind as dimers to CGG triplets with different orientations and spacing (21). Our analysis using the SCOPE program did not identify specific CSG repeated motifs in the Mrr1 bound fragments. Taken together, these results suggest that Mrr1 can bind to single CGG triplets (CCG on the opposite strand) flanked by A/T-rich sequences, potentially as a monomer, as already reported for some members of the zinc cluster factor family (2). However, the presence of multiple occurrences of the DCSGH motif in the Mrr1
promoter targets (Fig. S1 and data not shown) suggests that Mrr1 has to bind to more than one
site to activate transcription.
Discussion

The zinc cluster transcription factor Mrr1 is a central regulator of *MDR1* expression in *C. albicans*, because *MRR1* is required for the induction of *MDR1* by toxic chemicals and gain-of-function mutations in *MRR1* are responsible for the constitutive *MDR1* overexpression in fluconazole-resistant strains (10, 23). Nevertheless, additional transcription factors have been implicated in the control of *MDR1* expression, because they bind to the *MDR1* promoter (1, 11, 19, 26-27, 31, 39, 41). Here, we studied the role of two of these transcription factors, Upc2 and Cap1, for which gain-of-function mutations have been described that render them hyperactive (1, 11-12).

Upc2 binds to the *MDR1* promoter and modulates *MDR1* expression in response to environmental conditions in *C. albicans* wild-type strains (41). We found that Upc2 is not required for upregulation of *MDR1* by benomyl and hydrogen peroxide, two chemicals that are commonly used to induce expression of this efflux pump (16, 23, 27). To date, only three gain-of-function mutations that render Upc2 hyperactive have been found in fluconazole-resistant, clinical *C. albicans* isolates. Of these, the G648D mutation has a stronger effect on *ERG11* overexpression and fluconazole resistance than the A643T and A643V mutations (11-12, 14). However, even when present in both *UPC2* alleles, the G648D mutation caused only a moderate *MDR1* upregulation that did not contribute to drug resistance. This result suggests that gain-of-function mutations in Upc2 are unlikely to be a cause of Mdr1-mediated drug resistance in *C. albicans*.

In contrast to Upc2, Cap1 was essential for *MDR1* upregulation in the presence of hydrogen peroxide and also contributed to *MDR1* induction by benomyl. The former result confirms earlier observations showing the requirement of Cap1 for hydrogen peroxide-induced *MDR1* expression (27). Conflicting results were reported with respect to the involvement of Cap1 in *MDR1* induction by benomyl. One study found that Cap1 was largely dispensable for
benomyl-induced MDRI expression (27), while in another study MDRI transcript levels in response to benomyl were found to be fourfold reduced in a cap1Δ mutant compared to a wild-type control strain (39). In line with the latter results, we found that full activation of the MDRI promoter by benomyl depended on Cap1 (see Fig. 4). As Mrr1 is essential for MDRI upregulation in response to both inducing chemicals, Mrr1 cooperates with Cap1, but not Upc2, to mediate MDRI induction by these environmental stimuli.

While Cap1 requires Mrr1 to induce MDRI expression in response to benomyl and hydrogen peroxide, the C-terminally truncated, hyperactive CAP1ΔC333 allele caused a constitutive MDRI overexpression also in the absence of Mrr1, demonstrating that it could activate the MDRI promoter in an Mrr1-independent fashion. Similar gain-of-function mutations in Cap1 could also be a cause of MDRI overexpression and drug resistance in clinical C. albicans isolates. However, no such mutations have been found so far in fluconazole-resistant isolates from patients. Instead, all MDRI overexpressing strains contain gain-of-function mutations in Mrr1 (10, 23, 30). One reason for this could be that hyperactive MRR1 alleles cause a stronger increase in fluconazole resistance than the C-terminally truncated Cap1 used in our study.

Another possible explanation is that the C-terminal truncation in Cap1 also has a deleterious effect on some Cap1 functions, because the mutated Cap1 failed to complement the hydrogen peroxide hypersusceptibility of a cap1Δ mutant (1). As resistance to oxidative stress is likely to be important for the ability of C. albicans to persist within the host, the environmental conditions in vivo may select against Cap1 mutations as a cause of fluconazole resistance. It remains to be seen if other activating mutations in Cap1 exist that do not negatively affect its ability to induce expression of all its target genes.

The fluconazole resistance of strains expressing hyperactive Mrr1 was only reduced, but not abolished after deletion of MDRI, indicating that Mrr1 can mediate increased fluconazole resistance in an Mdr1-independent fashion. This is in line with earlier findings that deletion of MRR1 from MDRI overexpressing clinical isolates increased fluconazole susceptibility more
than did deletion of MDR1 (23). Therefore, some of the other genes that are upregulated by hyperactive Mrr1 are likely to contribute to drug resistance. The contribution of other Mrr1 target genes to fluconazole resistance was even higher than that of MDR1, as is evident from the still four- to eightfold increase in the MIC of strains expressing the hyperactive Mrr1 in an mdr1Δ background compared to the 16-fold increase in the parental wild-type background (Table 3). It is currently not known which of these genes are involved in Mrr1-mediated fluconazole resistance. As the drug resistance conferred by the hyperactive Mrr1 did not depend on the presence of CAP1 (Table 2), the most likely candidates are those genes that are upregulated by Mrr1 in a Cap1-independent fashion. As noted previously (23), many Mrr1 target genes encode putative oxidoreductases which may act to remedy fluconazole-induced cell damage (see Table 5).

Interestingly, the increased fluconazole resistance of strains expressing the hyperactive Cap1 was also not completely abolished after deletion of MDR1, indicating that Cap1, like Mrr1, can mediate fluconazole resistance in an Mdr1- (and Mrr1-) independent fashion (Tables 2 and 3). Some known Cap1 target genes that were not found to be upregulated by Mrr1, like PDR16, FLU1, and RTA2, have previously been associated with fluconazole resistance and may therefore be responsible for this phenotype (3, 15, 29, 39).

The in vivo localization studies showed that Mrr1 bound to about half of the 83 genes that were reproducibly upregulated by the hyperactive Mrr1P683S. Therefore, these genes are probably direct targets of Mrr1. Similar to MDR1, most other direct Mrr1 target genes were also bound and upregulated by the hyperactive Mrr1 independently of Cap1, including the known Cap1 targets orf19.251, orf19.3131, orf19.7042, and orf19.6586 (39). Even if Mrr1 cooperates with Cap1 to induce common target genes in response to environmental signals, gain-of-function mutations in Mrr1 enable the transcription factor to act independently of Cap1. However, some genes (e.g., OYE23) were bound but not upregulated by the hyperactive Mrr1 in a cap1Δ mutant background (Tables 5 and S2), indicating that a hyperactive Mrr1
requires Cap1 for the transcriptional upregulation of these genes. We found a surprisingly high number of additional Mrr1 binding sites in the genome that were not associated with genes whose expression was affected by the presence of the activated Mrr1. It is possible that some of the peaks are artefacts caused by unspecific binding of the overexpressed, HA-tagged Mrr1 and do not represent biologically relevant Mrr1 binding sites. But it is also conceivable that even a hyperactive Mrr1 can induce the expression of the corresponding genes only in cooperation with other regulators, which may not be active under the growth conditions used in our assays. In addition, Mrr1 may also be involved in the regulation of noncoding transcripts, as many Mrr1 binding sites were not in the vicinity of known ORFs.

The identification of the in vivo DNA binding sites of a hyperactive Mrr1 also allowed us to deduce a potential Mrr1 binding motif. Interestingly, the DCSGHD motif occurs repeatedly in the MDRI upstream region (Fig. S1). It remains to be established by in vitro DNA binding studies with purified Mrr1 and by in vivo transactivation assays with mutated versions of the MDRI promoter fused to a reporter if the transcription factor indeed binds to these sites. However, the presence of multiple Mrr1 binding motifs explains the previous observation that no single region in the MDRI promoter is absolutely required for the constitutive MDRI upregulation in strains carrying gain-of-function mutations in Mrr1, whereas Mrr1 itself is essential for MDRI overexpression (10, 13, 23, 26).

The physiological function of the Mdr1 efflux pump, which is not significantly expressed under standard growth conditions, is not known. Our results demonstrate that two transcription factors that bind to the MDRI promoter, Mrr1 and Cap1, cooperate to induce MDRI expression under certain environmental conditions. Mutations that activate these transcription factors enable each of them to independently upregulate MDRI. This effect is especially pronounced for Mrr1, which also results in the upregulation of additional genes that contribute to fluconazole resistance, explaining why gain-of-function mutations in Mrr1 are the cause of MDRI overexpression in fluconazole-resistant clinical C. albicans isolates.
future studies it will be interesting to determine the importance of other transcription factors that bind to the *MDR1* promoter (Mcm1, Ndt80, Wor1) for Mrr1-mediated upregulation of this multidrug efflux pump.
Acknowledgments

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References


Fig. 1. Activation of the MDRI promoter by hyperactive UPC2 and MRR1 alleles. C. albicans strains carrying a P_{MDRI}−GFP reporter fusion in the indicated genetic backgrounds were grown to log phase in YPD medium and the mean fluorescence of the cells was determined by flow cytometry. The results obtained with two independently generated reporter strains are shown in each case (means and standard deviations from three experiments). The following strains were used (see Table S1): SCMPG2A and -B (wild type), SCMRR1M4MPG2A and -B (mrr1\Delta), UPC2M4MPG2A and -B (upc2\Delta), SCUPC2R12MPG2A and -B (UPC2^{G648D}/UPC2^{G648D}, wild type), SCUPC2R14MPG2A and -B (UPC2^{G648D}/UPC2^{G648D}, mrr1\Delta), ∆mrr1UPC2R12MPG2A and -B (UPC2^{G648D}/UPC2^{G648D}, mrr1\Delta), ∆mrr1UPC2R14MPG2A and -B (UPC2^{G648D}/UPC2^{G648D}, mrr1\Delta), SCUPC2R12MPG2A and -B (MRR1/MRR1^{P683S}, wild type), SCUPC2R14MPG2A and -B (MRR1/MRR1^{P683S}, wild type), ∆upc2MRR1R32MPG2A and -B (MRR1/MRR1^{P683S}, upc2\Delta), ∆upc2MRR1R34MPG2A and -B (MRR1^{P683S}/MRR1^{P683S}, upc2\Delta). The background fluorescence of the parental strains, which do not contain the GFP gene, is indicated by the black part in each column (one measurement).

Fig. 2. Activation of the MDRI promoter by hyperactive CAP1 and MRR1 alleles. C. albicans strains carrying a P_{MDRI}−GFP reporter fusion in the indicated genetic backgrounds were grown to log phase in YPD medium and the mean fluorescence of the cells was determined by flow cytometry. The results obtained with two independently generated reporter strains are shown in each case (means and standard deviations from three experiments). The following strains were used (see Table S1): SCMPG2A and -B (wild type), SCMRR1M4MPG2A and -B (mrr1\Delta), SCCAP1M4MPG2A and -B (cap1\Delta), SCCAP1R12MPG2A and -B (CAP1/CAP1^{ΔC33}, wild type), SCCAP1R14MPG2A and -B (CAP1^{ΔC33}/CAP1^{ΔC33}, wild type).
Fig. 3. Effect of combining hyperactive \textit{MRR1} and \textit{CAP1} alleles on \textit{MDR1} promoter activity and drug resistance.

(A) \textit{C. albicans} strains that are homozygous for the indicated hyperactive \textit{MRR1} and \textit{CAP1} alleles and contain a P_{\textit{MDR1}}-\textit{GFP} reporter fusion were grown to log phase in YPD medium and the mean fluorescence of the cells was determined by flow cytometry. The results obtained with two independently generated reporter strains are shown in each case (means and standard deviations from three experiments). The following strains were used (see Table S1): SCMPG2A and -B (wild type), SCMRR1R32MPG2A and -B (\textit{MRR1}^{P683S}/\textit{MRR1}^{P683S}, wild type), \Delta cap1MRR1R32MPG2A and -B (\textit{MRR1}^{P683S}/\textit{MRR1}^{P683S}, \textit{cap1}Δ), \Delta cap1MRR1R34MPG2A and -B (\textit{MRR1}^{P683S}/\textit{MRR1}^{P683S}, \textit{cap1}Δ). The background fluorescence of the parental strains, which do not contain the \textit{GFP} gene, is indicated by the black part in each column (one measurement). Data are from the same experiments as in Fig. 1, and the values of the control strains are included for comparison.

(B) Susceptibilities to fluconazole and cerulenin of the wild-type parental strain SC5314 and mutant derivatives in which both resident \textit{MRR1} and/or \textit{CAP1} alleles were replaced by the hyperactive \textit{MRR1}^{P683S} and \textit{CAP1}^{AC333} alleles, respectively. The results obtained with two independently generated strains are shown in each case. The following strains were used:
SC5314 (wild type), SCMRR1R34A and -B \((MRR1^{P683S}}\), SCCAP1R14A and -B \((CAP1^{∆C333}}\).

SCMRR1R34CAP1R14A and -B \((MRR1^{P683S} CAP1^{∆C333}}\).

**Fig. 4.** Activation of the \(MDR1\) promoter by benomyl and \(H_2O_2\) in wild-type, \(mrr1Δ\), \(cap1Δ\), and \(upc2Δ\) strains. Overnight cultures of \(C. albicans\) strains carrying a \(P_{MDR1}\)-GFP reporter fusion in the indicated genetic backgrounds were diluted \(10^{-2}\) in three tubes with fresh YPD medium and grown to log phase. One culture was left untreated and 50 \(µg/ml\) benomyl or 0.005\% \(H_2O_2\) was added to the other cultures to induce \(MDR1\) expression. The cultures were incubated for 80 min and the mean fluorescence of the cells was determined by flow cytometry. The results obtained with two independently generated reporter strains are shown in each case (means and standard deviations from three experiments). The following strains were used (see Table S1): SCMPG2A and -B (wild type), SCMRR1M4MPG2A and -B \((mrr1Δ)\), SCCAP1M4MPG2A and -B \((cap1Δ)\), UPC2M4MPG2A and -B \((upc2Δ)\).

**Fig. 5.** (A) \(MDR1\) promoter activity in \(C. albicans\) \(mrr1Δ\) mutants expressing a wild-type \(MRR1\) allele or the \(MRR1^{P683S}\) allele, without or with a C-terminal 3xHA tag, from the endogenous \(MRR1\) promoter or the strong \(ADH1\) promoter. The strains were grown to log phase in YPD medium and the mean fluorescence of the cells was determined by flow cytometry. The results obtained with two independent transformants are shown in each case (means and standard deviations from three experiments). The following strains were used:

CAG48MRR1M4B \((mrr1Δ)\), CAG48MRR1M4K2B1 and -2 \((P_{MRR1}^{MRR1})\), CAG48MRR1M4H2B1 and -2 \((P_{MRR1}^{MRR1}-HA)\), CAG48MRR1M4K3B1 and -2 \((P_{MRR1}^{MRR1}^{P683S})\), CAG48MRR1M4H3B1 and -2 \((P_{MRR1}^{MRR1}^{P683S}-HA)\), CAG48MRR1M4E2B1 and -2 \((P_{ADH1}^{MRR1})\), CAG48MRR1M4EH2B1 and -2 \((P_{ADH1}^{MRR1}-HA)\).
(B) Expression of the HA-tagged Mrr1\textsuperscript{P683S} protein in mrr1\Delta single and mrr1\Delta cap1\Delta double mutants. Whole-cell protein extracts of the strains were analyzed by western immunoblotting with an anti-HA antibody. The following strains were used: 1, SC5314; 2, SC\Delta mrr1MEH3A; 3, SC\Delta mrr1\Delta cap1MEH3A; 4, SC\Delta mrr1MEH3B; 5, SC\Delta mrr1\Delta cap1MEH3B; 6, SC\Delta mrr1ME3A; 7, SC\Delta mrr1ME3B. The HA-tagged Mrr1\textsuperscript{P683S} was expressed at similar levels in independent transformants of the mrr1\Delta (lanes 2 and 4) and mrr1\Delta cap1\Delta mutants (lanes 3 and 5). No crossreacting proteins were detected in the parental strain SC5314 (lane 1) and in mrr1\Delta mutants expressing untagged Mrr1\textsuperscript{P683S} (lanes 6 and 7).

Fig. 6. Identification of putative Mrr1 DNA binding motifs. (A) Weblogo representation of the DCSGHD motif identified by SCOPE. (B) Enrichment of the DCSGHD motif in the 710 sequence dataset (filled circles), compared to what is expected randomly in the whole genome (open circles). Each of the 710 1-kb sequences was divided into 20 intervals of 50 bp and the number of DCSGHD motif occurrences was compiled and plotted for each interval. The maximum fold enrichment value (3.5) is observed in the center of the analyzed 1-kb sequences. (C) Enrichment of the DCSGHD motif in the sequences corresponding to 40 genes bound and regulated by Mrr1 (filled circles) as compared to all genes (open circles). The p-value (3.E-06) represents the probability of observing this motif distribution in random data sets.
Table 1: Susceptibilities to fluconazole and cerulenin of the wild-type parental strain SC5314 and mutant derivatives in which one or both resident \textit{UPC2} and \textit{MRR1} alleles were replaced by the hyperactive \textit{UPC2}^{G648D} and \textit{MRR1}^{P683S} alleles, respectively.

<table>
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<th>Strain</th>
<th>Relevant genotype</th>
<th>MIC Fluconazole (µg/ml)</th>
<th>MIC Cerulenin (µg/ml)</th>
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<tr>
<td>\textit{Δupc2}SCMRR1R34A and -B</td>
<td>\textit{upc2}Δ/\textit{upc2}Δ \textit{MRR1}^{P683S}/\textit{MRR1}^{P683S}</td>
<td>0.78</td>
<td>6.25</td>
</tr>
</tbody>
</table>
Table 2: Susceptibilities to fluconazole and cerulenin of the wild-type parental strain SC5314 and mutant derivatives in which one or both resident *CAP1* and *MRR1* alleles were replaced by the hyperactive *CAP1*<sup>ΔC333</sup> and *MRR1*<sup>P683S</sup> alleles, respectively.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>MIC Fluconazole (µg/ml)</th>
<th>MIC Cerulenin (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC5314</td>
<td>wild-type</td>
<td>0.2</td>
<td>0.39</td>
</tr>
<tr>
<td>SCMRR1M4A and -B</td>
<td>mrr1&lt;sup&gt;Δ&lt;/sup&gt;/mrr1&lt;sup&gt;Δ&lt;/sup&gt;</td>
<td>0.2</td>
<td>0.39</td>
</tr>
<tr>
<td>CAP1M4A and -B</td>
<td>cap1&lt;sup&gt;Δ&lt;/sup&gt;/cap1&lt;sup&gt;Δ&lt;/sup&gt;</td>
<td>0.2</td>
<td>0.39</td>
</tr>
<tr>
<td>SCCAP1R12A and -B</td>
<td>CAP1/CAP1&lt;sup&gt;ΔC333&lt;/sup&gt;</td>
<td>0.39</td>
<td>0.39</td>
</tr>
<tr>
<td>SCCAP1R14A and -B</td>
<td>CAP1&lt;sup&gt;ΔC333&lt;/sup&gt;/CAP1&lt;sup&gt;ΔC333&lt;/sup&gt;</td>
<td>0.78</td>
<td>0.78</td>
</tr>
<tr>
<td>∆mrr1/CAP1R12A and -B</td>
<td>mrr1&lt;sup&gt;Δ&lt;/sup&gt;/mrr1&lt;sup&gt;Δ&lt;/sup&gt; CAP1/CAP1&lt;sup&gt;ΔC333&lt;/sup&gt;</td>
<td>0.39</td>
<td>0.39</td>
</tr>
<tr>
<td>∆mrr1/CAP1R14A and -B</td>
<td>mrr1&lt;sup&gt;Δ&lt;/sup&gt;/mrr1&lt;sup&gt;Δ&lt;/sup&gt; CAP1&lt;sup&gt;ΔC333&lt;/sup&gt;/CAP1&lt;sup&gt;ΔC333&lt;/sup&gt;</td>
<td>0.39</td>
<td>0.78</td>
</tr>
<tr>
<td>SCMRR1R32A and -B</td>
<td>MRR1/MRR1&lt;sup&gt;P683S&lt;/sup&gt;</td>
<td>0.39</td>
<td>0.78</td>
</tr>
<tr>
<td>SCMRR1R34A and -B</td>
<td>MRR1&lt;sup&gt;P683S&lt;/sup&gt;/MRR1&lt;sup&gt;P683S&lt;/sup&gt;</td>
<td>3.13</td>
<td>6.25</td>
</tr>
<tr>
<td>∆cap1/MRR1R32A and -B</td>
<td>cap1&lt;sup&gt;Δ&lt;/sup&gt;/cap1&lt;sup&gt;Δ&lt;/sup&gt; MRR1/MRR1&lt;sup&gt;P683S&lt;/sup&gt;</td>
<td>0.39</td>
<td>0.78</td>
</tr>
<tr>
<td>∆cap1/MRR1R34A and -B</td>
<td>cap1&lt;sup&gt;Δ&lt;/sup&gt;/cap1&lt;sup&gt;Δ&lt;/sup&gt; MRR1&lt;sup&gt;P683S&lt;/sup&gt;/MRR1&lt;sup&gt;P683S&lt;/sup&gt;</td>
<td>3.13</td>
<td>6.25</td>
</tr>
</tbody>
</table>
Table 3: Contribution of the Mdr1 efflux pump to drug resistance conferred by hyperactive MRR1, CAP1, and UPC2 alleles. Shown are the susceptibilities to fluconazole and cerulenin of wild-type strains and mdr1Δ mutants in which both resident MRR1, CAP1, or UPC2 alleles were replaced by the MRR1<sup>P683S</sup>, CAP1<sup>ΔC333</sup>, and UPC2<sup>G648D</sup> alleles, respectively.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>MIC Fluconazole (µg/ml)</th>
<th>MIC Cerulenin (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SC5314</td>
<td>wild-type</td>
<td>0.2</td>
<td>0.39</td>
</tr>
<tr>
<td>SCMDR1M4A and -B</td>
<td>mdr1Δ/mdr1Δ</td>
<td>0.2</td>
<td>0.39</td>
</tr>
<tr>
<td>SCMRR1R34A and -B</td>
<td>MRR1&lt;sup&gt;P683S&lt;/sup&gt;/MRR1&lt;sup&gt;P683S&lt;/sup&gt;</td>
<td>3.13</td>
<td>6.25</td>
</tr>
<tr>
<td>∆mdr1/MRR1R34A and -B</td>
<td>mdr1Δ/mdr1Δ/MRR1&lt;sup&gt;P683S&lt;/sup&gt;/MRR1&lt;sup&gt;P683S&lt;/sup&gt;</td>
<td>0.78-1.56</td>
<td>0.78</td>
</tr>
<tr>
<td>SCCAP1R14A and -B</td>
<td>CAP1&lt;sup&gt;ΔC333&lt;/sup&gt;/CAP1&lt;sup&gt;ΔC333&lt;/sup&gt;</td>
<td>0.78</td>
<td>0.78</td>
</tr>
<tr>
<td>∆mdr1/CAP1R14A and -B</td>
<td>mdr1Δ/mdr1Δ/CAP1&lt;sup&gt;ΔC333&lt;/sup&gt;/CAP1&lt;sup&gt;ΔC333&lt;/sup&gt;</td>
<td>0.39</td>
<td>0.39</td>
</tr>
<tr>
<td>SCUPC2R14A and -B</td>
<td>UPC2&lt;sup&gt;G648D&lt;/sup&gt;/UPC2&lt;sup&gt;G648D&lt;/sup&gt;</td>
<td>1.56</td>
<td>0.39</td>
</tr>
<tr>
<td>∆mdr1/UPC2R14A and -B</td>
<td>mdr1Δ/mdr1Δ/UPC2&lt;sup&gt;G648D&lt;/sup&gt;/UPC2&lt;sup&gt;G648D&lt;/sup&gt;</td>
<td>1.56</td>
<td>0.39</td>
</tr>
</tbody>
</table>
**Table 4:** Fluconazole susceptibilities of *mrr1Δ* mutants expressing a wild-type *MRR1* allele or the *MRR1<sup>P683S</sup>* allele, without or with a C-terminal 3xHA tag, from the endogenous *MRR1* promoter or the strong *ADH1* promoter.

<table>
<thead>
<tr>
<th>Strain Description</th>
<th>MIC Fluconazole (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCMRR1M4A and -B</td>
<td><em>mrr1Δ</em></td>
</tr>
<tr>
<td>SCMRR1M4K2A and -B</td>
<td><em>P&lt;sub&gt;MRR1&lt;/sub&gt;</em>&lt;sup&gt;-&lt;/sup&gt;<em>MRR1</em></td>
</tr>
<tr>
<td>SCMRR1M4K3A and -B</td>
<td><em>P&lt;sub&gt;MRR1&lt;/sub&gt;</em>&lt;sup&gt;-&lt;/sup&gt;*MRR1&lt;sup&gt;P683S&lt;/sup&gt;-HA</td>
</tr>
<tr>
<td>SCM&lt;sub&gt;Δ&lt;/sub&gt;mrr1MH3A and -B</td>
<td><em>P&lt;sub&gt;MRR1&lt;/sub&gt;</em>&lt;sup&gt;-&lt;/sup&gt;*MRR1&lt;sup&gt;P683S&lt;/sup&gt;-HA</td>
</tr>
<tr>
<td>SCΔmrr1ME2A and -B</td>
<td><em>P&lt;sub&gt;ADH1&lt;/sub&gt;</em>&lt;sup&gt;-&lt;/sup&gt;<em>MRR1</em></td>
</tr>
<tr>
<td>SCΔmrr1MEH2A and -B</td>
<td><em>P&lt;sub&gt;ADH1&lt;/sub&gt;</em>&lt;sup&gt;-&lt;/sup&gt;*MRR1&lt;sup&gt;-HA&lt;/sup&gt;</td>
</tr>
<tr>
<td>SCΔmrr1ME3A and -B</td>
<td><em>P&lt;sub&gt;ADH1&lt;/sub&gt;</em>&lt;sup&gt;-&lt;/sup&gt;*MRR1&lt;sup&gt;P683S&lt;/sup&gt;</td>
</tr>
<tr>
<td>SCΔmrr1MEH3A and -B</td>
<td><em>P&lt;sub&gt;ADH1&lt;/sub&gt;</em>&lt;sup&gt;-&lt;/sup&gt;*MRR1&lt;sup&gt;P683S&lt;/sup&gt;-HA</td>
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Table 5: Genes upregulated and bound by activated Mrr1

<table>
<thead>
<tr>
<th>orf19 no.</th>
<th>Gene</th>
<th>Molecular function</th>
<th>Upregulation</th>
<th>Binding</th>
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<td></td>
<td></td>
<td></td>
<td>WT</td>
<td>cap1Δ</td>
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<td>orf19.4476</td>
<td>aryl-alcohol dehydrogenase activity</td>
<td>15424.4</td>
<td>2019.7</td>
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<td>orf19.1048</td>
<td>IFD6</td>
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<td>422.9</td>
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<td>orf19.271</td>
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<td>2,4-dienoyl-CoA reductase (NADPH) activity</td>
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<td>90.4</td>
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<tr>
<td>orf19.5604</td>
<td>MDR1</td>
<td>multidrug efflux pump activity</td>
<td>263.1</td>
<td>829.5</td>
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<td>orf19.629</td>
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<td>orf19.7306</td>
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<td>orf19.4309</td>
<td>GRP2</td>
<td>oxidoreductase activity</td>
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<td>32.8</td>
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<td>orf19.251</td>
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<td>orf19.3131</td>
<td>OYE32</td>
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<td>arginase activity</td>
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<td>orf19.3433</td>
<td>OYE23</td>
<td>NADPH dehydrogenase activity</td>
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<td>5.4</td>
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<tr>
<td>orf19.6993</td>
<td>GAP2</td>
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<td>29.9</td>
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<td>HG72</td>
<td>glucose transmembrane transporter activity</td>
<td>5.1</td>
<td>2.1</td>
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<tr>
<td>orf19.780</td>
<td>DUR1,2</td>
<td>aliphophanate hydrolase activity, urea carboxylase activity</td>
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<td>3.6</td>
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<td>ALS1</td>
<td>cell adhesion molecule binding</td>
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<td>pyruvate carboxylase activity</td>
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<td>2.5</td>
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<td>drug transmembrane transporter activity</td>
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<td>5.8</td>
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<td>transcription factor activity</td>
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<td>YMX6</td>
<td>NADH dehydrogenase activity</td>
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<tr>
<td>orf19.7372</td>
<td>MRR1</td>
<td>specific RNA polymerase II transcription factor activity</td>
<td>2.3</td>
<td>2.7</td>
</tr>
<tr>
<td>orf19.4778</td>
<td>LYS142</td>
<td>transcription factor activity</td>
<td>2.3</td>
<td>3.7</td>
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<tr>
<td>orf19.5282</td>
<td>unknown</td>
<td></td>
<td>2.2</td>
<td>3.6</td>
</tr>
</tbody>
</table>

1 Average values from two experiments with independently constructed strains. Values for the cap1Δ mutants are omitted when the gene was not upregulated by at least twofold or no reliable hybridization signals were obtained in one or both experiments (see Table S2).
Fig. 1

![Graph showing mean fluorescence levels for different genetic backgrounds.]

- **UPC2**/**UPC2**
- **G648D**
- **wild type mrr1**
- **upc2Δ**

The graph compares mean fluorescence levels for various genetic backgrounds, including wild type, mrr1Δ, upc2Δ, and various combinations involving UPC2, MRR1, and G648D mutations.
Fig. 2

Mean fluorescence

- CAP1/CAP1
- C333
- CAP1/C333
- C333

wild type
mrr1

CAP1/CAP1
C333
CAP1/C333
C333

wild type
mrr1

CAP1/CAP1
C333
CAP1/C333
C333

wild type
mrr1

CAP1/CAP1
C333
CAP1/C333
C333
Fig. 4

Mean fluorescence
Benomyl
H$_2$O$_2$

--- + --- + --- + --- + --- + - -+ --- + --- +
- +-- +-- +-- +-- +-- +-- +-- +-

wild type
mrr1'
upc2'
cap1'

0
20
40
60
80
100
120
Fig. 6

A

B

Fold enrichment = 3.5

C

p-value = 5.0E-06