The Transcription Factor Ndt80 Does Not Contribute to Mrr1-, Tac1-, and Upc2-Mediated Fluconazole Resistance in Candida albicans

Christoph Sasse1, Rebecca Schillig1, Franziska Dierolf1, Michael Weyler1, Sabrina Schneider1, Selene Mogavero1,2, P. David Rogers3,4,5,6, Joachim Morschhäuser1

1 Institut für Molekulare Infektionsbiologie, Universität Würzburg, Würzburg, Germany, 2 Department of Biology, University of Pisa, Pisa, Italy, 3 Children’s Foundation Research Center, Le Bonheur Children’s Hospital, Memphis, Tennessee, United States of America, 4 Department of Pharmaceutical Sciences, College of Pharmacy, University of Tennessee Health Science Center, Memphis, Tennessee, United States of America, 5 Department of Clinical Pharmacy, College of Pharmacy, University of Tennessee Health Science Center, Memphis, Tennessee, United States of America, 6 Department of Pediatrics, College of Medicine, University of Tennessee Health Science Center, Memphis, Tennessee, United States of America

Abstract

The pathogenic yeast Candida albicans can develop resistance to the widely used antifungal agent fluconazole, which inhibits ergosterol biosynthesis, by the overexpression of genes encoding multidrug efflux pumps or ergosterol biosynthesis enzymes. Zinc cluster transcription factors play a central role in the transcriptional regulation of drug resistance. Mrr1 regulates the expression of the major facilitator MDR1, Tac1 controls the expression of the ABC transporters CDR1 and CDR2, and Upc2 regulates ergosterol biosynthesis (ERG) genes. Gain-of-function mutations in these transcription factors result in constitutive overexpression of their target genes and are responsible for fluconazole resistance in many clinical C. albicans isolates. The transcription factor Ndt80 contributes to the drug-induced upregulation of CDR1 and ERG genes and also binds to the MDR1 and CDR2 promoters, suggesting that it is an important component of all major transcriptional mechanisms of fluconazole resistance. However, we found that Ndt80 is not required for the induction of MDR1 and CDR2 expression by inducing chemicals. CDR2 was even partially derepressed in ndt80Δ mutants, indicating that Ndt80 is a repressor of CDR2 expression. Hyperactive forms of Mrr1, Tac1, and Upc2 promoted overexpression of MDR1, CDR1/CDR2, and ERG11, respectively, with the same efficiency in the presence and absence of Ndt80. Mrr1- and Tac1-mediated fluconazole resistance was even slightly enhanced in ndt80Δ mutants compared to wild-type cells. These results demonstrate that Ndt80 is dispensable for the constitutive overexpression of Mrr1, Tac1, and Upc2 target genes and the increased fluconazole resistance of strains that have acquired activating mutations in these transcription factors.

Introduction

Infections by the pathogenic yeast Candida albicans are commonly treated with the antifungal agent fluconazole, which blocks ergosterol biosynthesis by inhibiting sterol 14α-demethylase, a key enzyme in the ergosterol biosynthetic pathway. C. albicans can develop resistance to fluconazole by various mechanisms, including mutations in the target enzyme that decrease its affinity to the drug, increased expression of the ERG11 gene encoding sterol 14α-demethylase, or overexpression of multidrug efflux pumps of the ABC transporter and major facilitator superfamilies [1]. Many details of the molecular basis of drug resistance in C. albicans have been elucidated in recent years, especially with the identification of transcription factors that regulate the expression of ergosterol biosynthesis genes and multidrug efflux pumps. Transcription factors of the zinc cluster family, which is specific for fungi, have central roles in the transcriptional control of fluconazole resistance in C. albicans. Upc2 regulates the expression of ERG11 and other ERG genes [2,3], Tac1 controls the expression of the ABC transporters CDR1 and CDR2 [4], and Mrr1 regulates the expression of the major facilitator MDR1 [5]. Mutants lacking these transcription factors cannot upregulate their target genes in response to inducing stimuli. In addition, fluconazole-resistant, clinical C. albicans isolates that overexpress CDR1 and CDR2, MDR1, or ERG11 contain gain-of-function mutations in Tac1, Mrr1, and Upc2, respectively, which render the transcription factors constitutively active even under noninducing conditions and are responsible for the increased drug resistance of these strains [4,5,6,7,8,9,10,11,12,13,14].

While Mrr1, Tac1, and Upc2 each contribute in different and specific ways to the development of fluconazole resistance, another transcription factor, Ndt80, seems to be involved in the transcriptional regulation of Mrr1, Tac1, and Upc2 target genes and therefore have a much broader role in fluconazole resistance and many other cellular functions in C. albicans [15,16]. NDT80 was originally identified as a gene that increased CDR1 promoter
activity when it was overexpressed from a multicopy plasmid in the heterologous host Saccharomyces cerevisiae [17]. Deletion of \textit{NDT80} in \textit{C. albicans} resulted in reduced basal \textit{CDR1} expression levels, and induction of \textit{CDR1} expression by miconazole was abolished in the \textit{ndt80Δ} mutants, which became hypersusceptible to fluconazole and voriconazole [17,18]. Expression of \textit{NDT80} itself was found to be induced upon treatment of \textit{C. albicans} cells with itraconazole or miconazole [17,19]. The involvement of Ndt80 in \textit{CDR1} regulation was corroborated by the finding that Ndt80 binds to the \textit{CDR1} promoter in vivo [16]. Interestingly, Ndt80 also binds to the promoters of the efflux pumps \textit{CDR2} and \textit{MDR1} and those of genes encoding ergosterol biosynthesis enzymes, including \textit{ERG11} [16]. Ndt80 therefore seems to be involved in the transcriptional regulation of all major mechanisms of azole resistance in \textit{C. albicans}.

While a role of Ndt80 in \textit{CDR1} expression and fluconazole-induced upregulation of \textit{ERG} genes has been demonstrated [16,17], the relevance of Ndt80 binding to the promoters of \textit{MDR1} and \textit{CDR2} for the expression of these efflux pumps has not yet been addressed. We therefore investigated if Ndt80 is required for \textit{MDR1} and \textit{CDR2} upregulation by chemicals that are known to induce the expression of these drug transporters. In addition, we studied the importance of Ndt80 for the constitutive overexpression of \textit{MDR1}, \textit{CDR1}/\textit{CDR2}, and \textit{ERG11} in strains containing hyperactive \textit{MRR1}, \textit{TAC1}, and \textit{UPC2} alleles, respectively, which confer increased fluconazole resistance in clinical \textit{C. albicans} isolates.

**Results**

**Role of Ndt80 in \textit{MDR1} induction and constitutive \textit{MDR1} overexpression**

To evaluate the importance of Ndt80 for \textit{MDR1} expression, we constructed two independent \textit{ndt80Δ} mutants of the wild-type strain SC5314 by the \textit{SAT1} flipping method (see Materials and methods). We then integrated the \textit{GFP} reporter gene under the control of the native \textit{MDR1} promoter in the wild type and the \textit{ndt80Δ} mutants to quantify \textit{MDR1} promoter activity. For comparison, the reporter fusion was also introduced in two independently generated \textit{mrr1Δ} mutants of strain SC5314. The \textit{MDR1} gene is not significantly expressed under standard growth conditions, but it is activated in the presence of benomyl [5,20,21]. FACS analysis of the cells showed that Ndt80 was not required for the induction of the \textit{MDR1} promoter by benomyl, whereas \textit{MDR1} induction was abolished in cells lacking \textit{Mrr1} (Figure 1A). As the \textit{ndt80Δ} mutants displayed a higher background fluorescence than the wild type, we also detected GFP expression by Western immunoblotting (Figure 1B). These experiments confirmed that comparable amounts of GFP were produced in the presence and absence of \textit{NDT80}, i.e., Ndt80 was dispensable for the induction of the \textit{MDR1} promoter by benomyl.

A more important question with respect to drug resistance was whether Ndt80 contributes to the constitutive \textit{MDR1} overexpression in strains containing a hyperactive Mrr1. To address this issue, we introduced a gain-of-function mutation that causes a P683S amino acid substitution into both endogenous \textit{MRR1} alleles of the wild-type strain SC5314 and the \textit{ndt80Δ} mutants. The \textit{P\textsubscript{MRR1}-GFP} reporter fusion was then integrated into these strains to compare their \textit{MDR1} promoter activities. FACS and Western immunoblot analysis of GFP expression demonstrated that the hyperactive Mrr1 induced the \textit{MDR1} promoter with similar efficiency in both strain backgrounds (Figure 2A and B).

To evaluate the phenotypic consequences of expression of a hyperactive Mrr1 in the absence of Ndt80, we determined the fluconazole susceptibilities of wild-type and \textit{ndt80Δ} strains containing native or hyperactive \textit{MRR1} alleles (Figure 2C). In line with previous results [22], expression of the hyperactive \textit{MRR1\textsuperscript{P683S}} allele in the wild-type background resulted in a 16-fold increase in fluconazole resistance (MIC increased from 0.39 to 6.25 \textmu g/ml). In accord with findings by other researchers [16,17], we observed an enhanced fluconazole sensitivity of the \textit{ndt80Δ} mutants. Surprisingly, however, the presence of the hyperactive \textit{MRR1\textsuperscript{P683S}} allele caused an even stronger increase in fluconazole resistance in the \textit{ndt80Δ} mutants (MIC increased from 0.2 to 12.5 \textmu g/ml) than in their wild-type parent. To confirm that this phenotype was indeed caused by the deletion of \textit{NDT80}, we reinserted a functional \textit{NDT80} copy into the homozygous \textit{ndt80Δ} mutants. The reintroduction of \textit{NDT80} reverted the fluconazole susceptibilities of the \textit{ndt80Δ} mutants expressing native of hyperactive \textit{MRR1} alleles to the levels observed in the corresponding wild-type strains.

Altogether, these results demonstrated that Ndt80 is dispensable for \textit{MDR1} upregulation in response to the inducer benomyl and for the constitutive \textit{MDR1} overexpression caused by a hyperactive Mrr1. In addition, Ndt80 has a negative effect on Mrr1-mediated fluconazole resistance, presumably by reducing the expression of other Mrr1 target genes that also contribute to drug resistance [22].

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**Figure 1. Inducibility of the \textit{MDR1} promoter in wild-type, \textit{mrr1Δ}, and \textit{ndt80Δ} strains.** (A) Strains carrying a \textit{P\textsubscript{MDR1}-GFP} reporter fusion in the indicated genetic backgrounds were grown in the absence (-) or presence (+) of benomyl 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 are shown in each case (means and standard deviations from three experiments). The following strains were used (see Table S1): SCMG3A and -B (wild type), SCmrr1MG3A and -B (\textit{mrr1Δ}), SC\textit{Δndt80MG3A and -B (ndt80Δ)}. The background fluorescence of the parental strains, which do not contain the GFP gene, is indicated by the black part in each column. (B) GFP expression in the same strains was detected by Western immunoblotting with an anti-GFP antibody. The two blots show the results with the A and B series of strains. doi:10.1371/journal.pone.0025623.g001
Role of Ndt80 in CDR2 induction and constitutive CDR2 overexpression

As explained in the introduction, Ndt80 also binds to the promoter of the ABC transporter CDR2, which like CDR1 is regulated by the zinc cluster transcription factor Tac1. CDR2 is not significantly expressed under standard growth conditions, but its expression is strongly induced by fluphenazine [21,23,24]. To investigate if Ndt80 is required for the activation of the CDR2 promoter in response to this compound, we integrated a P\textsubscript{CDR2}\textsubscript{-}GFP reporter fusion into the endogenous CDR2 locus of the wild-type strain SC5314 and the ndt80\textalpha mutants. For comparison, the reporter fusion was also introduced in two independently generated tac1\textDelta mutants of strain SC5314. FACS analysis of the cells (Figure 3A) and Western immunoblot analysis of GFP expression (Figure 3B) demonstrated that the CDR2 promoter was efficiently induced by fluphenazine in both the wild type and the ndt80\textalpha mutants, while no induction was observed in tac1\textDelta mutants, in agreement with previous findings [4]. Interestingly, the ndt80\textalpha mutants also exhibited a basal CDR2 promoter activity in the absence of the inducer, in contrast to the wild type, in which no CDR2 expression was detectable under noninducing conditions. These results indicated that Ndt80 is a repressor of CDR2 expression.

Activating mutations in Tac1 result in constitutive CDR2 overexpression in fluconazole-resistant strains. To investigate whether Ndt80 affects CDR2 induction by a hyperactive Tac1, we introduced a gain-of-function mutation that results in a G980E amino acid substitution into both endogenous TAC1 alleles of the wild-type strain SC5314 and the ndt80\textalpha mutants. The P\textsubscript{CDR2}\textsubscript{-}GFP reporter fusion was then integrated into these strains to compare their CDR2 promoter activities. FACS analysis of the cells (Figure 4A) and Western immunoblot analysis of GFP expression (Figure 4B) demonstrated that the hyperactive Tac1 induced the CDR2 promoter in the ndt80\textalpha mutants with similar efficiency as in wild-type cells.

In order to determine if a hyperactive Tac1 can mediate increased drug resistance in ndt80\textalpha mutants, we compared the fluconazole susceptibilities of wild-type and ndt80\textalpha strains containing native or hyperactive TAC1 alleles (Figure 4C). In the wild-type background, the TAC1\textsuperscript{G980E} allele caused an 8-fold increased fluconazole resistance [MIC increased from 0.39 to 3.13 μg/ml]. An even stronger increase in resistance (32-fold) was observed in the ndt80\textalpha mutants, in which the MIC rose from 0.2 to 6.25 μg/ml. When a single ND\textsubscript{T}780 copy was reintegrated into the homozygous ndt80\textalpha mutants containing the TAC1\textsuperscript{G980E} alleles, the MIC of fluconazole remained at 6.25 μg/ml, but the strains showed reduced growth at 3.13 μg/ml, indicating that their resistance was intermediate between that of cells containing two ND\textsubscript{T}780 copies or none. These results demonstrated that Ndt80 also has a negative effect on Tac1-mediated fluconazole resistance.

Ndt80 is not required for Tac1-mediated CDR1 overexpression

CDR1 and CDR2 are usually coregulated by Tac1 [4,6,7,14]. In the light of previous reports that Ndt80 is an activator of CDR1 expression [17,18], our findings that ND\textsubscript{T}780 is not required for the inducible or constitutive upregulation of CDR2 and even inhibits CDR2 expression under noninducing conditions therefore came as a surprise. As CDR1 makes a stronger contribution than CDR2 to the increased drug resistance of strains in which these ABC transporters are overexpressed [25,26], we compared the CDR1 promoter activities of strains containing native or hyperactive TAC1 alleles in wild-type and ndt80\textalpha backgrounds. As can be seen...
in Figure 5, the hyperactive Tac1 caused a similar constitutive CDR1 overexpression in the presence and absence of Ndt80, explaining our finding that Ndt80 is not required for Tac1-mediated fluconazole resistance.

**Ndt80 is dispensable for ERG11 upregulation by a hyperactive Upc2**

Ndt80 has been found to contribute to the fluconazole-induced upregulation of ergosterol biosynthesis genes, including ERG11 [16]. Gain-of-function mutations in the zinc cluster transcription factor Upc2 result in constitutive ERG11 overexpression and increased fluconazole resistance [10,11,12]. To evaluate the importance of Ndt80 for the Upc2-mediated constitutive ERG11 upregulation, we introduced a gain-of-function mutation that results in a G648D amino acid substitution into both endogenous UPc2 alleles of the wild-type strain SC5314 and the ndt80Δ mutants. The P_{ERG11}-GFP reporter fusion was then integrated into these strains to compare their ERG11 promoter activities. FACS analysis of the cells (Figure 6A) and Western immunoblot analysis of GFP expression (Figure 6B) demonstrated that the hyperactive Upc2 caused a constitutive ERG11 overexpression in the presence and absence of Ndt80. To assess whether the hyperactive Upc2 could mediate increased fluconazole resistance in ndt80Δ mutants, we determined the MIC of fluconazole for the strains. Expression of the hyperactive Upc2 resulted in a 4-fold increase in fluconazole resistance in both the wild type and the ndt80Δ mutants, although the hypersusceptibility of the ndt80Δ mutants as compared with the wild type was conserved in the presence of the UPC2_{G648D} allele (Figure 6C). Reintroduction of a single copy of NDT80 in the ndt80Δ mutants restored fluconazole resistance to the level of the corresponding wild-type strains. These results demonstrate that Ndt80 is not required for the constitutive ERG11 overexpression and the resulting increase in fluconazole resistance caused by a hyperactive Upc2.

**Discussion**

Previous findings that Ndt80 contributes to the drug-induced upregulation of CDR1 and ERG11 and that it also binds to the promoters of MDR1 and CDR2 suggested that Ndt80 is involved in all major transcriptional mechanisms of fluconazole resistance in *C. albicans* [16,17]. We found that Ndt80 was dispensable for the upregulation of MDR1 in response to the inducer benomyl and for the induction of CDR2 expression by fluphenazine. In fact, CDR2 expression was partially derepressed in ndt80Δ mutants, arguing that Ndt80 binds to the CDR2 promoter to act as a repressor of this efflux pump under noninducing conditions. In contrast to a previous report [17], we did not observe a reduced basal expression of CDR1 in the absence of Ndt80 (Figure 5). However, this result concurs with another study in which CDR1 was not found among the genes that were differentially expressed in an ndt80Δ mutant [16].

It is likely that Ndt80 is required only under specific conditions for the expression of the genes to which it binds. Ndt80 was shown to bind to the promoter regions of 23% of *C. albicans* genes, but the expression of many of these genes was not altered after deletion of *NDT80* under the conditions tested [16]. Similarly, the zinc cluster transcription factor Upc2, which regulates the expression of ergosterol biosynthesis genes, also binds to the MDR1 promoter, but it is required neither for the benomyl-induced upregulation of the efflux pump nor for its constitutive upregulation by a hyperactive Mrr1 [22]. Nevertheless, under certain environmental conditions Upc2 can act either as an activator or a repressor of MDR1 expression [27]. Ndt80 may therefore also modulate CDR1 and CDR2 expression levels under other conditions than those used in our present study.

Apart from the role of Ndt80 in modulating gene expression in response to environmental signals, which may vary depending on the conditions, a more important question with respect to drug resistance was whether Ndt80 is required for the constitutive overexpression of genes mediating azole resistance in strains that have acquired activating mutations in the transcription factors Mrr1, Tac1, or Upc2. To address this issue, we compared the promoter activities of relevant target genes in strains expressing activated forms of the transcription factors in the presence and absence of Ndt80. Our results demonstrate that Ndt80 is not required for the constitutive overexpression of MDR1, CDR1/CDR2, and ERG11 and the resulting increase in fluconazole resistance caused by hyperactive Mrr1, Tac1, and Upc2, respectively. In fact, strains expressing hyperactive Mrr1 or Tac1 even displayed somewhat higher fluconazole resistance in the absence of Ndt80 than in its presence. Besides the efflux pumps, additional genes are upregulated in strains containing gain-of-function mutations in Mrr1 or Tac1, and these genes also contribute to the increased drug resistance of such strains [22,28]. Apparently, the overexpression of Mrr1 and Tac1 target genes overcomes defects of the ndt80Δ mutants which are responsible for
their fluconazole hypersusceptibility, and the absence of Ndt80 augments the capacity of constitutively active Mrr1 and Tac1 to mediate fluconazole resistance.

Our finding that Ndt80 is dispensable for the constitutive overexpression of MDRI, CDR1/CDR2, and ERG11 in strains with gain-of-function mutations in the zinc cluster transcription factors Mrr1, Tac1, and Upc2, respectively, also has potential practical implications. Combating drug resistance is an important goal to maintain the efficiency of currently used antifungal drugs, which could be achieved by inhibiting efflux pumps or their transcriptional regulators [29,30]. As Mdr1 and Gdr1/Gdr2 belong to different classes of transporters, their activities probably cannot be blocked by the same inhibitor. Similarly, the two types of efflux pumps and the ergosterol biosynthesis genes are specifically regulated by distinct zinc cluster transcription factors. A common mechanism that is used by Mrr1, Tac1, and Upc2 could provide a target for the simultaneous inhibition of several transcriptional mechanisms of drug resistance. As Ndt80 binds to the promoters of the major azole resistance genes and, at least under some conditions, contributes to theazole-induced expression of CDR1 and ergosterol biosynthesis genes, it seemed to be an attractive candidate for such an approach. However, as we have shown in this study, hyperactive forms of Mrr1, Tac1, and Upc2, which are often the cause of azole resistance in clinical C. albicans isolates, do not require Ndt80 to upregulate their target genes and thereby mediate drug resistance. Therefore, blocking Ndt80 would be of little use in attempts to override the azole resistance of clinical strains.

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 containing the NDT80 deletion cassette were grown overnight in YCB-BSA-YE medium (23.4 g yeast carbon base, 4 g bovine serum albumin, 2 g yeast extract per liter, pH 4.0) without selective pressure to induce the SAP2 promoter controlling caFLP expression. Alternatively, strains containing a SAT1 flipper cassette in which the caFLP gene is expressed from the MAL2 promoter (as in plasmids pTAC1M1, pMRRI1, pTAC1R3, and pUPC2R1) were grown overnight in YPM medium (10 g yeast extract, 20 g peptone, 20 g maltose per liter) instead of YCB-BSA-YE to induce the MAL2 promoter. 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 as described previously [31]. For the induction of the MDRI and CDR2 promoters, overnight cultures of the GFP reporter and parental strains were diluted 10−2 in 3 ml fresh YPD medium in glass tubes and grown for 3 h at 30°C. After adding 50 μg/ml benomyl or 10 μg/ml fluphenazine, the cultures were incubated for an additional hour and the fluorescence of the cells was quantified by fluorescence-activated cell sorter (FACS) analysis. For the detection of GFP by Western immunoblotting, cells
UPC2

(C) MIC of fluconazole for strains containing wild-type was detected by Western immunoblotting with an anti-GFP antibody. by the black part in each column. (B) GFP expression in the same strains of the parental strains, which do not contain the UPC2

doi:10.1371/journal.pone.0025623.g006

Figure 6. ERG11 promoter activity and fluconazole resistance of strains expressing the hyperactive UPC2G648D allele in wild-type and ndt80 backgrounds. (A) Reporter strains containing wild-type or hyperactive UPC2 alleles and expressing GFP under the control of the ERG11 promoter were grown to log phase in YPD medium. 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): SCCR2A and -B (wild type, UPC2), SCUPC2R14CG2A and -B (wild type, UPC2G648D), SCndt80EG2A and -B (ndt80Δ, UPC2) SCndt80UPC2R14EG2A and -B (ndt80Δ, UPC2G648D). The background fluorescence of the parental strains, which do not contain the GFP gene, is indicated by the black part in each column. (B) GFP expression in the same strains was detected by Western immunoblotting with an anti-GFP antibody. (C) MIC of fluconazole for strains containing wild-type UPC2 or UPC2G648D alleles in the indicated genetic backgrounds.

doi:10.1371/journal.pone.0025623.g006

were treated as described above, except that the cultures were grown in 50 ml volumes in Erlenmeyer flasks to obtain sufficient amounts of cells.

Plasmid constructions

For the deletion of NDT80, we first generated a modified SAT1 flipper cassette in which the MAL2 promoter controlling caFLP expression was replaced by a 4 kb fragment containing the promoter of the SAP2-1 allele of strain SC5314 [32]. For this purpose, a SalI site in the caSAT1 marker of pSFS2 [31] was first removed by inserting an Xhol-PstI fragment containing the caSAT1 marker from pNM16 [33] instead of the corresponding SalI-PstI fragment in pSFS2, resulting in pSFS4. The SAP2-1 upstream region was then amplified from genomic DNA of strain SC5314 with the primers SAP2P27 and IPC1 (all primers used in this study are listed in Table S2). The PCR product was digested at the introduced BamHI and SalI sites and used to replace the MAL2 promoter in pSFS4, thereby generating pSFS5. In contrast to previous versions of the SAT1 flipper cassette, in which caFLP expression is driven from the leaky MAL2 promoter or a shorter SAP2 promoter fragment, caFLP expression is tightly controlled and efficiently inducible after integration of the modified SAT1 flipper cassette from pSFS5 into the C. albicans genome. The NDT80 upstream and downstream regions were amplified with the primer pairs NDT80-4/NDT80-5 and NDT80-6/NDT80-7, respectively, and the SacI/SacII- and XhoI/ApaI-digested PCR products were cloned on both sides of the SAT1 flipper cassette of pSFS5 to generate pNDT80M3. For reintroduction of NDT80 into ndt80Δ mutants, the NDT80 coding region and ca. 0.5 kb of upstream and downstream sequences were amplified with the primers NDT80-4 and NDT80-3 and the PCR product was digested with SacI and SalI and used to replace the NDT80 upstream region in pNDT80M3, resulting in pNDT80K1.

Plasmid pERG11G2 contains a Candida-adapted GFP reporter gene [34] under the control of the ERG11 promoter [11]. Similar plasmids in which GFP expression is driven by the MDR1, CDR1, and CDR2 promoters were constructed by substituting the upstream and downstream regions of the corresponding genes for the ERG11 flanking sequences in pERG11G2, resulting in plasmids pMDR1G3, pCDR1G1, and pCDR2G3, respectively. The MDR1 upstream and downstream regions were amplified with the primer pairs MDR1p5/MDR1p7 and MDR1-3/MDR1-4, the CDR1 upstream and downstream regions were amplified with the primer pairs CDR1F/CDR1R and CDR29/CDR30, and the CDR2 upstream and downstream regions were amplified with the primer pairs CDR2-5/CDR2-6 and CDR2-3/CDR2-4.

Plasmids pUPC2R1 and pMRR1R3, which were used to replace the UPC2 and MRR1 wild-type alleles by the UPC2G648D and MRR1G648D alleles, respectively, with the help of the SAT1 flipper cassette, were described previously [11,22]. To obtain an analogous cassette for introduction of the TAC1G980E allele, the TAC1 downstream region was first amplified with the primers TAC1-6 and TAC1-7, and the XhoI/ApaI-digested PCR product was substituted for the UPC2 downstream fragment in pUPC2R1, yielding pTAC1R1. The C-terminal part of the TAC1 gene was then amplified with the primers TAC1-11 and TAC1hyp-2, thereby introducing a G2939A exchange that results in the G980E gain-of-function mutation in Tac1 [6]. The PCR product was digested with SacI and then used to replace the TAC1 downstream region in pUPC2R1, yielding pTAC1R1. The TAC1 deletion cassette was generated by amplifying the TAC1 upstream region with the primers TAC1-14 and TAC1-15 and substituting the SacI/SacII-digested PCR product for the UPC2 sequences in pTAC1R1 to obtain pTAC1M1.
Strain constructions

C. albicans strains were transformed by electroporation [35] with the following gel-purified linear DNA fragments: the SacI-Apal fragment from pMRR1R3 and pTAC1R3, and pUPC2R1 were used to introduce the hyperactive MRR1-GFP, TAC1-GFP, and UPC2-GFP allele fusions into the corresponding genomic loci in different strains (see Table S1). The correct integration of each construct was confirmed by direct sequencing of the PCR products. Isolation of genomic DNA and Southern hybridization

Genomic DNA from C. albicans strains was isolated as described previously [31]. The DNA was digested with appropriate restriction enzymes, separated on agarose gels, and transferred by Southern hybridization with gene-specific probes. 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 using logarithmic amplifiers. The mean fluorescence (arbitrary values) was determined with CellQuest Pro (Becton Dickinson) software.

Western immunoblotting

Cells were collected by centrifugation, washed in 2.5 ml of 0.5 mm glass beads in 500 μl TES buffer, and broken by vortexing for 10 min at 4°C. The supernatant was collected, and the protein concentration was quantified using the Bradford protein assay. Extracts were electrophoresed on a SDS-12% polyacrylamide gel. Proteins were transferred onto a nitrocellulose membrane with a Mini-Protean Tetra System (Bio-Rad, Munich, Germany) and stained with Ponceau S to control for equal loading.

Fluconazole susceptibility assays

The fluconazole susceptibility of the strains were determined using a previously described microdilution method [36], except that the assay was performed in SD medium [36] at 30°C. The MICs were determined after 16 to 24 h of incubation.

Supporting Information

Table S1 C. albicans strains used in this study.

Table S2 Primers used in this study.

Author Contributions

Conceived and designed the experiments: CS PDR JM. Performed the experiments: CS RS FD MW SS SM. Analyzed the data: CS JM. Wrote the paper: JM.
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