UNIVERSITÀ DI PISA
DOTTORATO DI RICERCA IN MICROBIOLOGIA E GENETICA
SETTORE SCIENTIFICO DISCIPLINARE MED/07

TESI DI DOTTORATO

MOLECULAR MECHANISMS OF DRUG RESISTANCE IN *CANDIDA ALBICANS*: ROLE OF TRANSCRIPTION FACTORS MCM1 AND ADA2

RELATORE
PROF. SSA SONIA SENESI

CANDIDATA
SELENE MOGAVERO

PRESIDENTE DEL CORSO
PROF. MARIO CAMPA

CORSO DI DOTTORATO 2008-2010

ABSTRACT

Candida albicans is an opportunistic fungal pathogen responsible for localized as well as disseminated infections. *C. albicans* is the most commonly isolated specie from blood cultures, accounting for over 60% of all *Candida* isolates. The fungistatic drug fluconazole is the most widely used to treat fungal infections, thanks to its favorable bio-availability and to its low toxicity. The frequent and widespread use of this antifungal has led to the outcome of drug resistant clinical isolates. Various drug resistance mechanisms are known that render *C. albicans* resistant to fluconazole, among those, the overexpression of efflux pumps, which extrude the drug out of the fungal cell, and the overexpression of the fluconazole target, the Erg11 enzyme. Overexpression of drug resistance genes is often associated to mutations in *trans*-acting transcription factors. The present study aimed at better understanding the role of known transcription factors involved in fluconazole resistance in *C. albicans*. In particular the role of TFs Mcm1 and Ada2 in resistance gene promoter activation has been evaluated. The transcription factors Mrr1 and Cap1 mediate *MDR1* upregulation in response to inducing stimuli, and gain-of-function mutations in Mrr1 or Cap1, which render the transcription factors hyperactive, result in constitutive *MDR1* overexpression. The essential MADS box transcription factor Mcm1 also binds to the *MDR1* promoter, but its role in inducible or constitutive *MDR1* upregulation is unknown. Using a conditional mutant in which Mcm1 can be depleted from the cells, the importance of Mcm1 for *MDR1* expression was investigated. The results obtained indicated that Mcm1 was dispensable for *MDR1* upregulation by H_2O_2, but required for full *MDR1* induction by benomyl. A C-terminally truncated, hyperactive Cap1 could upregulate *MDR1* expression both in the presence and absence of Mcm1. In contrast, a hyperactive Mrr1 containing a gain-of-function mutation depended on Mcm1 to cause

MDR1 overexpression. These results demonstrate a differential requirement of the co-regulator Mcm1 for Cap1- and Mrr1-mediated *MDR1* upregulation. When activated by oxidative stress or a gain-of-function mutation, Cap1 could induce *MDR1* expression independently of Mcm1, whereas Mrr1 required either Mcm1 or an active Cap1 to cause overexpression of the *MDR1* efflux pump.

Other transcription factors that mediate drug resistance gene regulation are Tac1, which regulates *CDR1* and *CDR2* expression, and Upc2, regulating *ERG11* gene expression. Also in this case, gain-of-function mutations, which render these transcription factors hyperactive, result in constitutive overexpression of the target genes.

Ada2 is part of the SAGA/ADA complex and has been shown to be recruited to 200 promoters upstream of genes involved in different stress-response functions and metabolic processes. As for Mcm1, the importance of Ada2 for *MDR1* expression was investigated, as well as for *CDR2* and *ERG11* expression. Ada2 was found to be dispensable for *MDR1* upregulation by H_2O_2, but required for *MDR1* activation by hyperactive Cap1. When activated by benomyl or a gain-of-function mutation, Mrr1 induced *MDR1* expression even better in the absence of Ada2. *CDR2* expression by hyperactive Tac1 was facilitated in the presence of Ada2 and an opposite behaviour was observed when *CDR2* expression was stimulated by the presence of fluphenazine in the medium. Finally, further experiments are required to better understand the role of Ada2 in the expression of *ERG11*.

Overall, these findings provide a more detailed insight into the molecular mechanisms of drug resistance in this important human fungal pathogen.

CONTENTS

1 INTRODUCTION

1.1 *Candida albicans*

The pathogenic fungus *Candida albicans* belongs to a genus composed of at least 150 other species, of whom only a few are able to cause pathology in the human host [**1**]. Those can cause superficial as well as invasive fungal infections. *Candida* spp. are the most common cause of invasive or systemic mycoses, which have dramatically increased over the last 3 decades, particularly in intensive care units (ICUs), but in the outpatient or non-hospital setting as well. Over the last 2 decades, the proportion of non-*C. albicans* infections has raised, nevertheless *C. albicans* remains the most commonly isolated specie from blood cultures, accounting for over 60% of all *Candida* isolates. These trends have heightened interest in newer antifungal agents, such as those of the echinocandin class, that exhibit activity against a broad range of *Candida spp*., including non-*albicans* species with reduced sensitivity or resistance to traditional first-line agents such as fluconazole and amphotericin B [**2**].

One of the reasons that the consequences of invasive candidiasis or candidemia are so severe is that the diagnosis is often made late in the course of the infection, thereby delaying initiation of appropriate treatment. By the time a patient with invasive candidiasis develops a positive blood culture or a person at risk has yeast in his or her bloodstream, the patient is at high risk for metastatic infection of visceral organs that is associated with increased morbidity/mortality. Because definitive diagnosis of candidemia is often delayed, most patients with suspected systemic candidiasis are treated empirically (e.g. prior to culture results when the patient has early signs/symptoms that are not specific for the infection) or prophylactically (in high-risk patients before they show evidence of

disease). The potential drawback of this strategy is that many patients will receive unnecessary courses of antifungal therapy, and this can lead to increased risks for drug toxicity and antifungal resistance [**2**].

Different classes of antifungal drugs are currently available to treat fungal infections, each of them having a specific mode of action [**3**] [**4**] [**5**]. Among these, 5-flucytosine inhibits fungal replication since it is a base analogue that is converted into 5-fluorouracil by fungus-specific enzymes, cytosine deaminase and uracil phosphoribosyltransferase, and this leads to the formation of non functional DNA and RNA [**6**] [**7**] [**8**]. The polyenes target ergosterol by binding to it and creating a pore in the fungal membrane, eventually resulting in cell death [**9**]. The azoles (imidazoles, such as ketoconazole or miconazole, and triazoles such as fluconazole or voriconazole) interrupt the conversion of lanosterol to ergosterol resulting in a sterol-depleted membrane and in the accumulation of toxic compounds [**10**]. The echinocandins (caspofungin, micafungin, anidulafungin) work by inhibiting β-1,3-glucan synthase, thus preventing the synthesis of glucan, an important component of *Candida* cell wall [**7**] [**11**].

1.2 Antifungal drug resistance mechanisms

1.2.1 5-Flucytosine

Fungi can become resistant to each of the above mentioned antifungal drugs by specific mechanisms. 5-Flucytosine (5FC) is an antifungal drug that targets nucleic acid synthesis. The drug is fungus specific, since fungi and plants have a cytosine deaminase that converts 5FC into 5-fluorouracil, which is incorporated into DNA and RNA and inhibits cellular function and division. Mammalian cells do not have cytosine deaminase, and thus 5FC is not effective in these cells. 5FC is usually used in combination with

polyenes or other antifungal agents in the treatment of fungal infections since it has a high frequency of inducing drug resistance [**5**] [**12**].

Common resistance mutations include mutation of the cytosine deaminase, which converts 5FC to 5-fluorouracil, and mutation in the uracil phosphoribosyltransferase (UPRT), an enzyme important for nucleic acid synthesis. In a study performed by Hope *et al*. [**13**], primary resistance to 5FC in 25 *C. albicans* strains was investigated by identifying and sequencing the genes *FCA1*, *FUR1*, *FCY21*, and *FCY22*, which code for cytosine deaminase, UPRT, and two purine-cytosine permeases, respectively. These proteins are involved in pyrimidine salvage and 5FC metabolism. An association between a polymorphic nucleotide and resistance to 5FC was found within *FUR1*, where the substitution of cytosine for thymine at nucleotide position 301 results in the replacement of arginine with cysteine at amino acid position 101 in UPRT. Isolates that were homozygous for this mutation display increased levels of resistance to 5FC, whereas heterozygous isolates showed reduced susceptibility. A single resistant isolate, lacking the above polymorphism in *FUR1*, had a homozygous polymorphism *FCA1* that results in a glycine-to-aspartate substitution at position 28 in cytosine deaminase [**13**]. Another resistance mutation can occur in the cytosine permease, which imports cytosine and 5FC into the cell. Recently, mutation in the permease demonstrated a correlation between permease mutation and fluconazole uptake, perhaps the competition between 5FC and fluconazole [**12**] [**14**].

1.2.2 Polyenes

The polyenes are a class of compounds with an amphipatic nature (one hydrophilic charged side of the molecule, and one hydrophobic uncharged side of the molecule). The polyenes target ergosterol, a sterol related to cholesterol, in fungal membranes. Ergosterol is the primary sterol in these fungal membranes, while cholesterol is the primary sterol in mammalian membranes. Polyenes work by binding to ergosterol and creating a pore that

allows small molecules to diffuse across the membrane, resulting in cell death. Hence, the polyenes are usually fungicidal at physiologic concentrations. There are two main polyenes: amphotericin B (AmB) and nystatin. AmB is the gold standard in the treatment of most fungal infections (*Candida*, *Cryptococcus*, and *Aspergillus*), especially in severe invasive infections where rapid response is needed. However, AmB has significant problems. Large doses are associated with nephrotoxicity, which limits its use. In addition, AmB is insoluble and so suspensions must be delivered intravenously. Use of nystatin is also limited by solubility issues. Both drugs can be used in a suspension for the treatment of oral fungal infections. Recently, lipid formulations of AmB have been developed which combine AmB with lipid structures to increase solubility and decrease toxicity. These formulations can be administered at higher AmB concentrations without toxicity, but they have a considerable expense [**12**].

Resistance to AmB has been identified in some clinical isolates, although the mechanism by which these isolates became resistant is not known. In addition, some laboratory strains with AmB resistance have been constructed [**15**]. Most of these isolates (clinical or laboratory) have a significant reduction of ergosterol in their plasma membrane, thus contributing to AmB resistance since the AmB target is absent [**12**].

1.2.3 Echinocandins

The echinocandins are the newest category of antifungal drug. The drugs work by inhibiting β-1,3-glucan synthase in the plasma membrane of the fungal cells [**16**]. Glucan synthase is important for the production of glucan, an important component of the fungal cell wall [**17**] [**18**]. Caspofungin (Cancidas), the first class member, received FDA approval in 2002 for treatment of *Candida* and *Aspergillus* infections, although it is not effective for *Cryptococcus* infections. Micafungin and anidulafungin followed in 2005 and 2006 respectively. Clinical resistance appears low with sporadic breakthrough cases

reported [**19**]. However, as patient exposure to echinocandin drugs broadens, the number of infecting strains with reduced susceptibility is expected to rise. Unfortunately, the relationship between reduced *in vitro* susceptibility to echinocandin drugs and clinical failure is ambiguous [**20**].

Early genetic studies by Myra Kurtz and Cameron Douglas (Merck Research Labs) with caspofungin in *S. cerevisiae* [**21**] [**22**] and *C. albicans* [**23**] indicated that Fks1, the major subunit of glucan synthase, is the presumed target of the echinocandins. These genetic studies suggested that target-site modification was a likely cause of reduced susceptibility. In fact, mutations that confer reduced echinocandin susceptibility in *S. cerevisiae* and *C. albicans* mapped to *FKS1* [**23**] [**24**]. Clinical isolates of *C. albicans* displaying highly elevated MIC values for caspofungin were found to contain *FKS1* mutations. Specific amino acid substitutions helped define two regions termed "hot-spot" 1 and 2 (HS1/HS2) that confer reduced susceptibility to caspofungin [**25**]. These regions are highly conserved amongst the Fks family [**11**].

1.2.4 Azoles

The azoles are the most important class of Ergosterol Biosynthesis Inhibitors (EBI). EBI are a diverse group of antifungal agents that include azoles, morpholines, thiocarbamates and allylamines. All of these drugs work by inhibiting the biosynthesis of ergosterol. Lack of ergosterol in the plasma membrane results in loss of membrane function and loss of fluidity that inhibits cell growth and division. Azoles work by inhibiting the product of the *ERG11* gene, which demethylates lanosterol, an intermediate in the pathway [**26**]. Inhibition of Erg11 results in the replacement of ergosterol with methylated sterols in the plasma membrane. There are two classes of azole drugs. The imidazoles, including ketoconazole, miconazole, and clotrimazole, have limited use for treatment of systemic infections but are used commonly for localized, surface infections.

The triazoles, including fluconazole, voriconazole, and itraconazole, are now used primarily for systemic infections. Because of its ability to cross the blood-brain barrier, fluconazole remains the best treatment for *Cryptococcus* infections of the brain and is important for long term prophylaxis against reactivation of those infections [**12**].

Yeast and fungal pathogens have developed several mechanisms enabling resistance to the azoles class of antifungal. These mechanisms involve at least four different types of alterations.

1.2.4.1 Alteration of antifungal transport enhanced ATP-dependent efflux

Failure to accumulate azole antifungals has been identified as a cause of azole resistance in several post-treatment clinical fungal isolates. These isolates include yeast species such as *C. albicans*, *C. glabrata*, *C. krusei*, *C. dubliniensis*, and *Cryptococcus neoformans*. In azole-resistant isolates, genes encoding ATP binding cassette (ABC) transporters were upregulated compared to the corresponding azole-susceptible isolates. ABC transporter genes that have been identified as upregulated in azole-resistant isolates include *CDR1* (for "*Candida* drug resistance 1") and *CDR2* in *C. albicans*, *CdCDR1* in *C. dubliniensis*, a *CDR1* homologue in *C. tropicalis*, *CgCDR1* and *CgCDR2* in *C. glabrata*, and *CnAFR* in *C. neoformans*. In *Aspergillus fumigatus*, itraconazole is able to induce an ABC transporter gene, *atrF* [**12**] [**27**] [**28**].

1.2.4.2 Alteration of antifungal transport by enhanced efflux dependent on the membrane proton gradient

Besides upregulation of ABC transporter genes, a multidrug transporter gene *CaMDR1* (for "multidrug resistance 1"), belonging to the family of major facilitators, is upregulated in some *C. albicans* azole-resistant yeast clinical isolates. In *C. albicans* and *C. dubliniensis* isolates with acquired azole resistance as a result of *MDR1* upregulation,

deletion of *CaMDR1* or *CdMDR1* results in a sharp increase in azole susceptibility, thus supporting the involvement of this specific gene in azole resistance [**29**] [**30**]. Deletion of *CaMDR1* in an azole-susceptible laboratory strain does not result in a significant increase in azole susceptibility, consistent with the fact that *CaMDR1* is almost never expressed in this type of strain and more generally in azole-susceptible clinical isolates [**12**] [**31**].

1.2.4.3 Alteration of the target enzyme

Azole resistance can also be the result of alterations in the target enzyme, Erg11, which demethylates lanosterol in the biosynthesis of ergosterol. Three types of alterations can occur with Erg11. First, the gene can be overexpressed. In one study, 35% of the resistant isolates had increased levels of *ERG11* mRNA [**32**]. Second, the gene can be mutated so that azoles are less active against the enzyme. Point mutations have been identified in three "hot spot" regions in the enzyme, such as those surrounding the active site [**33**]. A small number of those mutations have been proven to cause resistance by alteration in *Candida* or expression in other systems. Third, a gene conversion can occur in which allelic differences in *ERG11* are eliminated as resistance develops [**34**] [**35**]. It is postulated that this gene conversion occurs after one allele has been mutated to a resistant phenotype. Then drug pressure would select for a gene conversion event that resulted in the point mutation in both alleles of *ERG11* [**12**].

1.2.4.4 Alteration of the ergosterol biosynthetic pathway

Analysis of the sterol composition of some azole-resistant yeasts has revealed some alterations of enzymes involved in the ergosterol biosynthetic pathway. Accumulation of ergosta-7,22-dienol-3β-ol was observed in two separate azole-resistant *C. albicans* clinical isolates, which is a feature consistent with an absence of sterol $\Delta^{5,6}$-desaturase activity encoded by *ERG3* [**36**]. Interestingly, azole resistance was coupled with resistance to

AmB, which was expected because of the absence of ergosterol. The role of *ERG3* in azole resistance is suggested by the observation that exposure of yeast to azoles inhibits Erg11 and thus results in accumulation of 14α-methylated sterols and 14α-methylergosta-8,24(28)-dien-3β,6α-diol. Formation of this latter sterol metabolite is thought to be catalyzed by the *ERG3* gene product, and thus inactivation of *ERG3* suppresses toxicity and causes azole resistance. Loss-of-function mutations in *ERG3* alleles from the *C. albicans* azole-resistant Darlington strain have been characterized [**37**]. The deletion of *ERG3* in a *C. albicans* laboratory strain results in azole resistance [**15**], and thus one can assume that azole resistance in the Darlington strain is due to inactive *ERG3* alleles.

1.2.5 Transcriptional regulation of azole resistance

Antifungal resistance is often associated with changes in the transcription of genes, some of them being involved directly in resistance. For example, azole resistance is linked to the upregulation of multidrug transporters belonging to the class of ABC transporters and major facilitators and also to the upregulation of *ERG11* encoding the azole target. The molecular analysis of several azole-resistant isolates has revealed that these genes are in most cases not coordinately regulated but that each is regulated differentially [**34**] [**35**] [**38**] [**39**] [**40**]. This suggests that these genes are regulated by distinct regulatory pathways.

1.2.5.1 Regulation of ABC transporters

As mentioned above, *CDR1* and *CDR2* are major ABC transporters involved in azole resistance in *Candida albicans*. *TAC1*, a *C. albicans* transcription factor situated near the mating-type locus on chromosome 5, is necessary for the upregulation of the above mentioned ABC transporters mediating azole resistance [**41**]. It has been shown that a clinical azole-resistant strain that is homozygous at the mating-type locus contains a *TAC1* allele that is sufficient to confer fluconazole resistance to a laboratory strain lacking *TAC1*.

This type of allele was defined as "hyperactive" because it caused constitutive high expression of *CDR1* and *CDR2* in a *tac1*Δ/Δ mutant. In contrast, *TAC1* alleles of the matched azole-susceptible clinical strain or of a laboratory strain, heterozygous at the mating-type locus, were not able to confer azole resistance to a *tac1*Δ/Δ mutant. These alleles were defined as "wild-type" alleles. A wild-type *TAC1* allele also drives a high expression of *CDR1/2* in response to inducers. For instance, exposure to fluphenazine results in a specific upregulation of *CDR1* and *CDR2* [**42**].

1.2.5.2 Regulation of Mdr1 efflux pump

Constitutive overexpression of the *MDR1* gene, which encodes a multidrug efflux pump of the major facilitator superfamily, is a frequent cause of resistance to fluconazole and other toxic compounds in clinical *Candida albicans* strains [**32**] [**34**] [**38**] [**39**] [**43**] [**44**].

MRR1, a zinc cluster transcription factor, is responsible for increased expression of *MDR1* in drug-resistant clinical *C. albicans* isolates. Inactivation of *MRR1* in drug resistant isolates abolished both *MDR1* expression and multidrug resistance. Sequence analysis of the *MRR1* alleles of matched drug-sensitive and drug-resistant *C. albicans* isolate pairs showed that the resistant isolates had become homozygous for *MRR1* alleles that contained single nucleotide substitutions, such as a P683S change in one isolate or a G997V substitution in another isolate, but other substitutions have also been described [**45**]. The introduction of these mutated alleles into a drug-susceptible *C. albicans* strain resulted in constitutive *MDR1* overexpression and multidrug resistance [**46**] [**47**].

Another transcription factor that has been identified as a regulator of *MDR1* expression is the *CAP1* gene, which encodes for a bZip transcription factor. This TF has been shown to be involved in multidrug resistance and oxidative stress response in *C. albicans* [**48**]. The introduction of a hyperactive truncated allele of *CAP1* (*CAP1-TR*) in the

yeast genome results in resistance of the cell to many toxic compounds such as fluconazole, cerulenin, brefeldin A. The hyper-resistant phenotype displayed in the transformants was found to correlate with the overexpression of a number of potential *CAP1* transcriptional targets, including *MDR1*.

MDR1 expression can also be induced in the presence of certain chemical compounds, such as benomyl or hydrogen peroxide [**49**] [**50**]. Deletion constructs of the *MDR1* promoter helped identifying *cis*-acting elements in the *MDR1* promoter responsible for induction by benomyl [**51**]. These elements were localized between -399/299 upstream of the start codon. The *cis*-acting elements responsible for *MDR1* induction by peroxide were localized between -601/500 upstream of the start codon [**49**].

1.2.5.3 Regulation of Erg11 expression

In *Candida albicans*, the zinc cluster transcription factor Upc2 has been shown to regulate the expression of *ERG11* and other genes involved in ergosterol biosynthesis upon exposure to azole antifungals [**52**]. *ERG11* encodes lanosterol demethylase, the target enzyme of this antifungal class. Overexpression of *UPC2* reduces azole susceptibility, whereas its disruption results in hypersusceptibility to azoles and reduced accumulation of exogenous sterols. Overexpression of *ERG11* leads to the increased production of lanosterol demethylase, which contributes to azole resistance in clinical isolates of *C. albicans*. *UPC2* was found to be coordinately upregulated with *ERG11* in a fluconazole-resistant clinical isolate compared with a matched susceptible isolate from the same patient. Sequence analysis of the *UPC2* alleles of these isolates revealed that the resistant isolate contained a single nucleotide substitution in one *UPC2* allele that resulted in a G648D change in the encoded protein. Introduction of the mutated allele into a drug-susceptible strain resulted in constitutive upregulation of *ERG11* and increased resistance to fluconazole [**45**].

ERG11 expression can also be upregulated by the presence of azoles and other sterol biosynthesis inhibitors. In vitro studies demonstrate that azoles are nonfungicidal, with continued growth at strain-dependent rates even at high azole concentrations. RNA analysis revealed that *ERG11* expression in *C. albicans* is maximal during logarithmic-phase growth and decreases as the cells approach stationary phase. Incubation with fluconazole, however, results in a two- to five fold increase in *ERG11* RNA levels within 2 to 3 hours, and this increase is followed by resumption of culture growth. *ERG11* upregulation also occurs following treatment with other azoles (itraconazole, ketoconazole, clotrimazole, and miconazole) [**53**].

1.2.5.4 Mcm1

CaMCM1, a homologue of *S. cerevisiae MCM1*, is an essential gene of the MADS box transcription factor family involved in a variety of cellular processes, including chromatin remodelling, arginine response and mating as well as cell cycle regulation [**54**] [**55**]. In *C. albicans, CaMCM1* is an essential gene as well. Its protein levels are crucial for the determination of cell morphology and are regulated by an autoregulatory feedback mechanism. Depletion of CaMcm1 leads to constitutive induction of hyphae. However, overexpression under specific conditions also results in enhanced hyphae formation. CaMcm1 acts as a mediator recruiting regulatory factors required for morphogenesis in *C. albicans*. In a study that has been published in 2006 by Riggle and Kumamoto [**56**], a 35-bp *MDR1* promoter element has been identified, termed the MDRE, that mediates high-level *MDR1* transcription. The MDRE promotes transcription in an orientation-independent and dosage-dependent manner. Deletion of the MDRE in the full-lenght promoter does not abolish *MDR1 trans*-activation, indicating that elements upstream of the MDRE also contribute to transcription of *MDR1* in overexpressing strains. Analysis of the MDRE sequence indicates that it contains an Mcm1 binding site. Electrophoretic mobility

shift analysis demonstrated that both wild-type, FLC-sensitive and *MDR1 trans*-activated FLC-resistant strains contain a factor that binds the MDRE. Depletion of Mcm1, by use of strain in which *MCM1* expression in under the control of a regulated promoter [**57**], resulted in a loss of MDRE binding activity. Thus, the general transcription factor Mcm1 participates in the regulation of *MDR1* expression.

1.2.5.5 Ada2

The SAGA/ADA coactivator complex, which regulates numerous cellular processes by coordinating histone acetylation, is widely conserved in eukaryotes, and analysis of the *Candida albicans* genome identifies the components of this complex in the fungal pathogen [**58**]. Sellam *et al*. in 2009 investigated the multiple functions of SAGA/ADA in *C. albicans* by determining the genome-wide occupancy of Ada2 using chromatin immuneprecipitation (ChIP) [**59**]. Ada2 is recruited to 200 promoters upstream of genes involved in different stress-response functions and metabolic processes. Phenotypic and transcriptomic analysis of *ΔΔada2* mutant showed that Ada2 is required for the responses to oxidative stress, as well as to treatments with tunicamycin and fluconazole. Ada2 recruitment to the promoters of oxidative resistance genes is mediated by the transcription factor Cap1, and coactivator function were also established for Gal4, which recruits Ada2 to the promoters of glycolysis and pyruvate metabolism genes. Co-occupancy of Ada2 and the drug resistance regulator Mrr1 on the promoters of core resistance genes characterizing drug resistance in clinical strains was also demonstrated. Ada2 recruitment to the promoters of these genes were shown to be completely dependent on Mrr1. Furthermore, *ADA2* deletion causes a decrease in H3K9 acetylation levels of target genes, thus highlighting its importance for histone acetyl transferase activity [**59**].

1.3 Molecular tools available for *Candida albicans*

Genetic manipulation of *C. albicans* has been hindered by some of its own attributes. The construction of mutant strains is relatively laborious in comparison to *S. cerevisiae* due to the lack of an exploitable sexual cycle and the diploid nature of *C. albicans*. The expression of heterologous genes, like reporter genes, is also problematic because of the non-canonical decoding of the CUG codon [**60**] [**61**]. Until recently, genetic engineering in *C. albicans* largely depended on the use of auxotrophic strains because no dominant markers were available for the selection of transformants. Despite these limitations, powerful molecular methods for transformation, gene disruption and gene expression have been developed in recent years [**60**] [**61**]. This was facilitated by the recent availability of the complete *C. albicans* genome sequence. An optimized tool for gene disruption has been described by Reuß et al. in 2004 which has been used in this study [**62**].

1.3.1 Transformation vectors

The use of episomal plasmids results in relatively high transformation frequencies because they do not require a homologous recombination event. However, these plasmids need to contain one of the three autonomous replicating sequences identified for *C. albicans*. In most cases, transformants obtained by this method will contain both episomal and integrated plasmid copies in variable numbers [**63**]. This genetic variability is not conducive to accurate molecular manipulation. Specific integration vectors that allow high-frequency transformation into neutral chromosomal sites have been described [**64**] [**65**] [**66**]. These vectors rely on the homologous recombination between a *C. albicans* gene or a gene fragment present in the plasmid and its chromosomal counterpart. Integration

frequency is improved dramatically by linearising the plasmid within the region of homology to generate recombinogenic ends [**63**].

1.3.2 Gene inactivation

In general, specific deletions of *C. albicans* genomic regions are constructed by using a disruption cassette that contains a selectable marker sequence and flanking regions of homology to the target locus that promote genomic integration by homologous recombination [**63**]. The *URA*-blaster strategy was the most extensively used method for the sequential disruption of both copies of a gene in *C. albicans* [**67**]. This strategy is based on a *hisG-URA-hisG* cassette, which contains the *C. albicans URA3* gene flanked by direct repeats of *Salmonella typhimurium hisG*. Insertion is achieved by homologous recombination between the gene-specific regions flanking this disruption cassette and the chromosomal copy of the gene. Subsequent intrachromosomal homologous recombination between the *hisG* repeats results in the loss of the *URA3* marker. These *Ura*-revertants can be selected on medium containing 5-fluoroorotic acid (5-FOA). The same cassette can then be used to inactivate the second copy of the gene [**67**] [**68**]. More recently, several observations revealed that the use of the *URA3* marker in gene disruptions may lead to inaccurate interpretation of the results. Proper control strains expressing the *URA3* marker from the same locus as the mutant strains are required to exclude these "*URA3*-effects" [**69**] [**70**] [**71**] [**72**].

A Polymerase Chain Reaction-based disruption method employs long oligonucleotides with homology to the target gene [**73**] [**74**]. These oligonucleotides are used as primers to amplify the selectable marker from a vector, in order to create a disruption cassette comprising the selectable marker flanked by short regions of homology to the target locus. The disruption cassette is transformed directly into *C. albicans*. This strategy is quicker than the *URA*-blaster method because it does not require the

construction of the disruption cassette by cloning. However, a double auxotrophic strain is required because each allele must be disrupted with a different marker, since they cannot be recycled [**68**]. Alternative gene disruption methods have been developed more recently to allow marker recycling, make the process less time-consuming and avoid the use of 5-FOA, which might induce chromosome alterations [**75**]. In the *URA*-flipper method the *URA3* marker becomes excised by the Flp recombinase, which is also part of the disruption cassette [**76**]. The Cre-Lox system also relies on a recombinase, the site-specific (Cre) recombinase, to excise the selectable markers and allow further deletions of other genes in the same strain [**77**].

The *SAT1* flipping method relies on the use of a cassette that contains a dominant nourseothricin resistance marker (*caSAT1*) for the selection of integrative transformants and a *C. albicans*-adapted *FLP* gene that allows the subsequent excision of the cassette, which is flanked by *FLP* target sequences, from the genome [**62**]. Two rounds of integration/excision generate homozygous mutants that differ from the wild-type parent strain only by the absence of the target gene, and reintegration of an intact gene copy for complementation of mutant phenotypes is performed in the same way. Transformants are obtained after only one day of growth on a selective medium, and integration into the target locus occurs with high specificity after adding homologous flanking sequences on both sides of the cassette. *FLP*-recombinase is put under the control of the *MAL2* promoter and *FLP*-mediated excision of the *SAT1* flipper cassette can be achieved by simply growing the transformants for a few hours in medium containing maltose without selective pressure, and nourseothricin-sensitive (Nou^s) derivatives can easily be identified by their slower growth on indicator plates containing a low concentration of nourseothricin. Another way of inducing the expression of the *FLP*-recombinase is by putting this gene under the control of another inducible promoter, P_{SAP2}. This method is less “leaky” than the

previous one since the P_{MAL2} can be induced also in complete medium not containing maltose. In this way, the *FLP*-recombinase expression can be induced by the presence of BSA in the growth medium.

Fig. 1 shows a schematic model of the *SAT1* flipper cassette used in this study.

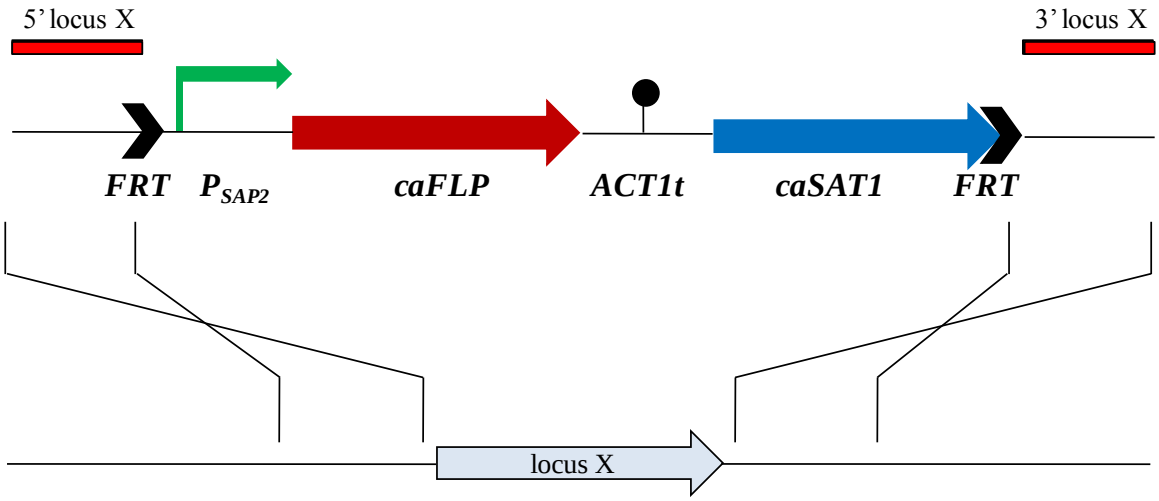

Fig. 1. Structure of the *SAT1* flipper cassette.
The *C. albicans*-adapted *FLP* gene (*caFLP*) is represented by the red arrow, the nourseothricin resistance marker (*caSAT1*) by the blue arrow, the *SAP2* promoter (P_{SAP2}) by the bent green arrow, and the transcription termination sequence of the *C. albicans ACT1* gene (*ACT1t*) by the filled circle. The 34-bp *FLP* recombination target sequences (*FRT*, black arrows) (5'-GAA GTT CCT ATA CTT TCT AGA GAA TAG GAA CTT C-3') flanking the cassette are not drawn to scale. The figure has been modified from Reuß *et al.* [**62**].

1.3.3 Reporter genes

Reporter genes are useful tools to study gene regulation, although they do not necessarily reflect transcript stability of the gene of interest [**60**] [**63**]. The *LAC4* gene of the yeast *Kluveromyces lactis*, which encodes β-galactosidase, was the first functional heterologous reporter gene for *C. albicans* [**78**]. The moderate sensitivity initially achieved with colorimetric substrates was dramatically increased using a bioluminescent substrate [**79**]. A second reporter system based on the enzymatic activity of the β-galactosidase was developed by Uhl *et al.* in 2001 [**80**]. In this case, the *lacZ* gene of *Streptococcus thermophilus* was cloned downstream of three different promoters, a constitutive one and

two inducible ones. Expression was readily detected in liquid assays using permeabilised cells and *in situ* colorimetric assays of colonies growing on plates. The luciferase gene from the sea pansy *Renilla reniformis* (*RrLUC*) does not contain CTG codons, and thus has been exploited as a useful reporter gene in *C. albicans* [**81**]. The bioluminescence based-assay proved an effective tool to study the activity of inducible, constitutive and phase-specific (white or opaque) promoters. However, the efficiency of this system with intact cells is significantly lower than with protein extracts. Reporter systems based on modified versions of the green fluorescent protein (*GFP*) gene from the jellyfish *Aequorea victoria* are also available [**82**] [**83**]. They have been genetically engineered to replace the non-canonical CTG serine codon for the TTG leucine codon and to carry mutations in the chromophore that enhance *GFP* fluorescence. These *yEGFP* (yeast enhanced *GFP*) genes offer major advantages in comparison to the β-galactosidase and luciferase enzymatic systems: no cofactors are required for the expression of *GFP* and the resulting fluorescence can be detected in living organisms, even at the level of single cells [**84**] [**85**]. This makes *yEGFP* an optimal reporter for studying the sub-cellular localization of a protein of interest [**60**]. More recently, cassettes for the PCR-mediated construction of green, yellow and cyan fluorescent protein fusions have also been developed [**86**].

1.4 Aims of the study

Fluconazole is the most widely used antimycotic to treat infections caused by *C. albicans*, the most common agent of human fungal infections, but the frequent and widespread use of this drug has caused an increased frequency of drug resistant clinical *C. albicans* strains. The present study was aimed to investigate new aspects of the underlying mechanisms that lead to fluconazole resistance in *C. albicans*. Previous studies have illustrated that common fluconazole resistance mechanisms in *C. albicans* are the overexpression of efflux pumps (such as *MDR1, CDR1* and *CDR2*) or the overexpression

of the target gene *ERG11*, which are regulated by known transcription factors (*CAP1*, *MRR1*, *TAC1*, *UPC2*). Recently, new transcription factors (TFs), *MCM1* and *ADA2* have been shown to be involved in drug resistance gene regulation. On this ground, the present study was focused to define the role of those two transcription factors in the azole resistance. To this aim, *MCM1* conditional mutants [**57**] and *ADA2* knock-out mutants, constructed in this study, were used to analyze the expression of drug resistance related genes in response to inducing chemicals. In addition, expression of drug resistance related genes has also been evaluated in *MCM1* or *ADA2* mutants containing hyperactive transcription factors alleles.

The present PhD project was carried out at the "Dipartimento di Biologia, sezione di Microbiologia" of the University of Pisa, Via San Zeno 35-39, under the supervision of Prof. ssa Sonia Senesi, and at the mycology unit of the "Institut für Molekulare Infektionsbiologie", Josef-Schneider Straße 2, Bau D15, 97080 Würzburg, under the supervision of Prof. Joachim Morschhäuser.

2 MATERIALS AND METHODS

For all microbiology and molecular biology procedures standard protocols were followed from Sambrook *et al*., (1989) [**87**] or Asubel *et al*., (1989) [**88**] with minor modifications.

All solutions and media were made in double distilled and distilled water, respectively. All solutions and media were sterilized by autoclaving or filter sterilized by passing through a 0.22 micron Millipore filter.

2.1 Bacterial strain

Escherichia coli K12: *E. coli* strain DH5α (F^-, *end*A1, *hsd*R17 [r_k^-, m_k^-], *sup*E44, *thi-1*, *rec*A1, *gyr*A96, *rel*A1, Δ[*arg*F-*lac*]U169, λ^-, Φ80d*lac*ZΔM15) (Bethesda Research Laboratories, 1986) was used for bacterial cloning experiments.

2.2 Primers

Details of primers used in this study are shown in Table 1. Primers were obtained from MWG (Ebersberg, Germany).

Table 1. List of primers used in this study

Primer name	Sequence (5'-3')	Gene	Gene sequences	Introduced restriction sites/ position
ACT1RT	AGTGTGACATGGATGTTAGAAAAGAATTATACGG	*ACT1*	+1512 +1545	
ACT2RT	ACAGAGTATTTTCTTTCTGGTGGAGCA	*ACT1*	+1677 +1651	
ADA2-3	GGGATGGTGTTCTCGAGGGGTAAATGTAG	*ADA2*	+1317 +1344	XhoI +1328
ADA2-4	GATCGGGCCCTACTTGATCGGTCATACTGGAGC	*ADA2*	+1753 +1721	ApaI +1748
ADA2-5	AACCTAGCAAACGAGCTCACGTGATGTAAGTG	*ADA2*	-529 -498	SacI -513
ADA2-6	GATAATCATGCCGCGGTTTATGATCTCCAG	*ADA2*	+160 +131	SacII +148
ADA2-7	GCAGGATTGACTACTGGAGCTCATAAACCATG	*ADA2*	+118 +149	SacI +138
ADA2-8	GGTAATGGTATATTTTTCCGCGGTTCTAATCGTTC	*ADA2*	+452 +418	SacII +433
ADA2-9	CCAAACACCAGGATTCTCGAGTGGTAATTCTT	*ADA2*	+987 +1018	XhoI +1002
ADA2-10	CTGAGAACACGGGCCCATATGCACAAA	*ADA2*	+1332 +1306	ApaI +1321

Primer name	Sequence (5'-3')	Gene	Gene sequences	Introduced restriction sites/ position
ADA2-11	ATATGAGCTCGGGCCCACTCACGTGATGTAAGTGC	*ADA2*	-518 -497	SacI-ApaI
ADA2-12	CTCCATCTCAACCGCGGCCAGAAGTATTGCC	*ADA2*	+1902 +1872	SacII +1889
CDR2-5	AGATGGTACCAGGTAGTGATAGTTAACACACC	*CDR2*	-464 -433	KpnI -456
CDR2-6	ATATGTCGACATTGTATGTGTTAATTAGTGAAATA	*CDR2*	+2 -33	SalI -8
EFB1A	ATTGAACGAATTCTTGGCTGAC	*EF1B*	+42 +63	
EFB1B	CATCTTCTTCAACAGCAGCTTG	*EF1B*	+960 +939	
MDR5RT	ATTTGTTCAGATCAGTCATTGCTTCAGTGT	*MDR1*	+1499 +1528	
MDR6RT	GGTCCGTTCAAGTAAAACAAAACTGGAATA	*MDR1*	+1661 +1632	

Restriction sites introduced into the primers are underlined.

2.3 Plasmids

The following abbreviations are used in the description of plasmids: *caFLP*: *C. albicans*-adapted *FLP* gene encoding the site specific recombinase *FLP*; *caSAT1*: *C. albicans*-adapted nourseothricin resistance marker (dominant selection marker); *FRT*: (*FLP*-recognition target) minimal recombination target sites of the *FLP* recombinase; *GFP*: Green fluorescent protein gene; P_x: Promoter of the given gene (*X*) ; P_{tet}: rtTA-dependent promoter; *ACT1t*: Transcription termination sequence of the actin (*ACT1*) gene.

Table 2. Plasmids used in this study

Plasmids	Relevant insert	Reference
Plasmids used for the *MCM1* study		
pCAP1R1	[*5'CAP1*$^{\Delta C333}$*-ACT1t-FRT-*P_{MAL2}*-caFLP-ACT1t-caSAT1-FRT-3'CAP1*]- fragment for introduction of hyperactive *CAP1* allele	Schubert *et al*., submitted
pMPG2S	[*3'ACT1-*P_{MDR1}*-GFP-ACT1t-caSAT1-*P_{ACT1}]- fragment for *MDR1* promoter analysis, integration on *ACT1* promoter	Morschhäuser *et al*., 2007 [**46**]
pMRR1R3	[*MRR1*^{P683S}*-ACT1t-FRT-*P_{MAL2}*-caFLP-ACT1t-caSAT1-FRT-3'MRR1*]- fragment for introduction of hyperactive *MRR1* allele	Schubert *et al*., submitted
Plasmids used for the *ADA2* study		
pADA2K1	[*ADA2-FRT-*P_{SAP2-1}*-caFLP-ACT1t-caSAT1-FRT-3'ADA2*]- fragment for gene reintegration	This study
pADA2M1	[5'*CAP1-FRT-*P_{SAP2-1}*-caFLP-ACT1t-caSAT1-FRT-3'ADA2*]- fragment	This study
pADA2M2	[5'*ADA2-FRT-*P_{SAP2-1}*-caFLP-ACT1t-caSAT1-FRT-3'ADA2*]- fragment for gene deletion (allele 1)	This study
pADA2M3	[5'*ADA2-FRT-*P_{SAP2-1}*-caFLP-ACT1t-caSAT1-FRT-3'ADA2*]- fragment	This study
pADA2M4	[5'*ADA2-FRT-*P_{MAL2}*-caFLP-ACT1t-caSAT1-FRT-3'ADA2*]- fragment for gene deletion (allele 2)	This study

Plasmids	Relevant insert	Reference
pCAP1R1	[*5'CAP1*$^{\Delta C333}$*- ACT1t -FRT-*P$_{MAL2}$*-caFLP-ACT1t-caSAT1-FRT-3'CAP1*]- fragment for introduction of hyperactive *CAP1* allele	Schubert *et al*., submitted
pCDR2G3	[P$_{CDR2}$*-GFP-ACT1t-caSAT1-3'CDR2*]- fragment for *CDR2* promoter analysis	This study
pERG11G2	[P$_{ERG11}$*-GFP-ACT1t-caSAT1-3'ERG11*]- fragment for *ERG11* promoter analysis	Heilmann *et al*., 2010 [**89**]
pMDR1G3	[P$_{MDR1}$*-GFP-ACT1t-caSAT1-3'MDR1*]- fragment for *MDR1* promoter analysis	Morschhäuser lab
pMRR1R3	[*MRR1*^{P683S}*-ACT1t-FRT-*P$_{MAL2}$*-caFLP-ACT1t-caSAT1-FRT-3'MRR1*]- fragment for introduction of hyperactive *MRR1* allele	Schubert *et al*., submitted
pTAC1R3	[*3'TAC1*^{G980E}*-ACT1t-FRT-*P$_{MAL2}$*-caFLP-ACT1t-caSAT1-FRT-3'TAC1*]- fragment for introduction of *TAC1* hyperactive allele	Morschhäuser lab
pUPC2R1	[*UPC2*^{G648D}*-ACT1t-FRT-*P$_{MAL2}$*-caFLP-ACT1t-caSAT1-FRT-3'UPC2*]- fragment for introduction of *UPC2* hyperactive allele	Heilmann *et al*., 2010 [**89**]

2.4 *C. albicans* strains

Parental and mutant *Candida albicans* strains used in this study are listed in Table 3. Some mutants used in this study where already present in the collection of Prof. Morschhäuser's group. MRcan42 and MRcan43 where kindly provided by Prof. Steffen Rupp (University of Stuttgart, Germany) and SC5314 is the *Candida albicans* reference strain.

Table 3. Strains used in this study

Strain	Parent	Relevant genotype	Reference
SC5314		Wild-type reference strain	Gillum *et al*., 1984 [**90**]
SCMPG2A	SC5314	*ACT1/act1::*P$_{MDR1}$*-GFP-caSAT1*	Morschhäuser *et al*., 2007 [**46**]
SCMPG2B	SC5314	*ACT1/act1*::P$_{MDR1}$*-GFP-caSAT1*	Morschhäuser *et al*., 2007 [**46**]
MRcan42	SC531	*pTR-MCM1-myc/mcm1Δ*	Rottmann *et al*., 2003 [**57**]
MRcan43	SC5314	*pTR-MCM1/MCM1-myc*	Rottmann *et al*., 2003 [**57**]
can42MPG2A	MRcan42	*ACT1/act1*::P$_{MDR1}$*-GFP-caSAT1*	this study
can42MPG2B	MRcan42	*ACT1/act1*::P$_{MDR1}$*-GFP-caSAT1*	this study
can43MPG2A	MRcan43	*ACT1/act1*::P$_{MDR1}$*-GFP-caSAT1*	this study
can43MPG2B	MRcan43	*ACT1/act1*::P$_{MDR1}$*-GFP-caSAT1*	this study
can42MRR1R31A	MRcan42	*MRR1/MRR1*^{P683S}*-SAT1-FLIP*	this study
can42MRR1R31B	MRcan42	*MRR1/MRR1*^{P683S}*-SAT1-FLIP*	this study
can42MRR1R32A	can42MRR1R31A	*MRR1/MRR1*^{P683S}*-FRT*	this study
can42MRR1R32B	can42MRR1R31B	*MRR1/MRR1*^{P683S}*-FRT*	this study
can42MRR1R33A	can42MRR1R32A	*MRR1*^{P683S}*-SAT1-FLIP/MRR1*^{P683S}*-FRT*	this study

Strain	Parent	Relevant genotype	Reference
can42MRR1R33B	can42MRR1R32B	*MRR1*^{P683S}*-SAT1-FLIP*/*MRR1*^{P683S}*-FRT*	this study
can42MRR1R34A	can42MRR1R33A	*MRR1*^{P683S}*-FRT*/*MRR1*^{P683S}*-FRT*	this study
can42MRR1R34B	can42MRR1R33B	*MRR1*^{P683S}*-FRT*/*MRR1*^{P683S}*-FRT*	this study
can43MRR1R31A	MRcan43	*MRR1*/*MRR1*^{P683S}*-SAT1-FLIP*	this study
can43MRR1R31B	MRcan43	*MRR1*/*MRR1*^{P683S}*-SAT1-FLIP*	this study
can43MRR1R32A	can43MRR1R31A	*MRR1*/*MRR1*^{P683S}*-FRT*	this study
can43MRR1R32B	can43MRR1R31B	*MRR1*/*MRR1*^{P683S}*-FRT*	this study
can43MRR1R33A	can43MRR1R32A	*MRR1*^{P683S}*-SAT1-FLIP*/*MRR1*^{P683S}*-FRT*	this study
can43MRR1R33B	can43MRR1R32B	*MRR1*^{P683S}*-SAT1-FLIP*/*MRR1*^{P683S}*-FRT*	this study
can43MRR1R34A	can43MRR1R33A	*MRR1*^{P683S}*-FRT*/*MRR1*^{P683S}*-FRT*	this study
can43MRR1R34B	can43MRR1R33B	*MRR1*^{P683S}*-FRT*/*MRR1*^{P683S}*-FRT*	this study
can42CAP1R11A	MRcan42	*CAP1*$^{\Delta C333}$*-SAT1-FLIP*/*CAP1-2*	this study
can42CAP1R11B	MRcan42	*CAP1-1*/*CAP1*$^{\Delta C333}$*-SAT1-FLIP*	this study
can42CAP1R12A	can42CAP1R11A	*CAP1*$^{\Delta C333}$*-FRT*/*CAP1-2*	this study
can42CAP1R12B	can42CAP1R11B	*CAP1-1*/*CAP1*$^{\Delta C333}$*-FRT*	this study
can42CAP1R13A	can42CAP1R12A	*CAP1*$^{\Delta C333}$*-FRT*/*CAP1*$^{\Delta C333}$*-SAT1-FLIP*	this study
can42CAP1R13B	can42CAP1R12B	*CAP1*$^{\Delta C333}$*-SAT1-FLIP*/*CAP1*$^{\Delta C333}$*-FRT*	this study
can42CAP1R14A	can42CAP1R13A	*CAP1*$^{\Delta C333}$*-FRT*/*CAP1*$^{\Delta C333}$*-FRT*	this study
can42CAP1R14B	can42CAP1R13B	*CAP1*$^{\Delta C333}$*-FRT*/*CAP1*$^{\Delta C333}$*-FRT*	this study
can43CAP1R11A	MRcan43	*CAP1-1*/*CAP1*$^{\Delta C333}$*-SAT1-FLIP*	this study
can43CAP1R11B	MRcan43	*CAP1-1*/*CAP1*$^{\Delta C333}$*-SAT1-FLIP*	this study
can43CAP1R12A	can43CAP1R11A	*CAP1-1*/*CAP1*$^{\Delta C333}$*-FRT*	this study
can43CAP1R12B	can43CAP1R11B	*CAP1-1*/*CAP1*$^{\Delta C333}$*-FRT*	this study
can43CAP1R13A	can43CAP1R12A	*CAP1*$^{\Delta C333}$*-SAT1-FLIP*/*CAP1*$^{\Delta C333}$*-FRT*	this study
can43CAP1R13B	can43CAP1R12B	*CAP1*$^{\Delta C333}$*-SAT1-FLIP*/*CAP1*$^{\Delta C333}$*-FRT*	this study
can43CAP1R14A	can43CAP1R13A	*CAP1*$^{\Delta C333}$*-FRT*/*CAP1*$^{\Delta C333}$*-FRT*	this study
can43CAP1R14B	can43CAP1R13B	*CAP1*$^{\Delta C333}$*-FRT*/*CAP1*$^{\Delta C333}$*-FRT*	this study
SCADA2M1A	SC5314	*ada2*Δ::*SAT1-FLIP*/*ADA2*	this study
SCADA2M1B	SC5314	*ada2*Δ::*SAT1-FLIP*/*ADA2*	this study
SCADA2M2A	SCADA2M1A	*ada2*Δ::*FRT*/*ADA2*	this study
SCADA2M2B	SCADA2M1B	*ada2*Δ::*FRT*/*ADA2*	this study
SCADA2M3A	SCADA2M2A	*ada2*Δ::*FRT*/*ada2*Δ::*SAT1-FLIP*	this study
SCADA2M3B	SCADA2M2B	*ada2*Δ::*FRT*/*ada2*Δ::*SAT1-FLIP*	this study
SCADA2M4A	SCADA2M3A	*ada2*Δ::*FRT*/*ada2*Δ::*FRT*	this study
SCADA2M4B	SCADA2M3B	*ada2*Δ::*FRT*/*ada2*Δ::*FRT*	this study
SCADA2MK1A	SCADA2M4A	*ADA2*::*SAT1-FLIP*/*ada2*Δ::*FRT*	this study
SCADA2MK1B	SCADA2M4B	*ADA2*::*SAT1-FLIP*/*ada2*Δ::*FRT*	this study
SCADA2MK2A	SCADA2MK1A	*ADA2*::*FRT*/*ada2*Δ::*FRT*	this study
SCADA2MK2B	SCADA2MK1B	*ADA2*::*FRT*/*ada2*Δ::*FRT*	this study

Strain	Parent	Relevant genotype	Reference
SCΔada2CG3A	SCADA2M4A	*CDR2*/*cdr2*::P_{CDR2}-*GFP-caSAT1*	this study
SCΔada2CG3B	SCADA2M4B	*CDR2*/*cdr2*::P_{CDR2}-*GFP-caSAT1*	this study
SCΔada2EG2A	SCADA2M4A	*ERG11*/*erg11*::P_{ERG11}-*GFP-caSAT1*	this study
SCΔada2EG2B	SCADA2M4B	*ERG11*/*erg11*::P_{ERG11}-*GFP-caSAT1*	this study
SCΔada2MG3A	SCADA2M4A	*MDR1*/*mdr1*::P_{MDR1}-*GFP-caSAT1*	this study
SCΔada2MG3B	SCADA2M4B	*MDR1*/*mdr1*::P_{MDR1}-*GFP-caSAT1*	this study
SCΔada2CAP1R11A	SCADA2M4A	$CAP1^{\Delta C333}$-*SAT1-FLIP*/*CAP1-2*	this study
SCΔada2CAP1R11B	SCADA2M4B	*CAP1-1*/$CAP1^{\Delta C333}$-*SAT1-FLIP*	this study
SCΔada2CAP1R12A	SCΔada2CAP1R11A	$CAP1^{\Delta C333}$-*FRT*/*CAP1-2*	this study
SCΔada2CAP1R12B	SCΔada2CAP1R11B	*CAP1-1*/$CAP1^{\Delta C333}$-*FRT*	this study
SCΔada2CAP1R13A	SCΔada2CAP1R12A	$CAP1^{\Delta C333}$-*FRT*/$CAP1^{\Delta C333}$-*SAT1-FLIP*	this study
SCΔada2CAP1R13B	SCΔada2CAP1R12B	$CAP1^{\Delta C333}$-*SAT1-FLIP*/$CAP1^{\Delta C333}$-*FRT*	this study
SCΔada2CAP1R14A	SCΔada2CAP1R13A	$CAP1^{\Delta C333}$-*FRT*/$CAP1^{\Delta C333}$-*FRT*	this study
SCΔada2CAP1R14B	SCΔada2CAP1R13B	$CAP1^{\Delta C333}$-*FRT*/$CAP1^{\Delta C333}$-*FRT*	this study
SCΔada2CAP1R14MG3A	SCΔada2CAP1R14A	*MDR1*/*mdr1*::P_{MDR1}-*GFP-caSAT1*	this study
SCΔada2CAP1R14MG3B	SCΔada2CAP1R14B	*MDR1*/*mdr1*::P_{MDR1}-*GFP-caSAT1*	this study
SCΔada2MRR1R31A	SCADA2M4A	$MRR1^{P683S}$-*SAT1-FLIP*/*MRR1*	this study
SCΔada2MRR1R31B	SCADA2M4B	$MRR1^{P683S}$-*SAT1-FLIP*/*MRR1*	this study
SCΔada2MRR1R32A	SCΔada2MRR1R31A	$MRR1^{P683S}$-*FRT*/*MRR1*	this study
SCΔada2MRR1R32B	SCΔada2MRR1R31B	$MRR1^{P683S}$-*FRT*/*MRR1*	this study
SCΔada2MRR1R33A	SCΔada2MRR1R32A	$MRR1^{P683S}$-*FRT*/$MRR1^{P683S}$-*SAT1-FLIP*	this study
SCΔada2MRR1R33B	SCΔada2MRR1R32B	$MRR1^{P683S}$-*FRT*/$MRR1^{P683S}$-*SAT1-FLIP*	this study
SCΔada2MRR1R34A	SCΔada2MRR1R33A	$MRR1^{P683S}$-*FRT*/$MRR1^{P683S}$-*FRT*	this study
SCΔada2MRR1R34B	SCΔada2MRR1R33B	$MRR1^{P683S}$-*FRT*/$MRR1^{P683S}$-*FRT*	this study
SCΔada2MRR1R34MG3A	SCΔada2MRR1R34A	*MDR1*/*mdr1*::P_{MDR1}-*GFP-caSAT1*	this study
SCΔada2MRR1R34MG3B	SCΔada2MRR1R34B	*MDR1*/*mdr1*::P_{MDR1}-*GFP-caSAT1*	this study
SCΔada2TAC1R31A	SCADA2M4A	$TAC1^{G980E}$-*SAT1-FLIP*/*TAC1-2*	this study
SCΔada2TAC1R31B	SCADA2M4B	*TAC1-1*/$TAC1^{G980E}$-*SAT1-FLIP*	this study
SCΔada2TAC1R32A	SCΔada2TAC1R31A	$TAC1^{G980E}$-*FRT*/*TAC1-2*	this study
SCΔada2TAC1R32B	SCΔada2TAC1R31B	*TAC1-1*/$TAC1^{G980E}$-*FRT*	this study
SCΔada2TAC1R33A	SCΔada2TAC1R32A	$TAC1^{G980E}$-*FRT*/$TAC1^{G980E}$-*SAT1-FLIP*	this study
SCΔada2TAC1R33B	SCΔada2TAC1R32B	$TAC1^{G980E}$-*SAT1-FLIP*/$TAC1^{G980E}$-*FRT*	this study
SCΔada2TAC1R34A	SCΔada2TAC1R33A	$TAC1^{G980E}$-*FRT*/$TAC1^{G980E}$-*FRT*	this study
SCΔada2TAC1R34B	SCΔada2TAC1R33B	$TAC1^{G980E}$-*FRT*/$TAC1^{G980E}$-*FRT*	this study
SCΔada2TAC1R34CG3A	SCΔada2TAC1R34A	*CDR2*/*cdr2*::P_{CDR2}-*GFP-caSAT1*	this study
SCΔada2TAC1R34CG3B	SCΔada2TAC1R34B	*CDR2*/*cdr2*::P_{CDR2}-*GFP-caSAT1*	this study
SCΔada2UPC2R11A	SCADA2M4A	$UPC2^{G648D}$-*SAT1-FLIP*/*UPC2*	this study
SCΔada2UPC2R11B	SCADA2M4B	$UPC2^{G648D}$-*SAT1-FLIP*/*UPC2*	this study
SCΔada2UPC2R12A	SCΔada2UPC2R11A	$UPC2^{G648D}$-*FRT*/*UPC2*	this study

Strain	Parent	Relevant genotype	Reference
SCΔada2UPC2R12B	SCΔada2UPC2R11B	*UPC2^{G648D}-FRT/UPC2*	this study
SCΔada2UPC2R13A	SCΔada2UPC2R12A	*UPC2^{G648D}-FRT/UPC2^{G648D}-SAT1-FLIP*	this study
SCΔada2UPC2R13B	SCΔada2UPC2R12B	*UPC2^{G648D}-FRT/UPC2^{G648D}-SAT1-FLIP*	this study
SCΔada2UPC2R14A	SCΔada2UPC2R13A	*UPC2^{G648D}-FRT/UPC2^{G648D}-FRT*	this study
SCΔada2UPC2R14B	SCΔada2UPC2R13B	*UPC2^{G648D}-FRT/UPC2^{G648D}-FRT*	this study
SCΔada2UPC2R14EG2A	SCΔada2UPC2R14A	*ERG11/erg11*::P$_{ERG11}$*-GFP-caSAT1*	this study
SCΔada2UPC2R14EG2B	SCΔada2UPC2R14B	*ERG11/erg11*::P$_{ERG11}$*-GFP-caSAT1*	this study
SCCG3A	SC5314	*CDR2/cdr2*::P$_{CDR2}$*-GFP-caSAT1*	this study
SCCG3B	SC5314	*CDR2/cdr2*::P$_{CDR2}$*-GFP-caSAT1*	this study
SCEG2A	SC5314	*ERG11/erg11*::P$_{ERG11}$*-GFP-caSAT1*	Heilmann *et al.*, 2010 [**89**]
SCEG2B	SC5314	*ERG11/erg11*::P$_{ERG11}$*-GFP-caSAT1*	Heilmann *et al.*, 2010 [**89**]
SCMG3A	SC5314	*MDR1/mdr1*::P$_{MDR1}$*-GFP-caSAT1*	Morschhäuser lab
SCMG3B	SC5314	*MDR1/mdr1*::P$_{MDR1}$*-GFP-caSAT1*	Morschhäuser lab
SCCAP1R12A	SC5314	*CAP1$^{\Delta C333}$-FRT/CAP1-2*	Schubert *et al.*, in revision
SCCAP1R12B	SC5314	*CAP1-1/CAP1$^{\Delta C333}$-FRT*	Schubert *et al.*, in revision
SCCAP1R14A	SCCAP1R12A	*CAP1$^{\Delta C333}$-FRT/CAP1$^{\Delta C333}$-FRT*	Schubert *et al.*, in revision
SCCAP1R14B	SCCAP1R12B	*CAP1$^{\Delta C333}$-FRT/CAP1$^{\Delta C333}$-FRT*	Schubert *et al.*, in revision
SCCAP1R14MG3A	SCCAP1R14A	*MDR1/mdr1*::P$_{MDR1}$*-GFP-caSAT1*	this study
SCCAP1R14MG3B	SCCAP1R14B	*MDR1/mdr1*::P$_{MDR1}$*-GFP-caSAT1*	this study
SCMRR1R32A	SC5314	*MRR1^{P683S}-FRT/MRR1*	Schubert *et al.*, in revision
SCMRR1R32B	SC5314	*MRR1^{P683S}-FRT/MRR1*	Schubert *et al.*, in revision
SCMRR1R34A	SCMRR1R32A	*MRR1^{P683S}-FRT/MRR1^{P683S}-FRT*	Schubert *et al.*, in revision
SCMRR1R34B	SCMRR1R32B	*MRR1^{P683S}-FRT/MRR1^{P683S}-FRT*	Schubert *et al.*, in revision
SCMRR1R34MG3A	SCMRR1R34A	*MDR1/mdr1*::P$_{MDR1}$*-GFP-caSAT1*	this study
SCMRR1R34MG3B	SCMRR1R34B	*MDR1/mdr1*::P$_{MDR1}$*-GFP-caSAT1*	this study
SCTAC1R32A	SC5314	*TAC1^{G980E}-FRT/TAC1-2*	Morschhäuser lab
SCTAC1R32B	SC5314	*TAC1-1/TAC1^{G980E}-FRT*	Morschhäuser lab
SCTAC1R34A	SCTAC1R32A	*TAC1^{G980E}-FRT/TAC1^{G980E}-FRT*	Morschhäuser lab
SCTAC1R34B	SCTAC1R32B	*TAC1^{G980E}-FRT/TAC1^{G980E}-FRT*	Morschhäuser lab
SCTAC1R34CG3A	SCTAC1R34A	*CDR2/cdr2*::P$_{CDR2}$*-GFP-caSAT1*	this study
SCTAC1R34CG3B	SCTAC1R34B	*CDR2/cdr2*::P$_{CDR2}$*-GFP-caSAT1*	this study
SCUPC2R12A	SC5314	*UPC2^{G648D}-FRT/UPC2*	Heilmann *et al.*, 2010 [**89**]
SCUPC2R12B	SC5314	*UPC2^{G648D}-FRT/UPC2*	Heilmann *et al.*, 2010 [**89**]
SCUPC2R14A	SCUPC2R12A	*UPC2^{G648D}-FRT/UPC2^{G648D}-FRT*	Heilmann *et al.*, 2010 [**89**]
SCUPC2R14B	SCUPC2R12B	*UPC2^{G648D}-FRT/UPC2^{G648D}-FRT*	Heilmann *et al.*, 2010 [**89**]

Strain	Parent	Relevant genotype	Reference
SCUPC2R14EG2A	SCUPC2R14A	*ERG11*/*erg11*::P_{ERG11}-*GFP-caSAT1*	Heilmann *et al.*, 2010 [**89**]
SCUPC2R14EG2B	SCUPC2R14B	*ERG11*/*erg11*::P_{ERG11}-*GFP-caSAT1*	Heilmann *et al.*, 2010 [**89**]

2.5 Growth and maintenance of *C. albicans* strains

All strains were stored as frozen stocks with 15% glycerol at -80°C and sub cultured on YPD agar plates (10 g yeast extract, 20 g peptone, 20 g dextrose, 15 g agar per liter) at 30°C. Strains were routinely grown in YPD liquid medium at 30°C in a shaking incubator at 250 rpm.

2.6 Selection media for *C. albicans* transformants

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 either YPM medium (10 g yeast extract, 20 g peptone, 20 g maltose per liter) without selective pressure to induce the MAL2 promoter, or in YCB-YE-BSA medium (23.4 g Yeast Carbon Base, 2 g Yeast Extract, 4 g Bovine Serum Albumin, pH 4) to induce the *SAP2-1* promoter that controls the expression of the caFLP gene in the *SAT1* flipper cassette. One hundred to 200 cells were then spread on YPD plates containing 10 μg/ml nourseothricin and grown for 2 days at 30°C. Nourseothricin-sensitive clones were identified by their small colony size and confirmed by restreaking on YPD plates containing 200 μg/ml nourseothricin. For the *ΔΔada2* mutants, cells were spread on normal YPD agar plates since they resulted hypersensitive to nourseothricin. In this case no difference in size was observed between the Nourseothricin-resistant and the nourseothricin-sensitive clones.

2.7 Growth and maintenance of *E. coli* strains

Recombinant *E. coli* strains were routinely grown in LB liquid medium (1% Peptone, 0.5% Yeast extract, 0.5% NaCl) under the selection pressure (100 µg/ml ampicillin). For growth on plates, 1.5% agar [Difco™ agar granulated, BD] was added to the media. The cells were grown at 37°C for 16-18 hours with shaking at 200 rpm and the plates were incubated at 37°C until the colonies appeared. While liquid cultures were processed to isolate plasmid DNA, the plates with streaked colonies were stored at 4ºC.

2.8 Small scale plasmid DNA isolation (Miniprep)

Miniprep was carried out using a commercial kit by Macherey-Nagel (Nucleospin® Plasmid) according to manufacturer's guideline. After cultivation of liquid cultures, cells were harvested by spinning at 11,000 x g for 30 sec. The surnatant was carefully removed and the pellet vigorously resuspended in 250 µl Buffer A1. 250 µl of Buffer A2 was then added and the mixture gently mixed by inverting the tube 6-8 times. The mixture was incubated at room temperature for 5 min until the lysate appeared clear. 300 µl of Buffer A3 was added to the lysate and the tube gently inverted 6-8 times. The lysate was so centrifuged for 5 min at 11,000 x g and the clearified supernatant was loaded on the kit columns placed on a collection tube. The column was spinned for 1 min at 11,000 x g, the flowthrough discarded and the column placed back on the collection tube. The column's silica membrane was then washed by adding 600 µl Buffer A4, the column spinned for 1 min at 11,000 x g and the flowthrough discarded. The silica membrane was then dried by spinning the column for further 2 minutes in the same collection tube. The plasmid was eluted by transferring the column on a clean 1.5 ml microcentrifuge tube, adding 50 µl of double distilled water directly on the membrane, incubating for 1 minute at room temperature and spinning down for 1 min at 11,000 x g.

2.9 Polymerase Chain Reaction (PCR)

A high fidelity DNA polymerase either Phusion (Finnzymes) or Elongase (Invitrogen) was used for all PCR applications. Usually the PCR reaction volume was 50 µl, which contained 1-2 ng plasmid DNA or 0.1-0.5 µg genomic DNA as template, 0.2 mM of each dNTP, 0.5 µM of each primer, 1 unit of enzyme and polymerase specific buffer. The PCR reaction was performed on the "Cyclone 25" Thermocycler (Peqlab) following the programme recommended by the enzyme manufacturers.

The cycling conditions when using Phusion enzyme were the following:

Initially the DNA was denatured at 98°C for 30 s followed by 30 cycles of amplification.

Denaturation at 98°C for 10 s

Annealing temperature (optimized) 45-55°C for 30 s

Extension at 72°C for 30 s /1 kb product

Final extension was done at 72°C for 10 minutes in order to fill the incomplete extension products. The annealing temperature was adjusted on the basis of the T_m of the primers used for amplification. The template amount, number of cycles and cycling conditions were varied to maximize the product yield. Successful amplification was confirmed by agarose gel electrophoresis and the PCR product was purified using a Macherey-Nagel kit (Macherey-Nagel GmbH, Düren, Germany).

2.10 Plasmid DNA digestion with restriction enzymes

Plasmid DNA digestions were performed using the appropriate enzyme from New England Biolabs (New England Biolabs GmbH, Frankfurt am Main, Germany) with the supplied digestion buffers. The amount of enzyme per µg of DNA used was according to manufacturer's instructions. All preparative digestion reactions were generally purified in a

50 µl reaction volume, at 37ºC for 6 h, containing 30-35 µl of plasmid DNA (inserts) or 10 µl of plasmid DNA (vectors). The digest products were then loaded on a 1% agarose gel along with 1 kb DNA ladder (Invitrogen).

2.11 Gel electrophoresis and gel elution of DNA fragments

Agarose gel electrophoresis of DNA was routinely carried out in 1x TAE (1 liter 50x TAE stock solution: 242 g Tris base, 57.1 ml glacial acetic acid and 100 ml 0.5 M EDTA pH 8.0 in distilled water). After electrophoresis, gels were stained in ethidium bromide (10mg/ml in water) and photographed using a gel documentation system (Bio-Rad). The sizes of the fragments were estimated by measuring the relative mobility of the bands in comparison to markers of known molecular size (1 kb DNA ladder, Invitrogen), run in a lane alongside.

To obtain the fragment of interest, digested DNA sample (transformation cassette from plasmid preparation, digested PCR products or plasmid inserts) was loaded on the agarose gel and run overnight at low voltage (38V) till the band of interest was well separated from the vector backbone. The required band was cut out with a scalpel. The DNA was eluted from the gel slice using the Macherey-Nagel "PCR cleanup/Gel extraction" kit (NucleoSpin Extract II) according to the manufacturer's guidelines.

Concentration of the eluted fragments was checked by "NanoDrop 1000" (Thermo Fisher Scientific, Wilmington, MA).

2.12 Cloning gene of interest in vectors

2.12.1 Ligation

The vector used in these studies was pBluescript KS II (Stratagene, Heidelberg, Germany). Ligation reactions were set up putting the appropriate volume of each fragment in order to have the same amount of molecules for each fragment, in a 20 µl reaction

volume containing 1 µl T4 ligase (Invitrogen) and 2 µl of 10x ligase buffer. A vector-only control ligation reaction was also set up during each ligation experiment. Ligation reactions were carried out over night in a cold water bath (starting temperature 10°C).

2.12.2 Preparation of *E. Coli DH5α* competent cells

E. coli competent cells were prepared using the calcium chloride method. A single colony of DH5α was inoculated in 10 ml LB medium and grown at 37°C with shaking at 200 rpm for 16-18 hours. The overnight grown culture was diluted 1:100 in 50 ml fresh LB medium and incubated at 37°C shaker for 3-4 hours (till OD_{600}= 0.7-0.9). The culture was then transferred to chilled 50 ml tubes and centrifuged at 3,000 rpm for 10 min at 4°C in a chilled rotor. The supernatant was discarded and the cell pellet was resuspended in 40 ml of ice cold 50 mM calcium chloride. The cell mix was incubated on ice for 30 minutes and centrifuged at 4,000 rpm for 10 minutes at 4°C. The pellet was then resuspended gently in 4 ml of 50 mM calcium chloride and 86% glycerol was added to a final concentration of 15% (v/v). Aliquotes of 100 µl competent cells were made in microcentrifuge tubes and stored at -80°C for later use.

2.12.3 Transformation of competent cells

For each transformation a frozen aliquot of competent cells was thawed on ice. The ligation reaction mixture (20 µl) was added to 100 µl competent *E. coli* cells and incubated for 30 minutes on ice. The cells were subjected to heat shock at 42°C for 45 seconds and then chilled on ice for 2 minutes. After adding 1 ml LB medium the cells were allowed to grow for 1h at 37°C. Finally, the cells were spread on LB selection plate (containing ampicillin). Plates were incubated at 37°C until the colonies appeared (~16 hours).

2.12.4 Screening of recombinants

Three µl of miniprep plasmid DNA of clones were digested with appropriate

restriction enzymes at 37°C in a 20 µl reaction mix for 1-2 h and separated on a 1% agarose gel. After staining in ethidium bromide the gel was observed in gel documentation system and correct recombinants were selected based on their expected band pattern on gel. Sequences of these clones were also verified, in order to avoid selecting recombinants with undesired nucleotide substitutions that can arise in PCR. For sequencing of the cloned fragments, 1 µl isolated plasmid DNA was mixed with 1 µl primer (20 µM) appropriate for the selected cloned fragment to be sequenced, and 5 µl distilled water, and sent to Sequence Laboratories Göttingen GmbH (Göttingen, Germany).

2.13 *C. albicans* transformation

C. albicans strains were transformed by electroporation as described by Köhler *et al.*, (1997), with minor modifications [**91**]. A single colony of the strain to be transformed was inoculated in 10 ml YPD medium, grown overnight at 30ºC shaker (250 rpm). Cells from the pre-culture were diluted 10^{-4} in 50 ml fresh YPD medium and grown at 30°C shaker till the culture reached mid log phase (OD_{600}= 1.6-2.2). Cells were collected by centrifugation at 4,000 rpm at 4°C for 5 minutes. The cell pellet was then suspended in 8 ml of sterile distilled water. After addition of 1 ml of 10x TE (100 mM Tris-HCl pH 7.5, 10 mM EDTA, pH 7.5) and 1 ml of 1M lithium acetate (pH 7.5), the suspension was incubated in a rotary shaker for 60 minutes at 30°C. 250 µl of 1 M dithiothreitol (DTT) was then added, and the cells were incubated again for 30 min at 30°C with shaking. After addition of 40 ml of water the cells were centrifuged (4,000 rpm, 4°C, 5 minutes), cell pellet was then washed sequentially in 25 ml of ice-cold water and 5 ml of ice-cold 1 M sorbitol. The supernatant was discarded and the washed cell pellet was resuspended in the residual liquid and kept on ice.

Five µl of the linear DNA fragment were mixed with 40 µl of electrocompetent cells in a 0.2 cm cuvette (PeqLab, Erlangen, Germany). For control, 5 µl of sterile distilled

water was used instead of DNA. Electroporation was carried out at 1.8 kV using an electroporation apparatus (Equibio, Kent, UK). After electroporation, the cells were transferred to 2 ml microcentrifuge tubes using 1 ml of YPD medium. The electroporated cells, after resuspension in 1 ml YPD medium, were divided into two aliquots so that two independent first round transformants could be obtained. The volume of each aliquot was adjusted to 1 ml by adding 500 µl YPD and the samples were then incubated for 4-6 h at 30°C. Following incubation, 100 µl from the sample was spread on YPD plates containing 200 µg/ml of nourseothricin. The plates were incubated at 30°C until colonies appeared. Individual colonies were streaked on respective selection media plates for further analysis.

2.14 Genomic DNA isolation from *C. albicans*

A single colony of the strain of which genomic DNA has to be isolated was inoculated in 10 ml YPD medium and grown overnight at 30ºC in a shaker (250 rpm). Cells were then pelletted by centrifugation at 3,000 rpm for 5 minutes, washed twice with 1 ml distilled water and then broken by vortexing for 5 minutes at maximum speed together with 200 µl Breaking Buffer (2% v/v TritonX 100, 1% v/v SDS, 100 mM NaCl, 10 mM Tris-Cl pH 7.5, 1 mM EDTA pH 7.5), 0.3 g Glass Beads (0.5 mm Φ) and 200 µl PCI (phenol/chloroform/IAA, 25:24:1). After addition of 200 µl of TE buffer (10 mM Tris-Cl pH 7.5, 1 mM EDTA pH 7.5) samples were centrifuged for 5 minutes at 13,000 rpm. The surnatant was then transferred in a new microcentrifuge tube and precipitated first with 1 ml 96% ethanol and then with 600 µl of 70% ethanol. The pellet was left to dry completely and then resuspended in 100 µl TE buffer with 0.1 mg/ml RNase.

2.15 Southern hybridization

Ten µg of genomic DNA from *C. albicans* transformants were digested overnight with appropriate restriction enzymes in a 30 µl reaction volume at 37°C. Digested samples,

along with 1 kb DNA ladder (Invitrogen) as marker, were loaded on a 1% agarose gel. The gel was run overnight at 36-40 V in 1x TAE buffer. After electrophoresis the gel was stained with ethidium bromide, and photographed.

For DNA transfer, a nylon membrane (15 cm x 14 cm, Schleicher & Schuell, Dassel, Germany) was pre-wet for few seconds in distilled water and equilibrated in 20x SSC (3 M NaCl, 0.3 M tri-sodium citrate dihydrate) for 5 minutes. The gel was rinsed with distilled water and aligned on top of the membrane in vacuum blot apparatus (Phamacia Biotech). In a sequential order, the gel was treated with different solutions (15 min each) under vacuum, Solution A (0.25 N HCl) for depurination of the DNA, Solution B (1.5 M NaCl, 0.5 N NaOH) for denaturing DNA and then with Solution C (1.5 M NaCl, 0.5 M Tris-Cl pH 7.5) for neutralization. Transfer was done with 20x SSC for 90 minutes. After the transfer was complete, the membrane was first soaked in 0.4N NaOH for 1 minute to denature the DNA and then treated with 0.2M Tris-Cl (pH 7.5) for 1 minute for neutralization. The wet membrane was placed on Whatmann filter paper and DNA was fixed to membrane by UV-cross linking using Stratalinker (Stratagene).

Hybridization was performed with the 'ECL labelling and detection kit' (GE Healthcare, Braunschweig, Germany) according to manufacturer's instructions. The membrane was prewet in 2x SSC for 1 minute and then placed in a hybridization bottle. For prehybridization, the membrane was incubated with 15 ml ECL hybridization buffer for 1 h at 42°C in a hybridization oven. The probe was prepared according to manufacturer's instruction using 100 ng DNA of interest (gel eluted) and 2 ng 1 kb DNA ladder (Invitrogen) and then was added to the prehybridization buffer. Hybridization was carried out at 42°C for 16-18 hours. After hybridization, the membrane was washed at 42°C three times with Wash Buffer I (6 M Urea, 0.4% SDS, 0.5x SSC) for 10 minutes and twice in 2x SSC at RT for 5 minutes. For signal detection, the washed membrane was

incubated in a mixture of detection solution 1 and 2 (1:1) for 1 min, then wrapped securely in saran wrap, and finally exposed to Amersham hyperfilm ECL in a film cassette for 1 h. For rehybridizing with a second probe the blot was washed in 2x SSC for 10 min and then steps for hybridization were followed as described above.

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

2.16.1 RNA isolation from *C. albicans*

All solutions used for RNA isolation were made either in DEPC treated water or treated with DEPC after preparation and RNase-free plastic ware was used in all procedures of RNA handling. For DEPC treatment 0.1% DEPC (v/v) was added and the solutions were incubated at 37°C overnight and autoclaved.

Total RNA was isolated from early log-phase *C. albicans* cultures in liquid YPD medium by the hot phenol method combined with a purification step with RNeasy kit (Qiagen, Hilden, Germany). A single colony of the *Candida* strain was inoculated in 10 ml YPD medium and grown overnight at 30ºC shaker (250 rpm). Cells from the preculture were diluted 10^{-2} in 50 ml fresh YPD medium and grown at 30°C shaker for 4 more hours. Where needed, doxycycline was added at a final concentration of 20 µg/ml. After 4 h of incubation, cultures were transferred to 50 ml centrifuge tubes and centrifuged for 3 minutes at 2500 x g at 4°C. Surnatant was discarded and the pellet resuspended in 1 ml ice-cold water and transferred in a 2 ml microcentrifuge tube. After centrifuging the samples for 10 seconds at 4°C, the pellet was resuspended in 400 µl TES solution (10 mM Tris-Cl pH 7.5; 10 mM EDTA pH 7.5; 0.5% SDS) and 400 µl of Acid Phenol (Aqua Roti Phenol, Roth, Germany) was added to the samples. The tubes were vigorously vortexed and incubated for 1 h at 65°C with occasional, brief vortexing. The samples were then chilled

on ice for 5 minutes and centrifuged at 11,000 x g for 5 minutes at 4°C. At this point, the aqueous top phase was transferred to a new 1.5 ml tube and mixed well with 1 vol buffer RLT (Qiagen kit) and 1 vol pure Ethanol. The mixture could so be transferred on the RNeasy mini column placed on a 2 ml collection tube. Columns were then centrifuged for 15 seconds at max speed, flowthrough discarded and 700 µl of buffer RW1 was added. Again columns were briefly centrifuged, flowthrough discarded and two steps with 500 µl of buffer RPE were performed. Columns were then placed on a new collection tube and spinned for 1 minute to be sure to get rid of all buffer. Columns were then placed on a 1.5 ml fresh microcentrifuge tube, 50 µl of RNase-free water was added directly to the columns membrane, and the total RNA was so eluted centrifuging the columns for 1 minute. Half the volume was immediately stored at -80°C and half was kept on ice for performing DNAse treatment

2.16.2 DNase treatment

Contaminating DNA was removed by treatment with the Ambion Turbo DNA-free kit (Applied Biosystems, Darmstadt, Germany). The RNA was incubated for 20 minutes at 37°C together with 0.1 vol 10X TURBO DNase buffer (3 µl) and 1 µl TURBO DNase. DNase was then inactivated by adding 0.1 vol resuspended DNase Inactivation Reagent (3 µl) and by mixing well. After incubating for 2 minutes at room temperature, samples were centrifuged at max speed for 2 minutes to spin down the inactivating reagent so that RNA could be collected from the upper phase and transferred to a clean tube. A second purification step was performed on the DNase treated RNA with the same RNeasy kit (Qiagen) as above, following the manufacturer's instructions.

2.16.3 RNA quantity and quality controls

The absence of genomic DNA was controlled by PCR, concentration was checked

with a NanoDrop 1000 analyser and the absence of RNA degradation was checked by letting the RNA run on a 1% agarose gel (TAE buffer) for 1 h at 70 V. Good quality RNA gave 3 bands, one for rRNA 18S, one for rRNA 28S and one for the complex of the two RNAs.

2.16.4 Conversion of RNA into cDNA

RNA was converted into cDNA with the SuperScript® III Reverse Transcriptase kit (Invitrogen, Karlsrhue, Germany) following the manufacturer's instructions. Generation of cDNA was checked by PCR using primers which bind outside an intron. Amplification of cDNA gave a smaller PCR product while amplification of genomic control DNA gave a bigger band.

2.16.5 Real Time PCR

qPCR was performed with the iQ^{TM} SYBR® Green Supermix (Bio-Rad) and the primer pairs MDR5RT and MDR6RT for *MDR1*, ACT1RT and ACT2RT for *ACT1*, which served as the reference, using the following conditions: an initial denaturation for 3 min at 95°C, 40 cycles of 30 sec 95°C, 40 sec 50°C, 10 sec 72°C, one cycle of 30 sec 95°C, 30 sec 57°C. Melt curves were generated and Ct values calculated by the Real Time program, Bio-Rad iQ5 V2.0 optical systems software. Ct values obtained by the software were then used to calculate the relative *MDR1* mRNA levels adjusted to the *ACT1* mRNA levels and using *MDR1* expression in the wild-type strain SC5314 as a reference (set to 1). Two independent RNA extractions, each with two technical replicates, were used to calculate mean and standard deviation of the final relative expression values.

2.17 Analysis of *MDR1* promoter activity by FACS analysis in the conditional *mcm1* mutants

YPD overnight cultures of *GFP* reporter and parental control strains were each

diluted 10^{-2} in six Erlenmeyer flasks containing 50 ml YPD medium (three without and three with 20 µg/ml doxycycline). After 3 h of growth at 30°C, 50 µg/ml benomyl or 0.005% H_2O_2 was added to two of the cultures (in both cases with and without doxycycline) to induce *MDR1* expression; the other two cultures were left untreated. Following 60 min of further incubation the mean fluorescence of the cells was determined by flow cytometry. Fluorescence-activated cell sorter (FACS) analysis was performed with a FACSCalibur cytometry system equipped with an argon laser emitting at 488 nm (Becton Dickinson, Heidelberg, Germany). Fluorescence was measured on the FL1 channel equipped with a 530-nm band-pass filter. Twenty thousand cells were analyzed per sample. Fluorescence data were collected by using logarithmic amplifiers. The mean fluorescence values were determined with CellQuest Pro (Becton Dickinson) software.

2.18 Analysis of *MDR1, CDR2* and *ERG11* promoter activity by FACS analysis in the *ΔΔada2* mutants

Procedures for *ΔΔada2* mutants were the same as for the *mcm1* mutants, with the difference that the volume in which the strains were grown was 3 ml instead of 50 ml, the presence of doxycycline was not needed and the other inducing chemicals, fluphenazine for *CDR2* induction and ketoconazole for *ERG11* induction, were used at a concentration of 10 µg/ml and 0.5 µg/ml respectively.

2.19 Confirmation of doxycycline-induced *MCM1* repression by Western immunoblotting

Strains can43MPG2A/B were grown as described above for determining *GFP* expression. Whole cell protein extracts were prepared from two cultures grown for 3 h in the presence or absence of doxycycline and from the remaining cultures after further 60 min incubation in the presence of benomyl or H_2O_2 (with and without doxycycline). Cells

were collected by centrifugation, washed twice in water, and broken by vortexing for 10 min at 4°C with 300 µl 0.5-mm glass beads in 300 µl breaking buffer (100mM Tris-Cl [pH 7.5], 200 mM NaCl, 20% glycerol, 5 mM EDTA, 4% of a Complete EDTA-free Protease Inhibitor Cocktail stock solution [Roche Diagnostics GmbH, Mannheim, Germany], 0.1% β-mercaptoethanol). Samples were centrifuged at 13,000 rpm for 5 min at 4°C, the supernatant collected, and the protein concentration quantified with a NanoDrop 1000 (Thermo Fisher Scientific, Wilmington, MA). Extracts were heated at 65°C for 10 min and 400 µg total protein of each sample was separated on an SDS-12% polyacrylamide gel. Proteins were transferred onto nitrocellulose membrane with a Trans-Blot SD Semi-Dry transfer apparatus (Bio-Rad, Munich, Germany). For detection of Mcm1-Myc, a monoclonal anti-c-Myc antibody (purified mouse immunoglobulin, clone 9E10, Sigma-Aldrich Chemie GmbH, product number M4439) was used as primary antibody at a dilution of 1:6000, and goat anti-mouse IgG (Fab-specific) peroxidase conjugate (Sigma A9917) was used as a secondary antibody at a dilution of 1:6000. Blots were developed using a chemiluminescence detection system (GE Healthcare UK Limited, Chalfont, UK) under conditions recommended by the manufacturer.

2.20 MIC test

Minimal inhibitory concentration of the *C. albicans* strains was determined by a microdilution assay in 96 well microtiter-plates. Strains were tested for fluconazole and cerulenin susceptibility. Stock solutions of the drugs were prepared, fluconazole was dissolved in water while cerulenin was dissolved DMSO, and both stock solutions were prepared at a concentration of 5 mg/ml and stored at 4°C. The medium used for the test was a minimal medium (SD) which was prepared as follows: YNB with the addition of ammonium sulfate (6.7 g) was solved in 800 ml water, pH was brought to 5.8 by adding NaOH, after autoclaving glucose was added (final concentration of 2%, 100 ml of a 20%

stock solution) and 100 ml of a 10X stock of CSM mix were added. CSM 10X mix was prepared by dissolving 7.9 g of CSM powder in 1 l water and sterile filtrating it. The test was performed by resuspending 1 fresh colony (2 days old) in 1 ml 0.9% NaCl, 4 µl of the cell suspension were then resuspended in 2 ml SD medium and 100 µl of the obtained suspension were then mixed on the microtiter-plate together with different dilutions of the drug to be tested. Starting concentrations of the drugs were 50 µg/ml and 1:2 serial dilutions were performed, diluting in SD medium, until concentration 0.02 µg/ml. The plates were incubated at 37°C for 48 h and MIC readings were then performed considering the MIC value as the first concentration where there was no visible growth.

2.21 Spot assays

Spot assays were performed on YPD agar plates or N10 agar plates (YPD + 10 µg/ml Nourseothricin). Overnight cultures in YPD liquid medium (10 ml) were diluted 10^{-2} and optical density was measured at a 600 nm wavelength. Starting cell concentration was adjusted to OD=10 and serial 1:10 dilutions were performed until OD=10^{-4}. Five µl of each cell concentration were then plated on the agar plates, the drop let to dry and the plates incubated at 30°C. After 48 h pictures were taken.

3 RESULTS

3.1 Role of Mcm1 in the regulation of *MDR1*

3.1.1 Introduction of a P_{MDR1}-*GFP* reporter fusion

To monitor the activation of the *MDR1* promoter in response to inducing chemicals in the presence and absence of Mcm1, a P_{MDR1}-*GFP* reporter fusion was introduced into the conditional *mcm1* mutant MRcan42, which contains a single, Myc-tagged *MCM1* allele under the control of a tetracycline-repressible promoter. In addition, the reporter fusion was introduced into the control strain MRcan43, which contains both a tetracycline-repressible *MCM1* copy and a Myc-tagged *MCM1* allele with its own promoter. To this aim, a *KpnI/SacII* fragment from plasmid pMPG2S (Fig. 2; Table 2) was used to transform the parental strains. This led to the replacement of one copy of the *ACT1* gene with the so formed cassette.

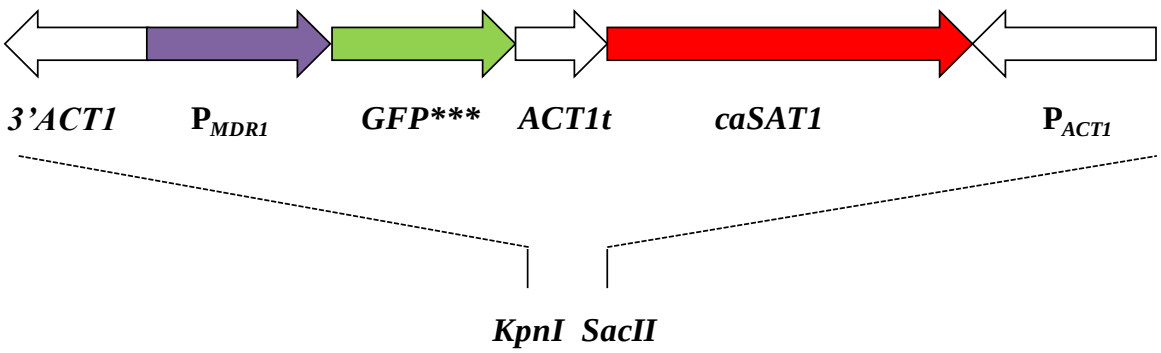

Fig. 2. Structure of the cassette from plasmid pMPG2S.
ACT1 coding region is replaced by the *MDR1* promoter (P_{MDR1}) fused with the *GFP* protein (*GFP****). The *ACT1* transcription termination sequence (*ACT1t*) was used for a correct termination of the *GFP* transcription. The cassette contains a selective resistance marker, *caSAT1*, which confers resistance to nourseothricin.

Two independent transformants of each parental strain were used for further analysis, named can42MPG2A/B and can43MPG2A/B respectively. SC5314 background

mutants where already present in the laboratory's collection, named SCMPG2A/B (Table 3).

3.1.2 Confirmation of Mcm1 depletion

To confirm that Mcm1 could be efficiently depleted by addition of doxycycline to the conditional mutants carrying the reporter fusion and that the presence of the *MDR1* inducers, benomyl and H_2O_2, did not affect doxycycline-mediated repression, Western Blot analysis was performed. As shown in Fig. 3, Mcm1 was not detectably expressed after 3 h of growth in the presence of doxycycline, and the addition of benomyl or H_2O_2 did not alleviate this repression.

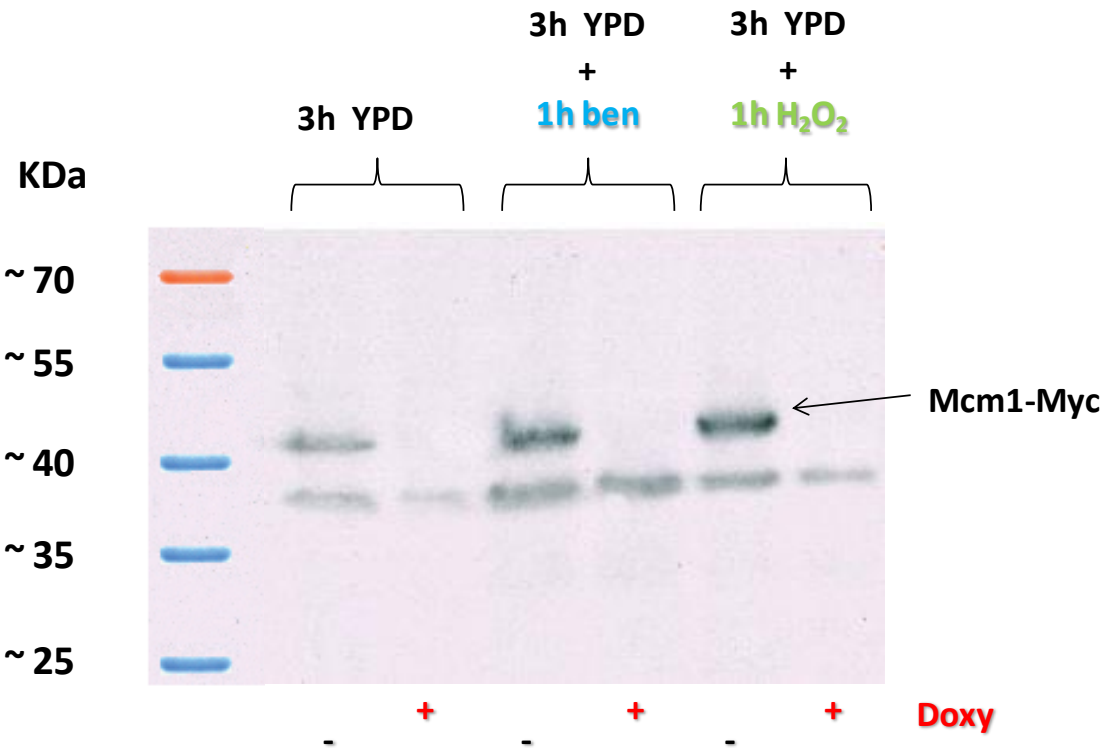

Fig. 3. Depletion of Mcm1 from reporter strains by treatment with doxycycline. Strains ca42MPG2A/B were grown in the absence (-) or presence (+) of doxycycline (Doxy) and treated with benomyl (Ben) or H_2O_2 as described in Materials and Methods. Whole cell protein extracts were prepared and analyzed by western immunoblotting with anti-Myc antibody. The position of Myc-tagged Mcm1 is indicated; an unspecific cross-reacting band served as loading control. The figure shows the results for strain cand42MPG2A; the same results were obtained with can42MPG2B.

3.1.3 Determination of *MDR1* promoter activity by FACS analysis

MDR1 promoter activity was determined by quantifying the fluorescence of the reporter strains (Fig. 4). As previously reported [**46**], expression of *MDR1* was not detectable in the wild-type strain SC5314 in YPD medium, but it could be induced by H_2O_2 treatment and, even more efficiently, by benomyl.

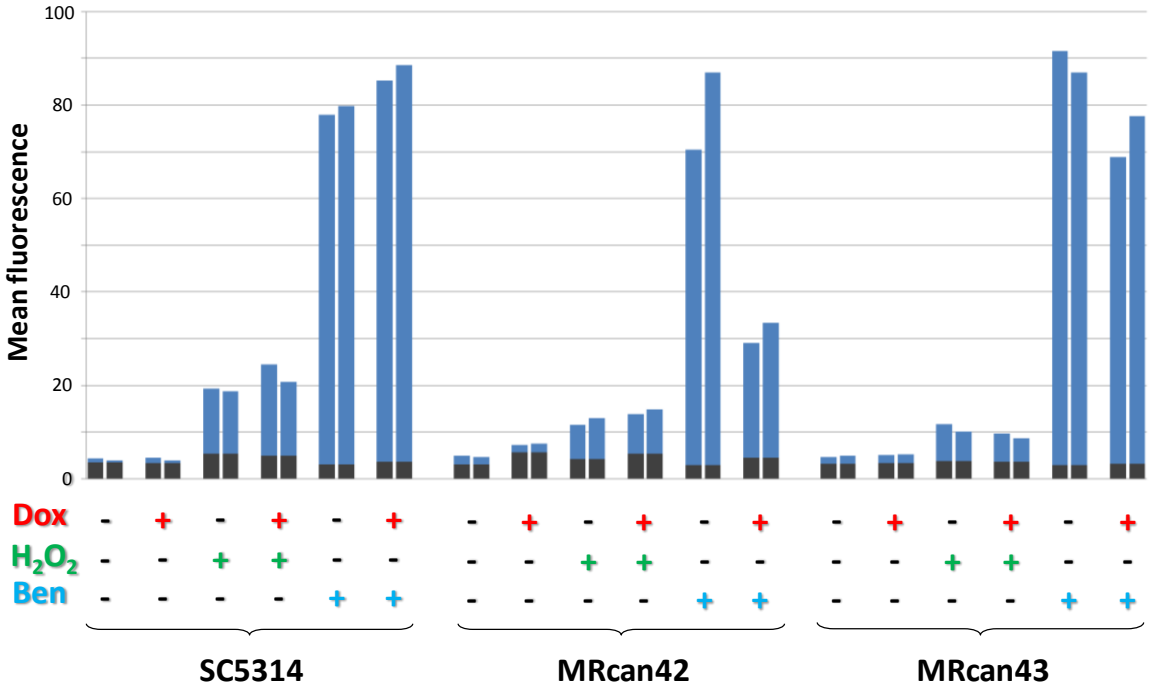

Fig. 4. Activation of the *MDR1* promoter.
Activation of the *MDR1* promoter by H_2O_2 and benomyl in the wild-type strain SC5314, the conditional mcm1 mutant MRcan42, and the control strain MRcan42. Parental strains and transformants carrying a P_{MDR1}-*GFP* reporter fusion were grown in the absence (-) or presence (+) of doxycycline (Dox) and treated with H_2O_2 or benomyl (Ben) as described in Materials and Methods. The mean fluorescence of the cells was determined by flow cytometry. The results obtained with two independently generated reporter strains (SCMPG2A/B, can42MPG2A/B, can43MPG2A/B) are shown in each case. The background fluorescence of the parental strains, which do not contain the *GFP* gene, is indicated by the black part in each column.

The presence of doxycycline did not affect the activity of the *MDR1* promoter under non-inducing or inducing conditions in the wild-type background. H_2O_2 also induced the *MDR1* promoter in the conditional mutant MRcan42, although less efficiently than in strain SC5314, and this induction was also observed after depletion of Mcm1. In contrast,

Mcm1 depletion resulted in a reduced induction of the *MDR1* promoter by benomyl, as the fluorescence of the conditional mutants was decreased in the presence of doxycycline. Doxycycline had a little effect on benomyl-induced *MDR1* expression in the control strain MRcan43. These results demonstrate that Mcm1 is dispensable for the induction of *MDR1* expression by H_2O_2, but it is required for full *MDR1* induction by benomyl.

3.1.4 Introduction of hyperactive transcription factors for constitutive *MDR1* overexpression

The induction of the *MDR1* promoter by H_2O_2 requires the bZIP transcription factor Cap1, which is activated by oxidative stress. Cap1 also contributes to, but is not essential for benomyl-induced *MDR1* expression. In contrast, the zinc cluster transcription factor Mrr1 is indispensable for *MDR1* expression in the presence of either of these inducers.

As Mcm1 was necessary for full benomyl-induced, but not for H_2O_2-induced *MDR1* expression, it was investigated whether Mcm1 was required for the constitutive *MDR1* overexpression caused by hyperactive forms of Mrr1 and Cap1. To this aim, the P683S gain-of-function mutation was introduced into both resident *MRR1* alleles of the conditional *mcm1* mutant MRcan42 and the control strain MRcan43. The transformation cassette used for this purpose is a SacI/ApaI fragment form plasmid pMRR1R3 (Fig. 5).

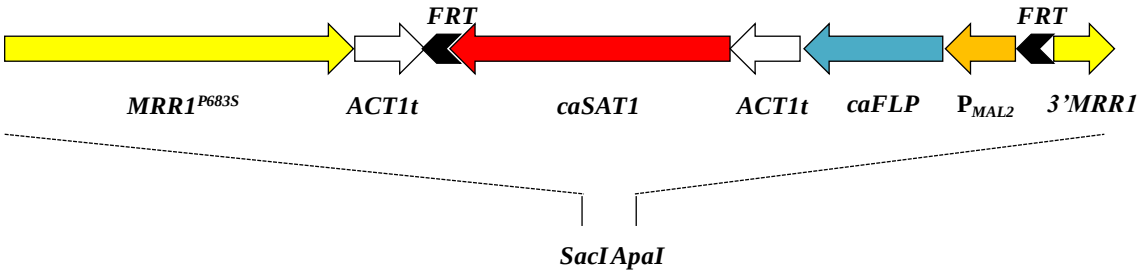

Fig. 5. Structure of the cassette from plasmid pMRR1R3.
MRR1 coding region is replaced by an *MRR1* allele containing a P683S substitution. The cassette contains the *FLP* recombinase gene (*caFLP*) under the control of the *MAL2* promoter (P_{MAL2}) and a selective resistance marker (*caSAT1*) which confers resistance to nourseothricin. *ACT1* transcription termination sequences (*ACT1t*) served for terminating both *MRR1* and *caFLP* transcription. *FLP* recombination target sequences (*FRT*) are not drawn to scale.

In addition, both wild-type *CAP1* alleles of these strains were replaced by the C-terminally truncated, hyperactive $CAP1^{\Delta C333}$ allele with a SacI/ApaI fragment from plasmid pCAP1R1 (Fig. 6).

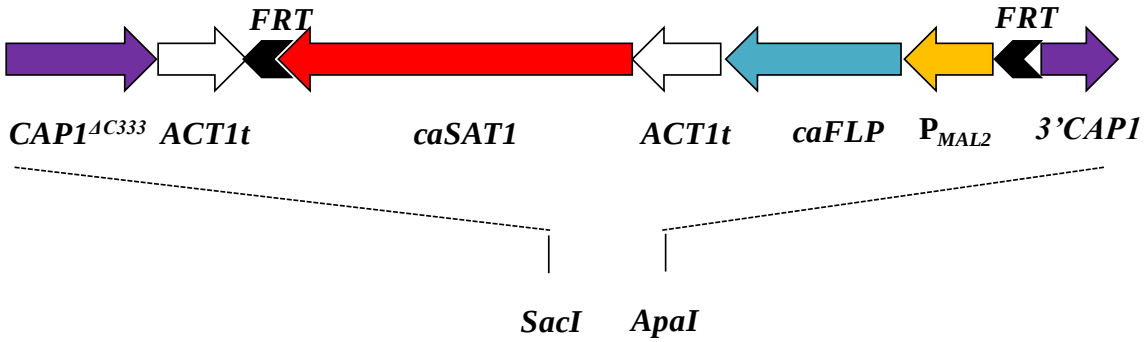

Fig. 6. Structure of the cassette from plasmid pCAP1R1.
CAP1 coding region is replaced by a truncated *CAP1* allele. The cassette contains the *FLP* recombinase gene (*caFLP*) under the control of the *MAL2* promoter (P_{MAL2}) and a selective resistance marker (*caSAT1*) which confers resistance to nourseothricin. *ACT1* transcription termination sequences (*ACT1t*) served for terminating both *CAP1* and *caFLP* transcription. *FLP* recombination target sequences (*FRT*) are not drawn to scale.

Homozygous strains were generated since the activating mutations in *MRR1* and *CAP1* have a stronger effect on *MDR1* expression when they are present in both alleles (Schubert *et al.*, submitted). Two independent series of mutants (A and B) were constructed in each case and used for further analysis.

3.1.5 Quantitative Real-Time PCR for determining *MDR1* expression

As the *MDR1* promoter is constitutively activated in strains expressing hyperactive Mrr1 or Cap1, *GFP* was not useful as a reporter to measure the dependence of *MDR1* expression on Mcm1, because considerable amounts of the relatively stable *GFP* protein would remain in the cells after shutting off *MCM1* expression by the addition of doxycycline. Therefore, *MDR1* mRNA levels were measured in the presence and absence of doxycycline by quantitative RT-PCR. As shown in Fig. 7, *MDR1* was constitutively expressed at high levels in strains carrying the $CAP1^{\Delta C333}$ allele (between 250- and 400-fold higher than in the control strain SC5314) and Mcm1 depletion in the conditional *mcm1* mutants by doxycycline did not significantly reduce *MDR1* mRNA levels. *MDR1* expression was even higher in strains containing the hyperactive $MRR1^{P683S}$ allele (between 1200- and 2200-fold higher than in the control strain SC5314). Mcm1 depletion by doxycycline resulted in a drastic reduction of *MDR1* mRNA levels in the conditional mutants (between 20- and 30-fold). A moderate reduction of *MDR1* mRNA levels (2.5-fold) was also observed in the control strains containing the hyperactive $MRR1^{P683S}$ allele after addition of doxycycline, but not in the control strains carrying the $CAP1^{\Delta C333}$ allele.

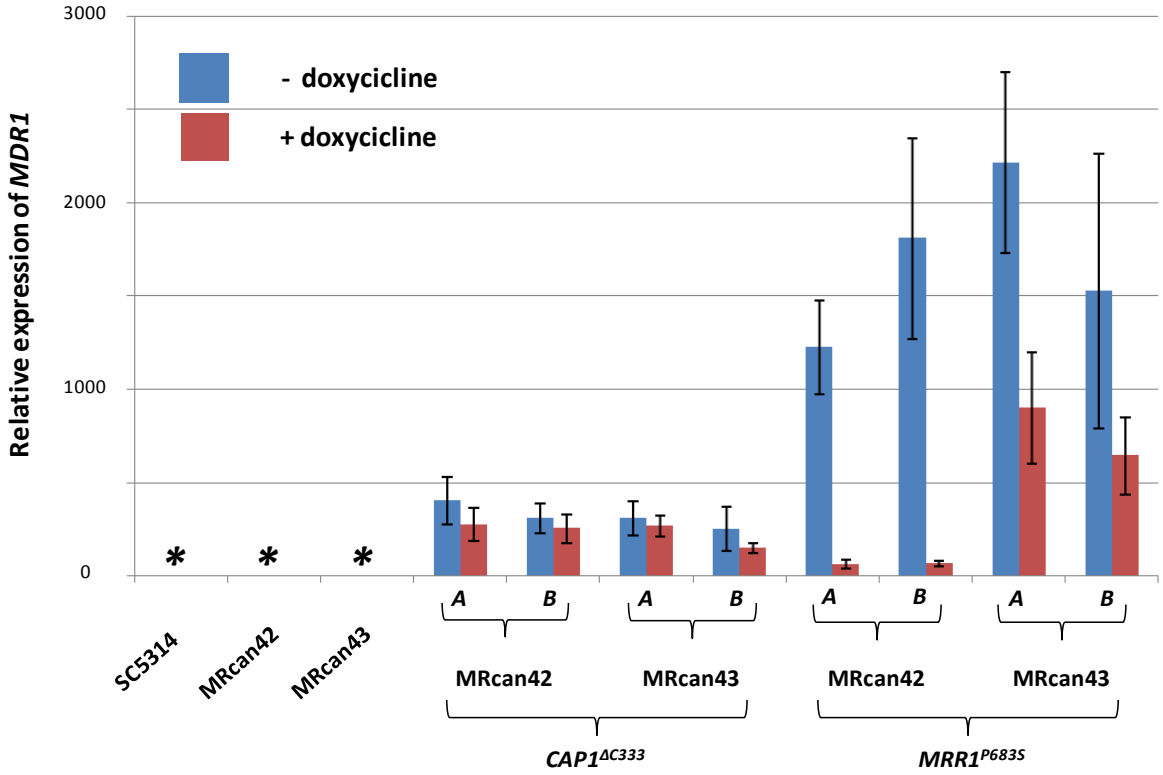

Fig. 7. Real-time analysis for *MDR1* expression.
Effect of Mcm1 depletion on *MDR1* overexpression mediated by hyperactive forms of Cap1 and Mrr1. The wild-type strain SC5314, the conditional mcm1 mutant MRcan42, control strain MRcan43, and independent derivatives (A and B) of MRcan42 and MRcan43 that were rendered homozygous for the $CAP1^{\Delta C333}$ or the $MRR1^{P683S}$ alleles were grown for 4 h in the absence (blue bars) or presence (red bars) of doxycycline as described in Materials and Methods. *MDR1* mRNA levels were determined by real time RT-PCR and are presented as relative expression levels compared to those of the reference strain SC5314 in the absence of doxycycline, which were set to 1. The graph shows the means and standard deviations of two independent experiments with duplicate measurements performed with each strain. **MDR1* expression levels in SC5314 and the parental strains MRcan42 and MRcan43 were too low to be visible.

These results indicate that a hyperactive Cap1 can mediate *MDR1* overexpression independently of Mcm1, whereas a hyperactive Mrr1 requires Mcm1 to promote *MDR1* expression.

3.2 Role of Ada2 in the regulation of *MDR1*, *CDR2* and *ERG11*

In order to investigate the role of Ada2 in the regulation of *MDR1*, *CDR2* and *ERG11*, genes known to be involved in drug resistance mechanisms, the gene was knocked out from the parental SC5314 *C. albicans* reference strain. The aim was to evaluate how *C.*

albicans behaves in different conditions in the absence of Ada2 and in the presence of hyperactive alleles of different transcription factors known to be involved in the regulation of the above mentioned genes. These include: *CAP1*$^{\Delta C333}$, *CAP1* truncated hyperactive allele, and *MRR1*^{P683S}, MRR1 hyperactive allele, which regulate *MDR1*, *TAC1*^{G980E}, hyperactive *TAC1* allele which regulates *CDR2*, or *UPC2*^{G648D}, hyperactive *UPC2* allele which regulates *ERG11*.

3.2.1 Knock out of *ADA2* gene

The first *ADA2* destruction cassette was produced as follows: one fragment of the downstream region of the *ADA2* gene was amplified with primers ADA2-3 and ADA2-4 (Table 1) from genomic SC5134 DNA. The PCR product was digested with *XhoI*/*ApaI* and cloned in the *XhoI*/*ApaI* digested pCAP1M4 plasmid. The produced plasmid was named pADA2M1 (Fig. 8) and contained only the downstream region of the *ADA2* gene.

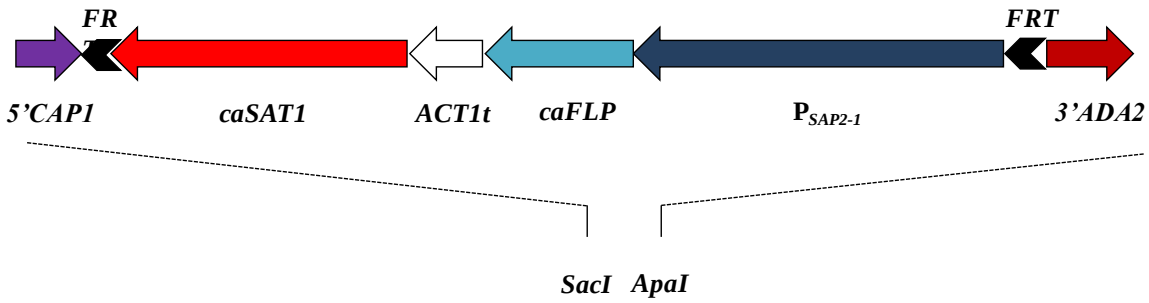

Fig. 8. Structure of the cassette from plasmid pADA2M1.
Downstream region of gene *ADA2* (*3'ADA2*) was cloned in plasmid pCAP1M4. The cassette contains the *FLP* recombinase gene (*caFLP*) under the control of the *SAP2-1* promoter (P_{SAP2-1}) and a selective resistance marker (*caSAT1*) which confers resistance to nourseothricin. *ACT1* transcription termination sequences (*ACT1t*) served for terminating *caFLP* transcription. *FLP* recombination target sequences (*FRT*) are not drawn to scale.

To introduce the upstream region of *ADA2* gene a PCR product was amplified with primers ADA2-5 and ADA2-6 (Table 1) from genomic SC5314 DNA. The PCR product was digested wit *SacI*/*SacII* and cloned in the *SacI*/*SacII* digested pADA2M1 to produce plasmid pADA2M2 (Fig. 9). Differences between pADA2M2 and the upstream sequence

of orf19.2331 are: G-498T, T-487Δ, A-458T, T-159Δ, T-158Δ, A-69Δ.

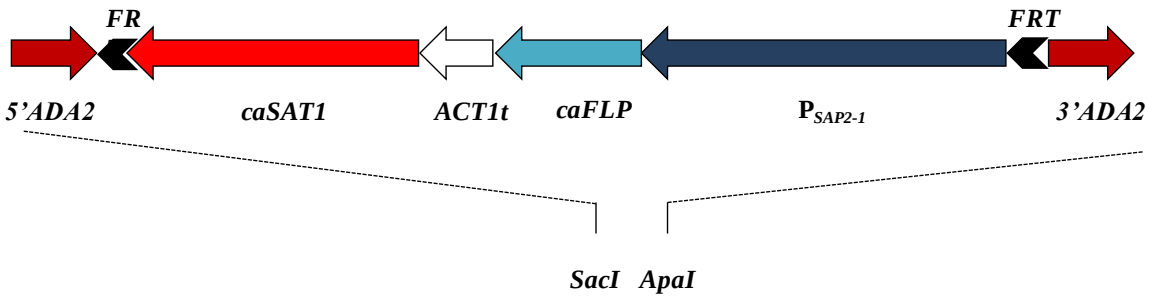

Fig. 9. Structure of the cassette from plasmid pADA2M2.
Upstream region of gene *ADA2* (*5'ADA2*) was cloned in plasmid pADA2M1. The cassette contains the *FLP* recombinase gene (*caFLP*) under the control of the *SAP2-1* promoter (P_{SAP2-1}) and a selective resistance marker (*caSAT1*) which confers resistance to nourseothricin. *ACT1* transcription termination sequences (*ACT1t*) served for terminating *caFLP* transcription. *FLP* recombination target sequences (*FRT*) are not drawn to scale.

A *SacI*/*ApaI* digested fragment of pADA2M2 was used to transform strain SC5314 in order to knock out one allele of the *ADA2* gene, generating strains SCADA2M1A/B. After recycling the nourseothricin resistance marker and generating strains SCADA2M2A/B, several attempts have been made to knock out the second allele with the same cassette from plasmid pADA2M2 without success. A new plasmid containing flanking regions internal to the previous ones was then constructed. Therefore, the resulting pADA2M3 was as follows: the N-terminal region of the *ADA2* gene was amplified with primers ADA2-7 and ADA2-8 (Table 1) from genomic SC5314 DNA. The C-terminal region of the *ADA2* gene was amplified with primers ADA2-9 and ADA2-10 from genomic SC5314 DNA. PCR products were digested with *SacI*/*SacII* and *XhoI*/*ApaI* respectively and cloned, together with the [*SacII*/*XhoI*] *SAT1*-Flipper-Fragment from pADA2M2, in the *SacI*/*ApaI* digested pADA2M2, to generate pADA2M3 (Fig. 10).

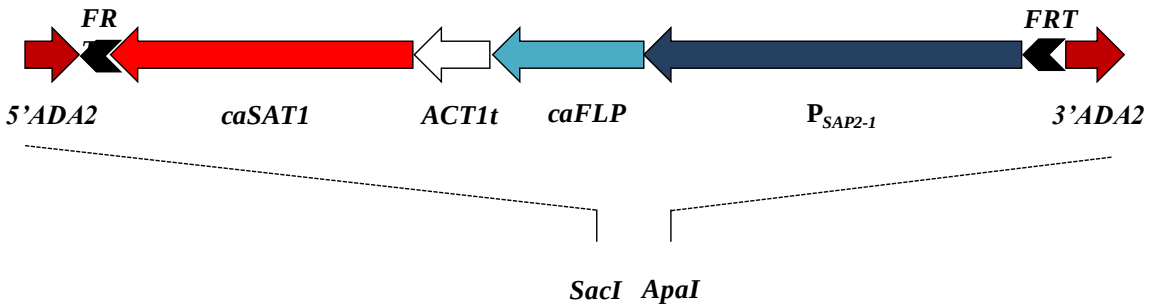

Fig. 10. Structure of the cassette from plasmid pADA2M3.
New upstream and downstream regions of gene *ADA2* (*5'ADA2, 3'ADA2*) were cloned in plasmid pADA2M2. The cassette contains the *FLP* recombinase gene (*caFLP*) under the control of the *SAP2-1* promoter (P_{SAP2-1}) and a selective resistance marker (*caSAT1*) which confers resistance to nourseothricin. *ACT1* transcription termination sequences (*ACT1t*) served for terminating *caFLP* transcription. *FLP* recombination target sequences (*FRT*) are not drawn to scale.

A *SacI*/*ApaI* fragment from pADA2M3 was used to transform the heterozygous *Δada2* mutants SCADA2M2A/B. The transformation led to the selection of clones which had a correctly integrated deletion cassette, but the nourseothricin resistance *SAT1* marker could apparently not be excised. Since Ada2 could be somehow required for the induction of the *SAP2* promoter, which should have induced the expression of the *FLP*-recombinase responsible for the excision of the whole cassette from the genome, another knock out cassette was constructed. In the new construct, the *FLP*-recombinase was under the control of the *MAL2* promoter, which is induced by the presence of maltose in the growth medium. For this purpose, a [SacII/XhoI]-Fragment with the *SAT1*-flipper and the *MAL2* promoter from plasmid pMRR1R3 was cloned in the SacII/XhoI digested pADA2M3 to form pADA2M4 (Fig. 11).

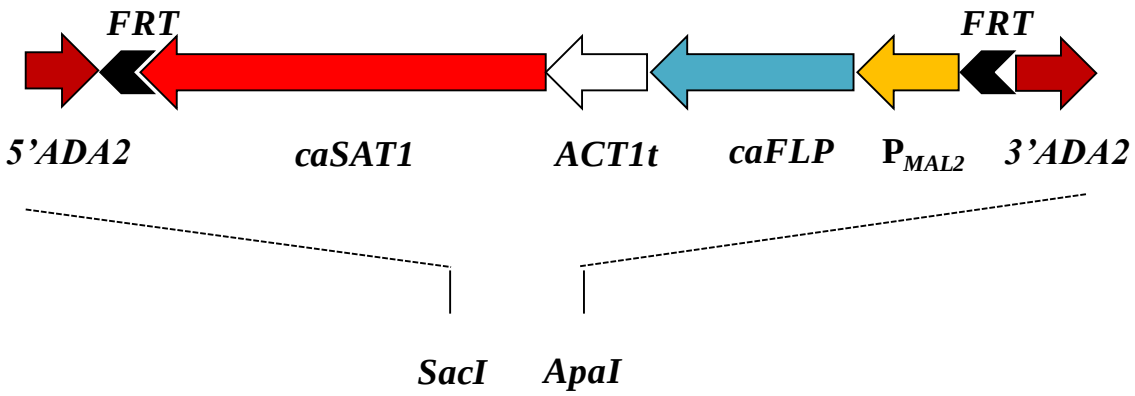

Fig. 11. Structure of the cassette from plasmid pADA2M4.
ADA2 knock-out cassette contains upstream and downstream regions of gene *ADA2* (*5'ADA2, 3'ADA2*), the *FLP* recombinase gene (*caFLP*) under the control of the *MAL2* promoter (P_{MAL2}) and a selective resistance marker (*caSAT1*) which confers resistance to nourseothricin. *ACT1* transcription termination sequences (*ACT1t*) served for terminating *caFLP* transcription. *FLP* recombination target sequences (*FRT*) are not drawn to scale.

The new transformation cassette from plasmid pADA2M4 was used to transform strains SCADA2M2A/B; clones were isolated but, once again, no derivatives in which the *SAT1* flipper cassette had been excised were obtained on plates containing 10 µg/ml nourseothricin. It was speculated that the double knock out *ΔΔada2* mutants were too sensitive towards nourseothricin and that could not grow on N10 (YPD + 10 µg/ml nourseothricin) plates, where normally the clones that had retained the *SAT1* resistance marker could be distinguished from the clones that had lost it by the size of the colony. So the strains which had been induced to excise the cassette by the presence of maltose in the medium were plated on normal YPD medium and then screened for the correct excision. Finally the double knock out mutants with the excised *SAT1* resistance marker were obtained and the newly cloned mutants were named SCADA2M3A/B and SCADA2M4A/B, with and without the resistance marker respectively.

3.2.2 Complementation of *ADA2* gene in *ΔΔada2* mutants

In order to demonstrate that the growth defect in the *ΔΔada2* mutant was indeed due to the absence of Ada2, one copy of the gene was reintegrated in both mutants. The

complementation cassette was constructed as follows: the *ADA2* gene including upstream and downstream regions was amplified from genomic SC5314 DNA with primers ADA2-11 and ADA2-12. The PCR product was digested with *SacI*/*XhoI* and with *XhoI*/*SacII* and both fragments were cloned in the *SacI*/*SacII* digested pADA2M2 to produce pADA2K1 (Fig. 12).

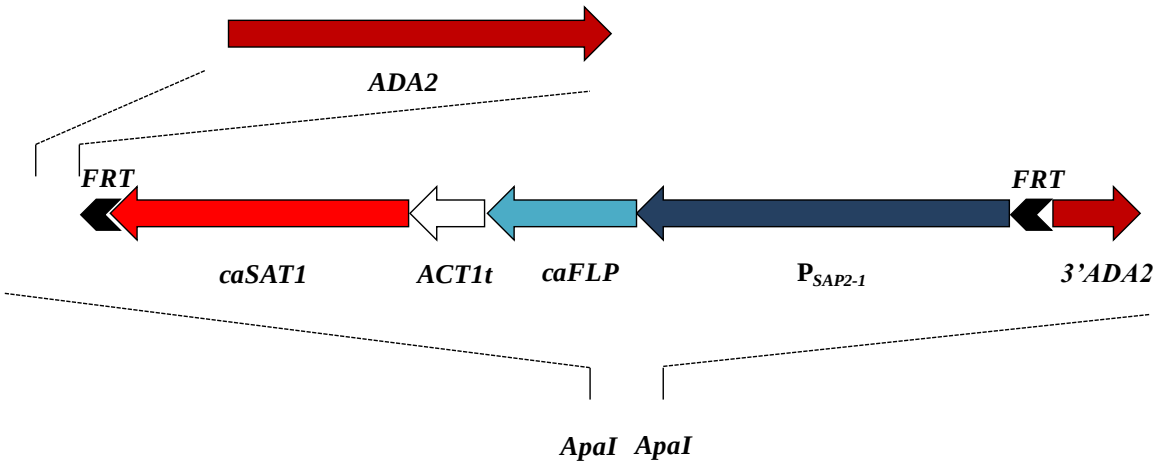

Fig. 12. Structure of the cassette from plasmid pADA2K1.
ADA2 complementation cassette contains *ADA2* complete coding region and the downstream region of gene *ADA2* (*3'ADA2*), the *FLP* recombinase gene (*caFLP*) under the control of the *SAP2-1* promoter (P_{SAP2-1}) and a selective resistance marker (*caSAT1*) which confers resistance to nourseothricin. *ACT1* transcription termination sequences (*ACT1t*) served for terminating *caFLP* transcription. *FLP* recombination target sequences (*FRT*) are not drawn to scale.

The plasmid was digested with *ApaI* and the complementation cassette was transformed in the *ΔΔada2* mutants SCADA2M4A/B. The resistance marker was then excised by induction of the *FLP*-recombinase in the presence of BSA. The newly generated complementation strains were named SCADA2MK1A/B and SCADA2MK2A/B, with and without the selection marker respectively.

3.2.3 Introduction of hyperactive alleles in the *ΔΔada2* mutants

Double knock out *ΔΔada2* mutants (SCADA2M4A/B) were transformed with different cassettes to introduce hyperactive alleles of *CAP1*, *MRR1*, *TAC1* and *UPC2* transcription factors.

A *SacI*/*ApaI* fragment from plasmid pCAP1R1 (Fig. 6) was transformed into the *ΔΔada2* mutants to create strains SCΔada2CAP1R11A/B. The selection marker was recycled to create strains SCΔada2CAP1R12A/B and a second round of transformation and recycling generated strains SCΔada2CAP1R13A/B and SCΔada2CAP1R14A/B. In these strains both original *CAP1* alleles were replaced with two hyperactive truncated ones.

Analogously, a *SacI*/*ApaI* fragment from plasmid pMRR1R3 (Fig. 5) was used to transform *ΔΔada2* mutants to generate, in two rounds of transformation and cassette recycling, strains SCΔada2MRR1R34A/B containing *MRR1* hyperactive allele.

A SacI/ApaI fragment from plasmid pTAC1R3 (Fig. 13) was used to introduce the *TAC1* hyperactive alleles producing strains SCΔada2TAC1R34A/B.

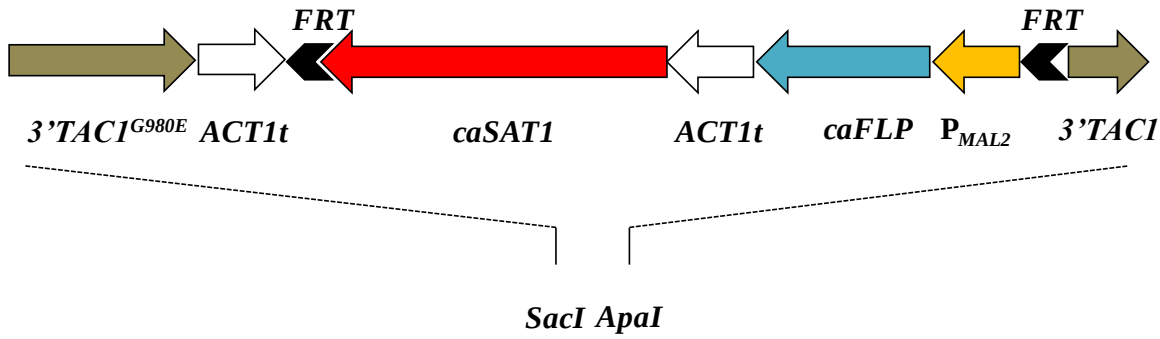

Fig. 13. Structure of the cassette from plasmid pTAC1R3.
TAC1 coding region is replaced by a *TAC1* allele containing a G980E substitution. The cassette contains the *FLP* recombinase gene (*caFLP*) under the control of the *MAL2* promoter (P_{MAL2}) and a selective resistance marker (*caSAT1*) which confers resistance to nourseothricin. *ACT1* transcription termination sequences (*ACT1t*) served for terminating both *TAC1* and *caFLP* transcription. *FLP* recombination target sequences (*FRT*) are not drawn to scale.

Finally, a *SacI*/*ApaI* fragment from plasmid pUPC2R1 (Fig. 14) was used to create strains SCΔada2UPC2R14A/B containing *UPC2* mutated alleles.

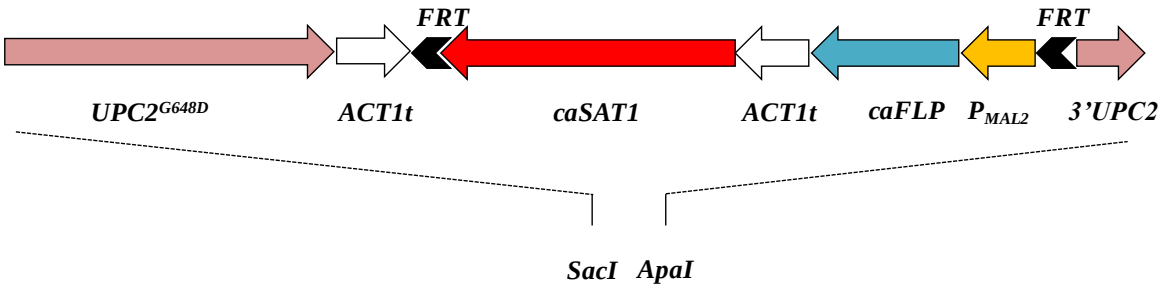

Fig. 14. Structure of the cassette from plasmid pUPC2R1.
UPC2 coding region is replaced by a *UPC2* hyperactive allele (*UPC*^{G648D}). The cassette contains the *FLP* recombinase gene (*caFLP*) under the control of the *MAL2* promoter (P_{MAL2}) and a selective resistance marker (*caSAT1*) which confers resistance to nourseothricin. *ACT1* transcription termination sequences (*ACT1t*) served for terminating both *TAC1* and *caFLP* transcription. *FLP* recombination target sequences (*FRT*) are not drawn to scale.

3.2.4 Introduction of *GFP* tagged promoter fusions

In order to monitor the activity of the hyperactive alleles of the four transcription factors Cap1, Mrr1, Tac1 and Upc2, a *GFP*-promoter fusion specific for each one of them was introduced in the previously created mutants.

While the plasmids containing *GFP*-tagged promoter fusions for genes *MDR1* and *ERG11*, targets of Cap1, Mrr1 and Upc2, were already present in the plasmid collection of Prof. Morschhäuser's group, the plasmid containing the *GFP*-tagged promoter fusion for gene *CDR2*, target of Tac1, was created in this study. The upstream region of the *CDR2* gene was amplified with primers CDR2-5 and CDR2-6 using genomic SC5314 DNA as template. The PCR product was digested with *KpnI*/*SalI* and cloned together with a [*SalI*/*ClaI*][*GFP*****-ACT1t-caSAT1*]-fragment from pERG11G2 in the *KpnI*/*ClaI* digested pCDR2G2. The newly cloned plasmid was named pCDR2G3 (Fig. 15).

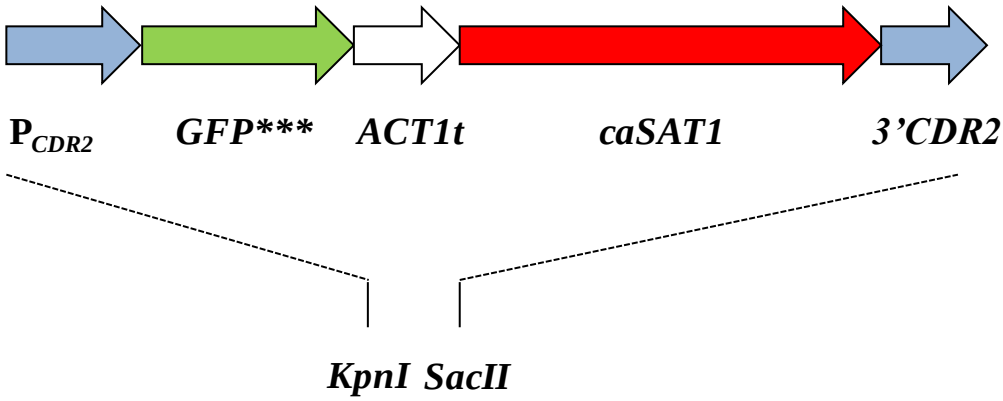

Fig. 15. Structure of the cassette from plasmid pCDR2G3.
CDR2 promoter (P$_{CDR2}$) is fused with the *GFP* protein (*GFP****). The *ACT1* transcription termination sequence (*ACT1t*) was used for a correct termination of the *GFP* transcription. The cassette contains a selective resistance marker, *caSAT1*, which confers resistance to nourseothricin.

Cap1 and Mrr1 are known to regulate Mdr1 expression, so strains SCΔada2CAP1R14A/B and SCΔada2MRR1R34A/B were transformed with a *XhoI*/*SacII* fragment from plasmid pMDR1G3 (Fig. 16) to introduce the *GFP*-tagged promoter fusion of gene *MDR1* in the place of one of the original *MDR1* promoters. The newly created mutants were named SCΔada2CAP1R14MG3A/B and SCΔada2MRR1R34MG3A/B.

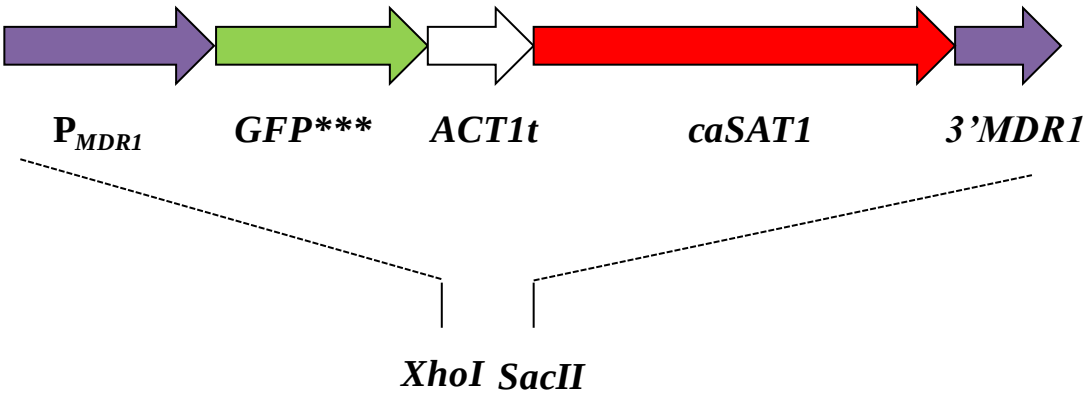

Fig. 16. Structure of the cassette from plasmid pMDR1G3.
MDR1 promoter (P$_{MDR1}$) is fused with the *GFP* protein (*GFP****). The *ACT1* transcription termination sequence (*ACT1t*) was used for a correct termination of the *GFP* transcription. The cassette contains a selective resistance marker, *caSAT1*, which confers resistance to nourseothricin.

Tac1 is a transcriptional activator of drug-responsive genes including *CDR1* and *CDR2*. A *GFP*-tagged promoter fusion of gene *CDR2* was transformed in strains

SCΔada2TAC1R34A/B. A *KpnI*/*SacII* fragment from plasmid pCDR2G3 (Fig. 15) was used to produce strains SCΔada2TAC1R34CG3A/B.

Upc2 is a transcriptional regulator of ergosterol biosynthetic genes and sterol uptake, including *ERG11*. A *GFP*-tagged promoter fusion of gene *ERG11* was transformed in strains SCΔada2UPC2R14A/B using a *ApaI*/*SacI* fragment from plasmid pERG11G2 (Fig. 17) to generate strains SCΔada2UPC2R14EG2A and B.

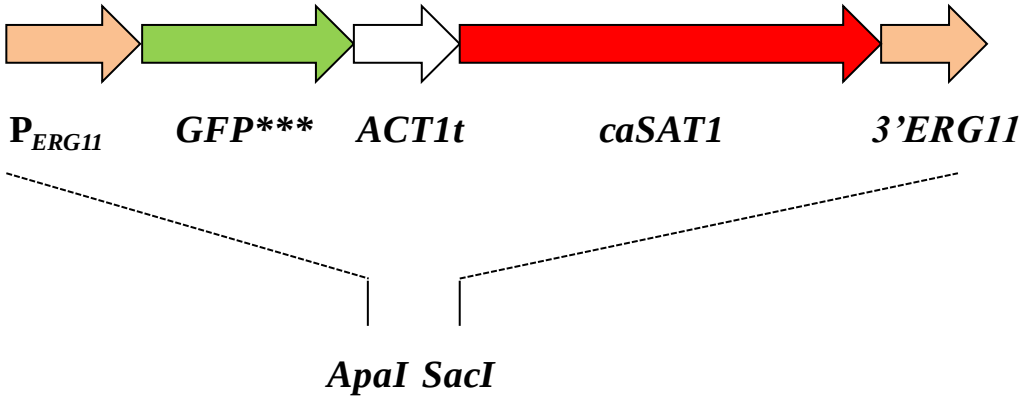

Fig. 17. Structure of the cassette from plasmid pERG11G2.
ERG11 promoter (P_{ERG11}) is fused with the *GFP* protein (GFP***). The *ACT1* transcription termination sequence (*ACT1t*) was used for a correct termination of the *GFP* transcription. The cassette contains a selective resistance marker, *caSAT1*, which confers resistance to nourseothricin.

All the above mentioned *GFP*-promoter fusions were also transformed in the original *ΔΔada2* mutants SCADA2M4A/B to create strains SCΔada2MG3A/B, SCΔada2CG3A/B, SCΔada2EG2A/B.

3.2.5 Matched mutants in the SC5314 background

For each one of the mutants generating from the *ΔΔada2* mutants, the matched mutant that still contained both *ADA2* alleles was required. Those mutants were named in the same way as the *ΔΔada2* mutants with the difference that the first part of the name, indicating the background strain, did not have the "Δada2" part in it.

With the exception of mutants SCCAP1R14MG3A/B, SCMRR1R34MG3A/B,

SCTAC1R34CG3A/B, and SCCG3A/B, the matched SC background mutants (Table 3) were already present in the strain collection of Prof. Morschhäuser's group. The eight remaining ones were produced in this study in an analogous fashion as for the *ΔΔada2* mutants. SCCAP1R14MG3A/B, and SCMRR1R34MG3A/B were created from strain SCCAP1R14A/B, and SCMRR1R34A/B (already present in the strain collection) and transforming them with a *XhoI*/*SacII* fragment from plasmid pMDR1G3. Strains SCCG3A/B, and SCTAC1R34CG3A/B were created transforming a *KpnI*/*SacII* fragment from plasmid pCDR2G3 into the reference strain SC5314, and SCTAC1R34A/B (already present in the strain collection) respectively.

3.2.6 FACS analysis for determining promoter activity

Promoter activity was determined by quantifying the fluorescence of the reporter strains by Fluorescence-activated Cell Sorter (FACS). All the results were obtained from two independently generated reporter strains.

3.2.6.1 *MDR1* promoter activity

To monitor activation of the *MDR1* promoter in response to inducing chemicals (hydrogen peroxide and benomyl) or to constitutively active transcription factors, a P_{MDR1}-*GFP* reporter fusion was introduced into the knockout *ΔΔada2* mutants. In addition, the reporter fusion was also added to the reference strain SC5314. Furthermore, the reporter fusion was introduced into the *ΔΔada2* mutants and SC5314 background strains containing hyperactive copies of *CAP1* and *MRR1*.

The first *CAP1* mutant generated on *ΔΔada2* background (SCΔada2CAP1R14MG3A) showed different phenotypes (growth rate and fluconazole susceptibility) from the second one (SCΔada2CAP1R14MG3B). Further analysis indicated that the “wrong” mutant was the B strain and not the A strain, so mutant B was excluded

from the analysis.

It was confirmed that Mdr1 activity is not detectable in the wild-type strain SC5314 in YPD medium, but it could be induced by H_2O_2 and, even more efficiently by benomyl (Fig. 18). Minor differences were observable in the *ΔΔada2* knockouts for what concerned Mdr1 induction by the used inducing chemicals. In those mutants, benomyl could induce Mdr1 activity about two fold as well as in the wild-type, while peroxide had the same effect in the *ΔΔada2* mutants and in the wild-type. Interestingly, in the only *CAP1* mutant included in the analysis (SCΔada2CAP1R14MG3A) any *GFP* expression above the background level was barely detectable. In contrast, the hyperactive *MRR1* allele, in the *ΔΔada2* background strains, seemed to have much more effect (twice as much) than in the wild-type.

Of note is the fact that the background fluorescence in all *ΔΔada2* mutants is slightly higher than in the SC5314 background strains.

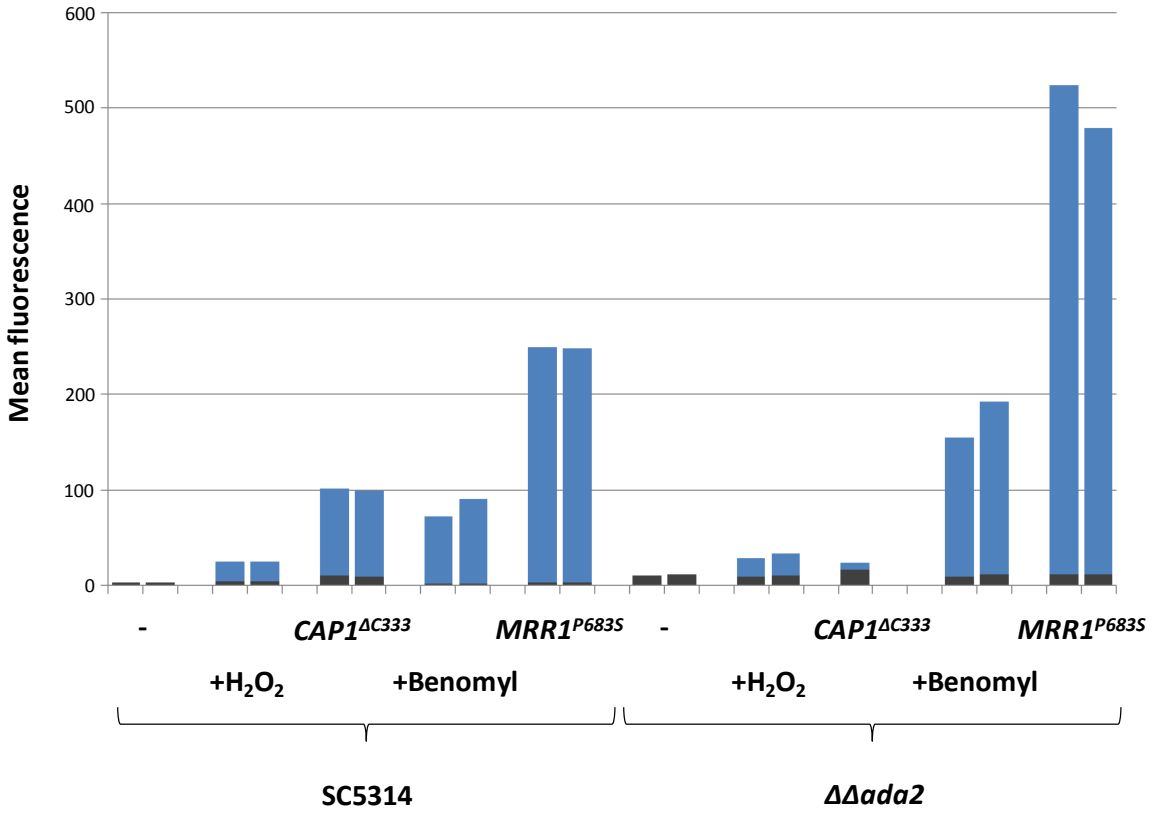

Fig. 18. Activation of the *MDR1* promoter.
Activation of the *MDR1* promoter by H_2O_2 and benomyl or the hyperactive alleles of *CAP1* and *MRR1* in the wild type SC5314 strain and in the double knock-out mutant *ΔΔada2*. Parental strains and transformants carrying a P_{MDR1}-*GFP* reporter fusion, and the independent derivatives A and B that were rendered homozygous for the *CAP1* and the *MRR1* hyperactive alleles, with and without the reporter fusion, were grown as described in Materials and Methods. The mean fluorescence of the cells was determined by flow cytometry. The results obtained with two independently generated strains are shown in each case. The background fluorescence of the parental strains, which do not contain the *GFP* gene, is indicated by the black part in each column.

3.2.6.2 *CDR2* promoter activity

CDR2 activation by fluphenazine and *TAC1* constitutively active allele was monitored, both in the wild-type SC5314 background and in the *ΔΔada2* mutant. Analogously to what has been described for the *MDR1* promoter, a P_{CDR2}-*GFP* reporter fusion was introduced in all mutant combinations, SC5314, *ΔΔada2* knockouts, SC and *ΔΔada2* background containing the hyperactive *TAC1* allele.

As shown in Fig. 19, induction of the *CDR2* promoter by fluphenazine, both in SC5314 and in *ΔΔada2* knockout backgrounds, was very efficient, although in *ΔΔada2*

mutants almost three time as much efficient than in the SC5314 background strains. On the contrary, the hyperactive *TAC1* alleles seem to be less efficient in the *ΔΔada2* mutants, with a mean fluorescence still detectable but almost half than the one observed in the SC background.

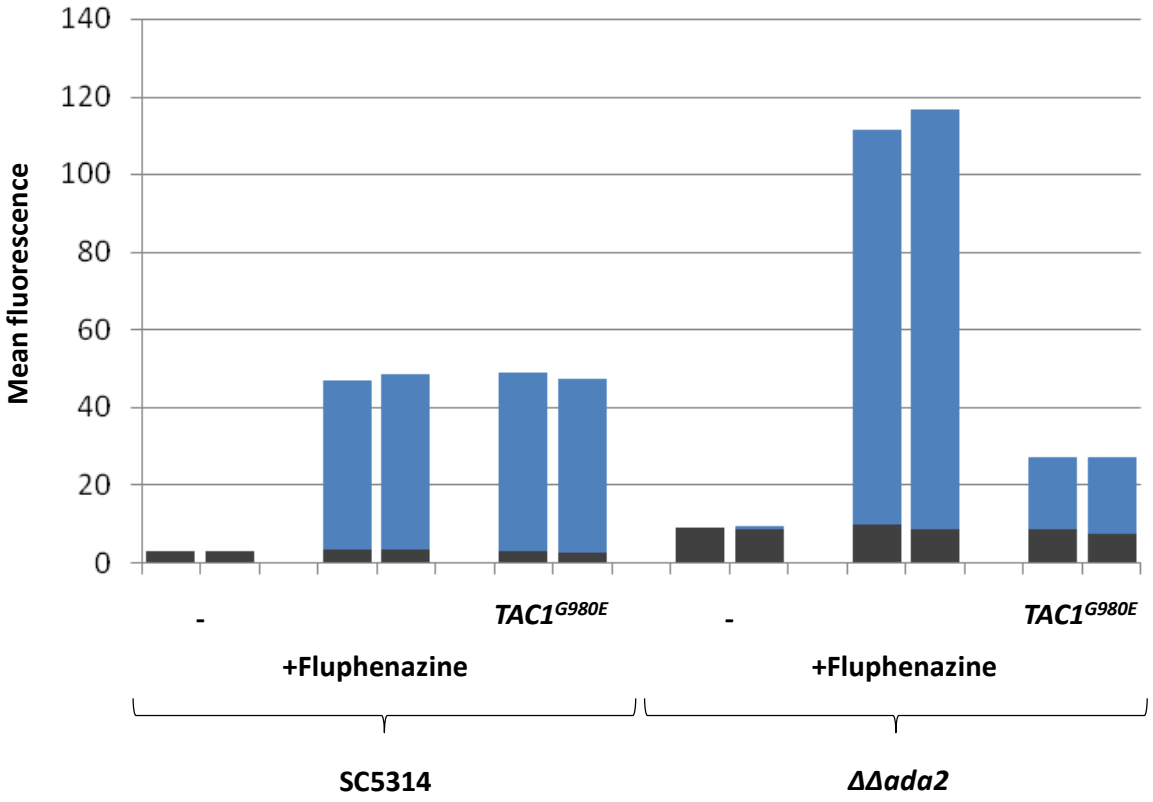

Fig. 19. Activation of the *CDR2* promoter.
Activation of the *CDR2* promoter by fluphenazine or the hyperactive allele of *TAC1* in the wild type SC5314 strain and in the double knock-out mutant *ΔΔada2*. Parental strains and transformants carrying a P_{CDR2}-*GFP* reporter fusion, and the independent derivatives A and B that were rendered homozygous for the *TAC1* hyperactive allele, with and without the reporter fusion, were grown as described in Materials and Methods. The mean fluorescence of the cells was determined by flow cytometry. The results obtained with two independently generated strains are shown in each case. The background fluorescence of the parental strains, which do not contain the *GFP* gene, is indicated by the black part in each column.

3.2.6.3 *ERG11* promoter activity

ERG11 promoter activity can be induced by chemicals such as ketoconazole or by hyperactive alleles of *UPC2* transcription factor. *ERG11* promoter activity was monitored following introduction of a P_{ERG11}-*GFP* promoter fusion in SC5314 and *ΔΔada2*

knockouts, and in mutants containing *UPC2* mutated allele which is constitutively active on the *ERG11* promoter.

Because ketoconazole requires a longer time to induce promoter activity compared to H_2O_2 and benomyl, in this case incubation time with the inducing chemical was increased to 4 hours.

As shown in Fig. 20, an higher basal fluorescence level was detectable under non inducing conditions in the SC5314 background strains compared to the activity of the other promoter fusions (P_{MDR1} and P_{CDR2}). This was even more marked in the *ΔΔada2* background strains, where a very high mean fluorescence, at least five times higher than with the other promoter fusions, was detected. Nevertheless, an increased activity of the promoter could be observed when ketoconazole was added to the growth medium, or when hyperactive alleles of *UPC2* transcription factor were present, both in the wild-type background and in the *ΔΔada2* mutant background. Interestingly, an increased *ERG11* expression is detected even in the absence of any inducing stimuli in the *ΔΔada2* mutant.

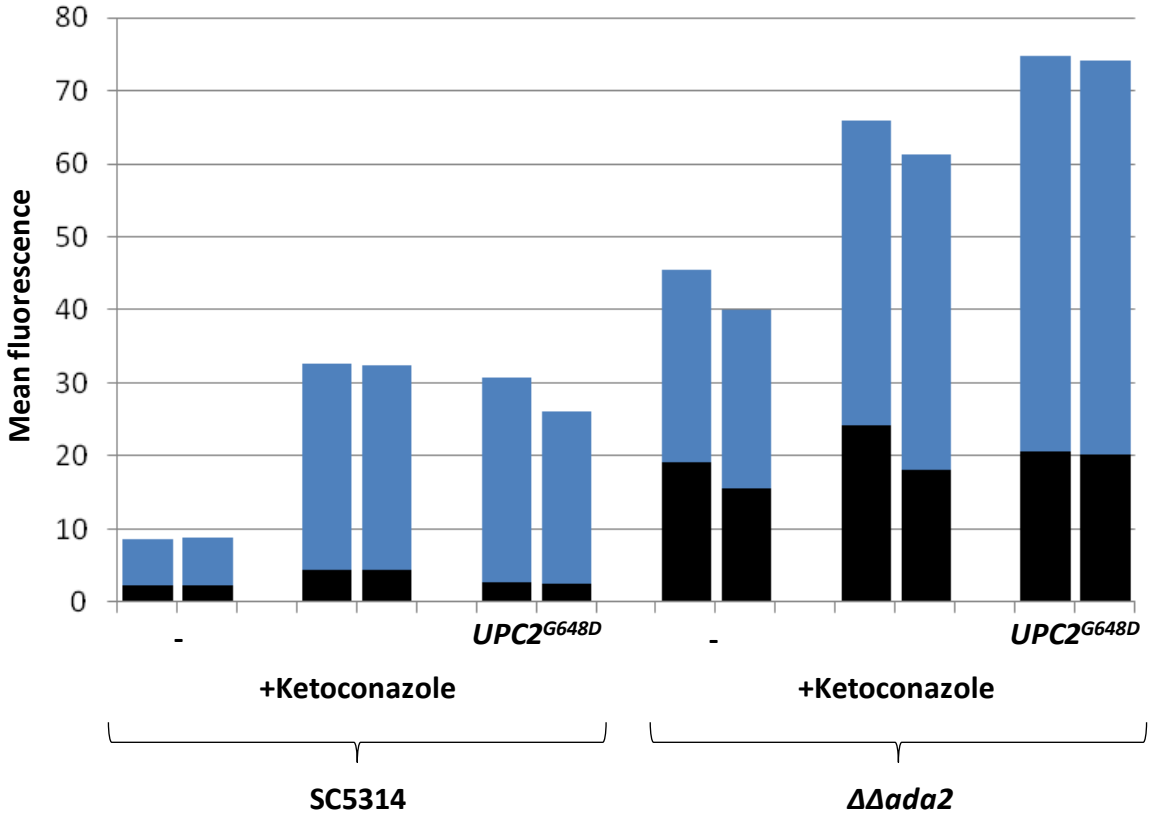

Fig. 20. Activation of the *ERG11* promoter.
Activation of the *ERG11* promoter by ketoconazole or the hyperactive allele of *UPC2* in the wild type SC5314 strain and in the double knock-out mutant *ΔΔada2*. Parental strains and transformants carrying a P_{ERG11}-*GFP* reporter fusion, and the independent derivatives A and B that were rendered homozygous for the *UPC2* hyperactive allele, with and without the reporter fusion, were grown as described in Materials and Methods. The mean fluorescence of the cells was determined by flow cytometry. The results obtained with two independently generated strains are shown in each case. The background fluorescence of the parental strains, which do not contain the *GFP* gene, is indicated by the black part in each column.

3.2.7 Analysis of fluconazole and cerulenin susceptibility by MIC tests

MIC susceptibility tests were performed in order to evaluate whether the results obtained by the FACS analysis could be confirmed by a phenotypic test that directly shows a strain's susceptibility to fluconazole or cerulenin, compared to its matched wild-type background mutant. Fluconazole susceptibility MIC tests (Fig. 21) showed that the double *ΔΔada2* knockouts (SCADA2M4A/B) were 3 $\log_2$ more susceptible to this drug, compared to the wild-type SC5314 or even to the heterozygous *ΔΔada2* mutant. Complementation of one *ADA2* allele in the double knockouts re-established the original MIC values,

demonstrating that the higher susceptibility of the double *ΔΔada2* mutants was due to the absence of Ada2 and not to an unspecific mutation that might have occurred during transformation.

The truncated *CAP1* hyperactive alleles that have been introduced in the only *ΔΔada2* mutant SCΔada2CAP1R14A only slightly affected its susceptibility to fluconazole, as MIC values were one dilution step higher than those obtained for the double *ΔΔada2* mutants. When the wild-type SC5314 was considered, MIC values in SCCAP1R14A/B were 2 dilutions higher compared to SC5314.

As already described [**47**], two hyperactive *MRR1* alleles, containing the single C-T mutation at position 2047, which results in a proline-serine substitution at position 683, confer a marked increased resistance when transformed into the wild-type *C. albicans* SC5314 strain, with MIC values 4 dilutions higher compared to the wild-type. In the absence of Ada2 the same behavior was observed, MIC values with hyperactive *MRR1* alleles were 4 dilutions higher compared to the values obtained for the mutants without those mutated alleles, although MIC values varied from 0.1 μg/ml to 1.56 μg/ml.

An analogous behavior was observed in the presence of constitutively active *TAC1*, with MIC values 3 dilutions higher, both in the *ΔΔada2* mutants and in the wild-type strain.

Only in the presence of *UPC2* activated alleles a different behavior was observed between *ΔΔada2* mutants and wild-type. Hyperactive *UPC2* alleles did not seem to confer any increased resistance in the *ΔΔada2* mutants as MIC values remained the same with or without them, while in the SC5314 background strain they increased drug resistance by 2 dilutions.

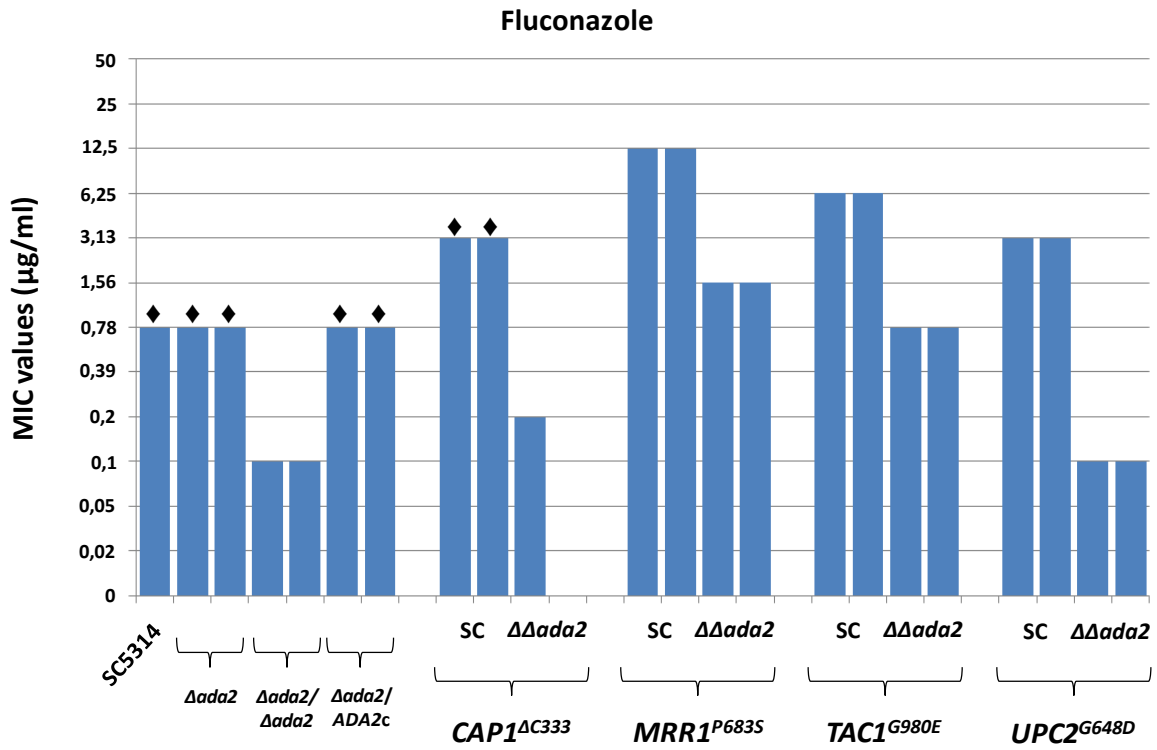

Fig. 21. Fluconazole susceptibility MIC test.
Wild-type strain SC5314, heterozygous null *Δada2* mutants, homozygous null *ΔΔada2* mutants, complementation *ada2* mutants, wild-type and double knock-out background strains which were rendered homozygous for the hyperactive alleles of *CAP1*, *MRR1*, *TAC1* and *UPC2*, were tested for fluconazole in a microdilution MIC assay as described in Materials and Methods. For each strain two independent transformants were tested. For the double knock-out *ΔΔada2* mutant containing the hyperactive *CAP1* allele only one transformant was available. ♦ Strains showing a remarkable decrease in growth at a lower drug concentration equivalent to one-step-dilution.

In addition to fluconazole, the same mutant strains were tested for susceptibility towards cerulenin, a substrate for both the ABC transporters Cdr1 and Cdr2 and the major facilitator Mdr1 (Fig. 22).

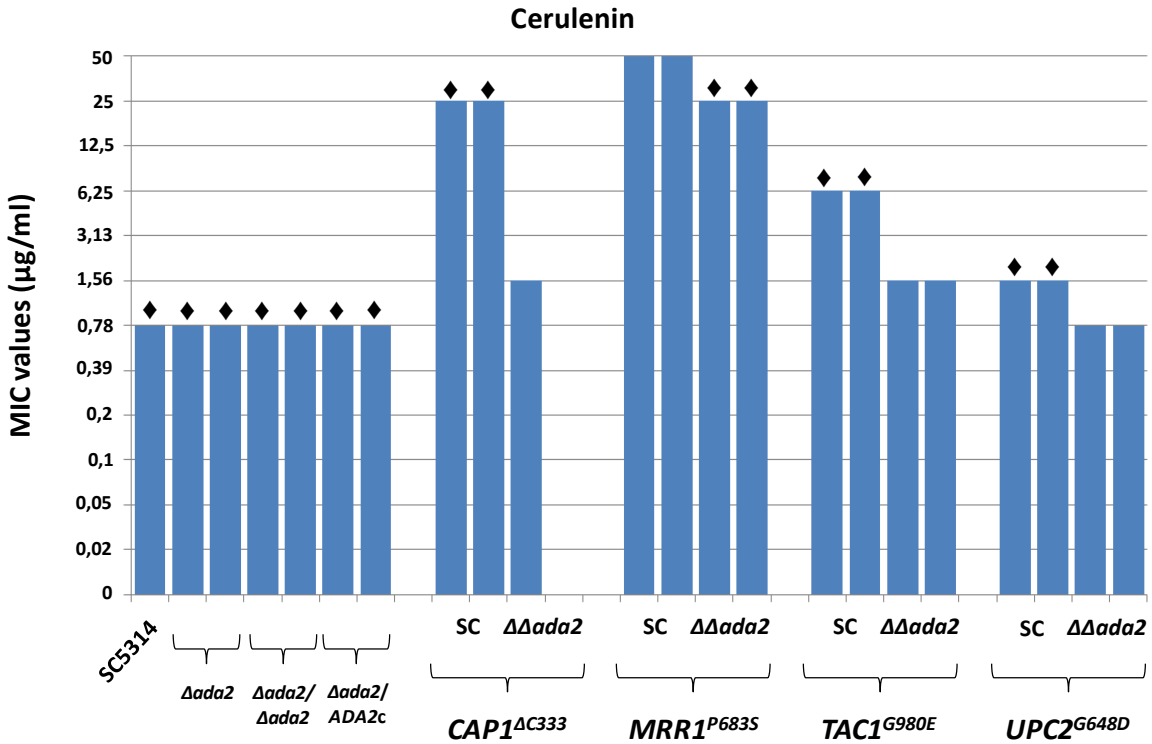

Fig. 22. Cerulenin susceptibility MIC test.
Wild-type strain SC5314, heterozygous null *Δada2* mutants, homozygous null *ΔΔada2* mutants, complementation *ada2* mutants, wild-type and double knock-out background strains which were rendered homozygous for the hyperactive alleles of *CAP1*, *MRR1*, *TAC1* and *UPC2*, were tested for cerulenin in a microdilution MIC assay as described in Materials and Methods. For each strain two independent transformants were tested. For the double knock-out *ΔΔada2* mutant containing the hyperactive *CAP1* allele only one transformant was available. ♦ Strains showing a remarkable decrease in growth at a lower drug concentration equivalent to one-step-dilution.

In this case, both heterozygous *Δada2* and homozygous knockout *ΔΔada2* mutants showed the same trend: MIC values against cerulenin were identical to those of the wild-type SC5314 strain.

In the presence of *CAP1* hyperactive alleles, the only mutant on the *ΔΔada2* double mutant background analyzed had a MIC value only one dilution step higher compared to the strains which did not have the hyperactive allele, in contrast to the SC5314 (1.56 µg/ml) background strain, where a strong increase in drug resistance could be observed in the presence of *CAP1* truncated allele, with MIC values 5 dilution steps higher than the wild-type (25 µg/ml), confirming what has previously been described [**92**].

If Cap1 seems to require the presence of Ada2 to confer resistance to cerulenin, this

does not seem to be true for Mrr1. Indeed, a very high MIC value was observed for mutant strains containing the *MRR1* hyperactive alleles, both in the *ΔΔada2* knockouts and in the wild-type, with MIC values of 25 and 50 µg/ml respectively, meaning 5 and 6 dilution steps higher than the strains without any constitutively active allele (Fig. 22).

When *TAC1* was analyzed, a minor increase in cerulenin resistance was still detectable in *ΔΔada2* mutants, one dilution step higher than in the mutant without *TAC1* hyperactive allele, while in the wild-type SC strain, hyperactive allele conferred resistance of 3 dilution steps higher (Fig. 22).

As could be expected, since cerulenin is a target of the efflux pumps and does not directly interfere on the ergosterol biosynthesis, only a slight effect was observed in the SC background when hyperactive alleles of *UPC2* were integrated in its genome. No effect on MIC values was observed in the *ΔΔada2* mutants.

3.2.8 Confirmation of *ADA2* complementation by spot assay

Following the destruction of the second *ADA2* allele, the obtained mutant strains showed a growth defect which was clearly visible when a spot assay was performed.

The growth rate difference between wild-type or heterozygous *Δada2* mutants and the homozygous *ΔΔada2* mutants was even more pronounced when the strains were grown on nourseothricin containing media. Fig. 23 shows the results obtained in a spot assay, where the different strains were compared on two different media, the complete rich media, YPD agar, and the N10 agar, YPD additioned with 10 µg/ml nourseothricin. The double knock-out mutants (SCADA2M4A/B) showed a growth defect, which was even more noticeable on the nourseothricin containing medium. While the heterozygous mutants did not show a different phenotype compared to the wild-type, the double knock-outs had a clear growth defect: on YPD media they grew as smaller colonies and they formed colonies only from a starting cell concentration 10 fold higher than the wild-type. This

behavior was more evident on N10 plates, since the colonies were formed only starting from a cell concentration equal to OD_{600nm} = 10, 10^3 times higher than the cell concentration required to form a colony in the case of SC5314.

To confirm that the observed phenotype was due to the lack of Ada2 and not to an unspecific mutation which could have occurred during the second round of transformation, one Ada2 allele was reintroduced in the double mutants SCADA2M4A/B, generating strains SCADA2MK2A/B. The wild-type phenotype was restored, the growth rate was normal and the nourseothricin sensibility was back to normal levels.

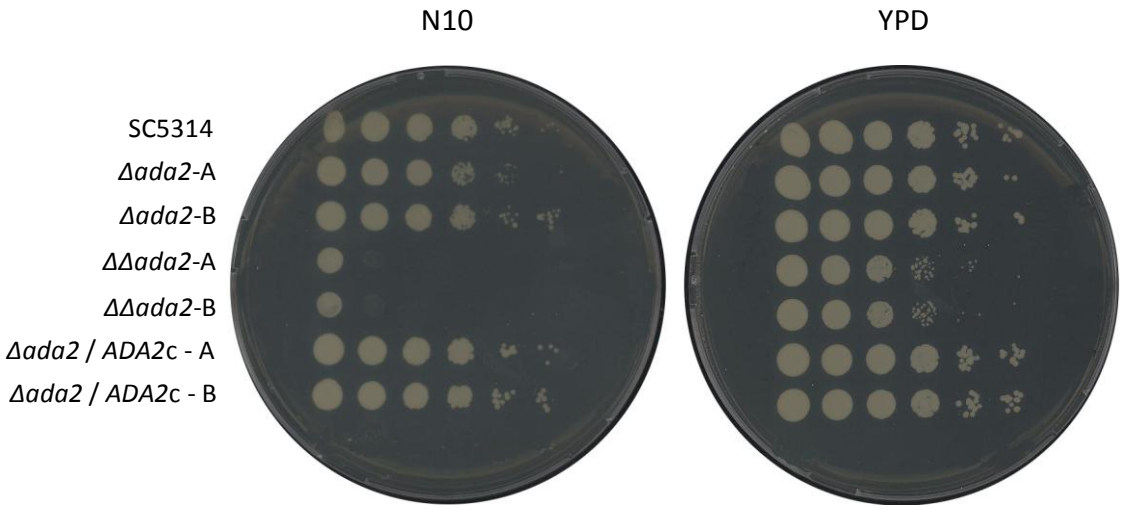

Fig. 23. Spot assay.
Wild-type strain SC5314, heterozygous *Δada2* and homozygous *ΔΔada2* knock-out deletion mutants and complementation mutants were spotted on nourseothricin containing YPD (10 µg/ml – N10) and normal YPD as described in Materials and Methods. Picture was taken after 24 h of growth.

4 DISCUSSION

4.1 Differential requirement of the transcription factor Mcm1 for activation of the *Candida albicans* multidrug efflux pump *MDR1*.

The transcription factors Mrr1 and Cap1 are both involved in the induction of *MDR1* expression in response to inducing chemicals; in addition, activating mutations in either of the two transcription factors result in constitutive overexpression of the efflux pump [**7**] [**47**] [**50**] [**92**] [**93**]. However, little is known about how Mrr1 and Cap1 activate *MDR1* transcription and which additional regulatory factors may be required. In this study the role of the MADS box transcription factor Mcm1 has been addressed, which has previously been implicated in the regulation of *MDR1* expression and was recently shown to bind to the *MDR1* promoter *in vivo* [**50**] [**56**] [**94**] [**95**].

Mcm1 can act as both a positive and a negative regulator of transcription [**57**] [**95**]. Depletion of Mcm1 did not result in constitutive activity of the *MDR1* promoter, indicating that the role of Mcm1 is not that of a repressor of *MDR1* expression in the absence of inducing conditions. Moreover, depletion of Mcm1 did not affect the inducibility of the *MDR1* promoter by H_2O_2 or the constitutive *MDR1* overexpression caused by a hyperactive Cap1. On the other hand, benomyl-induced *MDR1* expression was reduced, but not abolished, following depletion of Mcm1 and a hyperactive Mrr1 could not upregulate in the absence of Mcm1. The *MDR1* transcripts that were still present after Mcm1 depletion in strains expressing the hyperactive Mrr1 may represent mRNAs that were produced before Mcm1 depletion and not yet degraded. Alternatively, the cells might have contained residual amounts of Mcm1 that were not detectable by western blot analysis. These results suggest that the induction of *MDR1* expression by H_2O_2 occurs

mainly via activation of Cap1, which acts independently of Mcm1, and the induction by benomyl occurs partially via Mrr1, which depends on Mcm1 to activate the *MDR1* promoter, and partially via Cap1. Like H_2O_2, benomyl causes oxidative stress and activates Yap1, the homolog of Cap1 in *Saccharomyces cerevisiae* [**96**]. In response to oxidative stress, conserved cysteine residues in the C-terminus of Yap1 form disulphide bonds, resulting in conformational changes that interrupt the interaction of Yap1 with the nuclear export protein Crm1, so that Yap1 can accumulate in the nucleus and activate its target genes [**97**]. In *C. albicans*, Cap1 activity is also regulated by nuclear-cytoplasmic shuttling and mutation of one of the conserved cysteines in Cap1 or removal of the C-terminus results in constitutive Cap1 activity [**92**] [**98**].

Based on the results of this and previous studies, the model shown in Fig. 24 depicts the involvement of Mrr1, Cap1, and Mcm1 in four scenarios in which *MDR1* expression is upregulated.

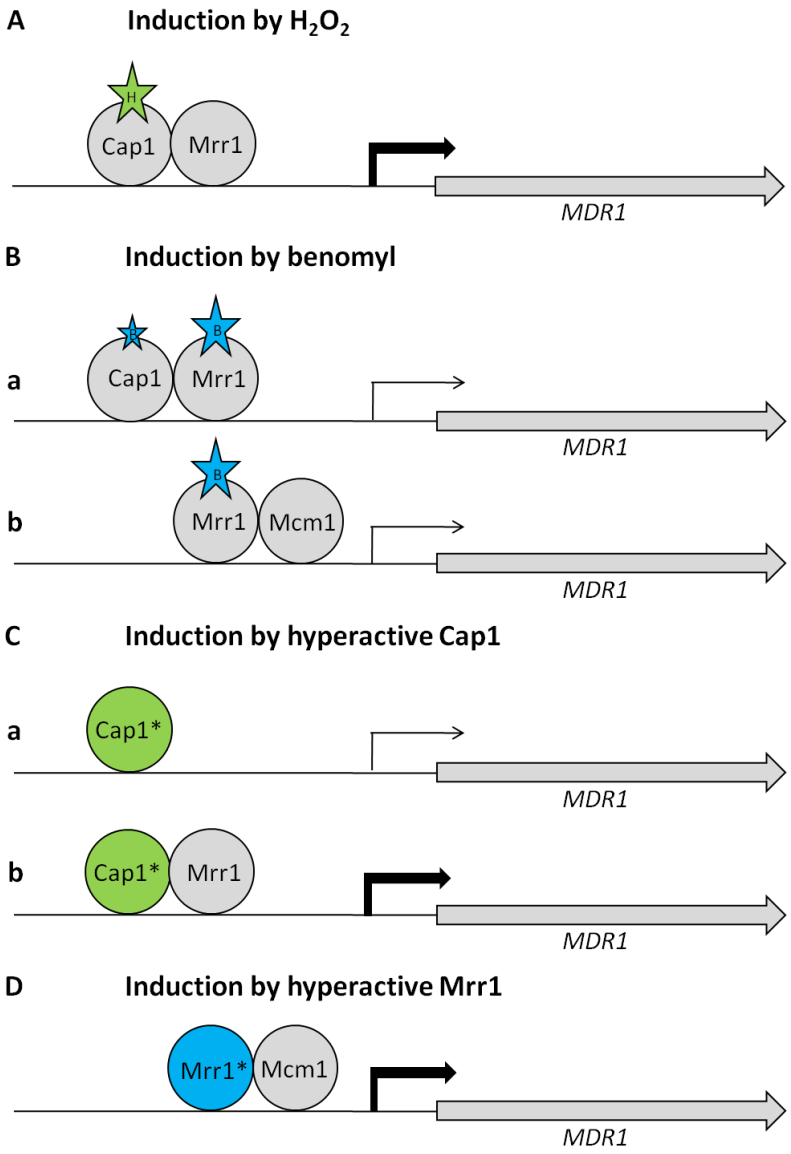

Fig. 24. Model of the role of Cap1, Mrr1 and Mcm1 in *MDR1* upregulation.
Role of the transcription factors Cap1, Mrr1 and Mcm1 in *MDR1* upregulation by inducing chemicals or gain-of-function mutations in Cap1 and Mrr1. Thinner bent arrows indicate reduced *MDR1* promoter activity in the absence of the missing transcription factor. (A) H_2O_2 activates Cap1, resulting in accumulation of the transcription factor in the nucleus, where it can induce *MDR1* expression together with Mrr1 in an Mcm1-independent fashion. (B) Benomyl activates Mrr1 and partially also Cap1 in an unknown way. When Cap1 is available at the *MDR1* promoter (a), Mrr1 and Cap1 can induce *MDR1* expression independently of Mcm1. In the absence of Cap1 (b), Mrr1 requires Mcm1 to induce *MDR1* expression. (C) A hyperactive form of Cap1 (labeled Cap1*) can induce the *MDR1* promoter in the absence of inducing stimuli and independently of Mcm1 and Mrr1 (a), but full induction requires the presence of Mrr1 (b). (D) A hyperactive Mrr1 containing a gain-of-function mutation (labeled Mrr1*) can induce the *MDR1* promoter independently of Cap1 (which is localized in the cytoplasm in the absence of inducing stimuli), but requires the co-regulator Mcm1.

In the presence of H_2O_2 (Fig. 24A), Cap1 is activated, accumulates in the nucleus, and induces *MDR1* expression. Under these conditions, Cap1 requires Mrr1 to activate the *MDR1* promoter, but Mcm1 is dispensable. In the presence of benomyl (Fig. 24B), Cap1 is also activated by oxidative stress and can induce *MDR1* expression together with Mrr1 independently of Mcm1 (a). However, *MDR1* induction by benomyl can also occur at a reduced level in the absence of Cap1 in an Mrr1-dependent fashion, and this requires the presence of Mcm1 (b). How benomyl activates Mrr1 is not known. It is possible that Mrr1 is activated by direct binding of the drug, similar to the zinc cluster transcription factors Pdr1 and Pdr3, which regulate efflux pump expression in *S. cerevisiae* and *Candida glabrata* [**99**]; however benomyl may also activate Mrr1 in an indirect fashion. The C-terminally truncated, hyperactive Cap1 can upregulate *MDR1* expression even in the absence of Mrr1, but not as efficiently as in a wild type background (Fig. 24C). In this respect, the hyperactive Cap1 differs from activated wild-type Cap1, which requires Mrr1 to induce the *MDR1* promoter in response to H_2O_2 or benomyl. However, similar to oxidative stress-activated Cap1, the hyperactive Cap1 does not depend on Mcm1 to induce *MDR1* expression. Finally, a hyperactive Mrr1 can promote *MDR1* overexpression independently of Cap1, but in this case it requires Mcm1 (Fig. 24D). Altogether, these results indicate that Mrr1 can upregulate *MDR1* expression in cooperation with either Cap1 or Mcm1. In response to oxidative stress, Mrr1 cooperates mainly with activated Cap1 to induce the *MDR1* promoter and Mcm1 is largely dispensable. In the absence of inducers, Cap1 remains in the cytoplasm and a hyperactive Mrr1 depends on the presence of Mcm1 to cause *MDR1* overexpression. These observations are in accordance with previous findings indicating that deletion of the putative Mcm1 binding sequence (the BRE/MDRE) from a truncated *MDR1* promoter abolished benomyl-induced or constitutive *MDR1* overexpression [**50**] [**56**]. However, deletion of the BRE/MDRE from the full-lenght

MDR1 promoter did not affect its constitutive activity in strains that contained gain-of-function mutations in Mrr1 [**47**] [**51**] [**56**]. The result of the present study, showing that a hyperactive Mrr1 requires Mcm1 to mediate *MDR1* overexpression, indicates that Mcm1 can contribute to *MDR1* expression in a manner that is independent of its binding site in the BRE/MDRE, either by binding to an additional region in the *MDR1* promoter or by interacting with bound Mrr1 without being directly bound to the DNA. Indeed, a recent paper by Askew and coworkers demonstrates that Mcm1 binds to the *MDR1* promoter in two ways: 1) on its canonical motif independently of any other co-factor; 2) on a non canonical motif through Ahr1 [**100**]. This protein is a DNA binding factor that targets promoters regulating several key adhesion genes, and it has also been shown to be involved in hyphal growth and virulence. The Ahr1 motif is located upstream of the Mcm1 motif in a region containing a *cis*-acting sequence of *MDR1* [**49**] [**51**]. Therefore, the proposed model is that Mcm1 mediates *MDR1* overexpression together with hyperactive Mrr1 by binding to the Ahr1 motif further upstream, even in the absence of its canonical binding motif, the BRE/MDRE.

4.2 Differential requirement of the transcription factor Ada2

The transcription factor Ada2 has a central role in cell wall integrity [**101**] which explains the growth defect phenotype in the double knock-out mutant. Higher basal autofluorescence of the *ΔΔada2* mutants containing *GFP* protein compared to wild-type background strains containing *GFP* protein can also be explained by the role that Ada2 occupies in cell wall integrity. It is widely recognized that high cell autofluorescence indicates cell death [**102**] [**103**]. Ada2 is not an essential protein in *Candida albicans*, but its complete removal induces in the cell a very susceptible state, most likely because of a perturbation of the cell wall integrity.

It has been showed in previous studies that Ada2 is recruited to at least 200 promoters upstream of genes involved in different stress-response functions and metabolic processes. In particular, Ada2 binds the promoters of several drug transporters, such as *MDR1*, *CDR1*, *CDR4*, *QDR1*, *YCF1*, *FLU1*, *ORF19.4531*, and *ORF19.301* as well as the phosphatidylinositol transfer gene *PDR16* [**59**]. In this study it was confirmed by MIC assay that an increase in sensitivity to fluconazole could be observed in cells lacking *ADA2*. This suggests that Ada2 plays a role in *C. albicans* azole tolerance, most likely by its recruitment as a co-activator by TFs that control the expression of several drug transporters. Constitutive activation of the transcriptional regulators of efflux pumps such as Tac1, Upc2, Mrr1 or Cap1, have been associated with clinical azole resistance in *C. albicans* [**46**] [**104**] [**105**], and therefore those TFs could use Ada2 as a coactivator to activate their targets.

4.2.1 Role of Ada2 in the activation of *MDR1* expression

As already mentioned, Ada2 recruitment to the promoters of oxidative resistance genes is mediated by the transcription factor Cap1. In addition, Ada2 co-occupies together with the drug resistance regulator Mrr1 the promoters of core resistance genes involved in drug resistance. Sellam and coworkers have shown that Mrr1 and Ada2 cooccupied six gene promoters among the eight core resistance genes, including *MDR1* [**59**]. They speculated that Ada2, since occupancy of these genes was completely dependent on Mrr1, functions as a co-activator for Mrr1. Results obtained in the present study demonstrate that Ada2 plays a role in the Mrr1-dependent activation of *MDR1* promoter, but it acts more like a repressor since its removal leads to a higher *MDR1* expression in the presence of an hyperactive or a benomyl-activated Mrr1 allele (Fig. 25).

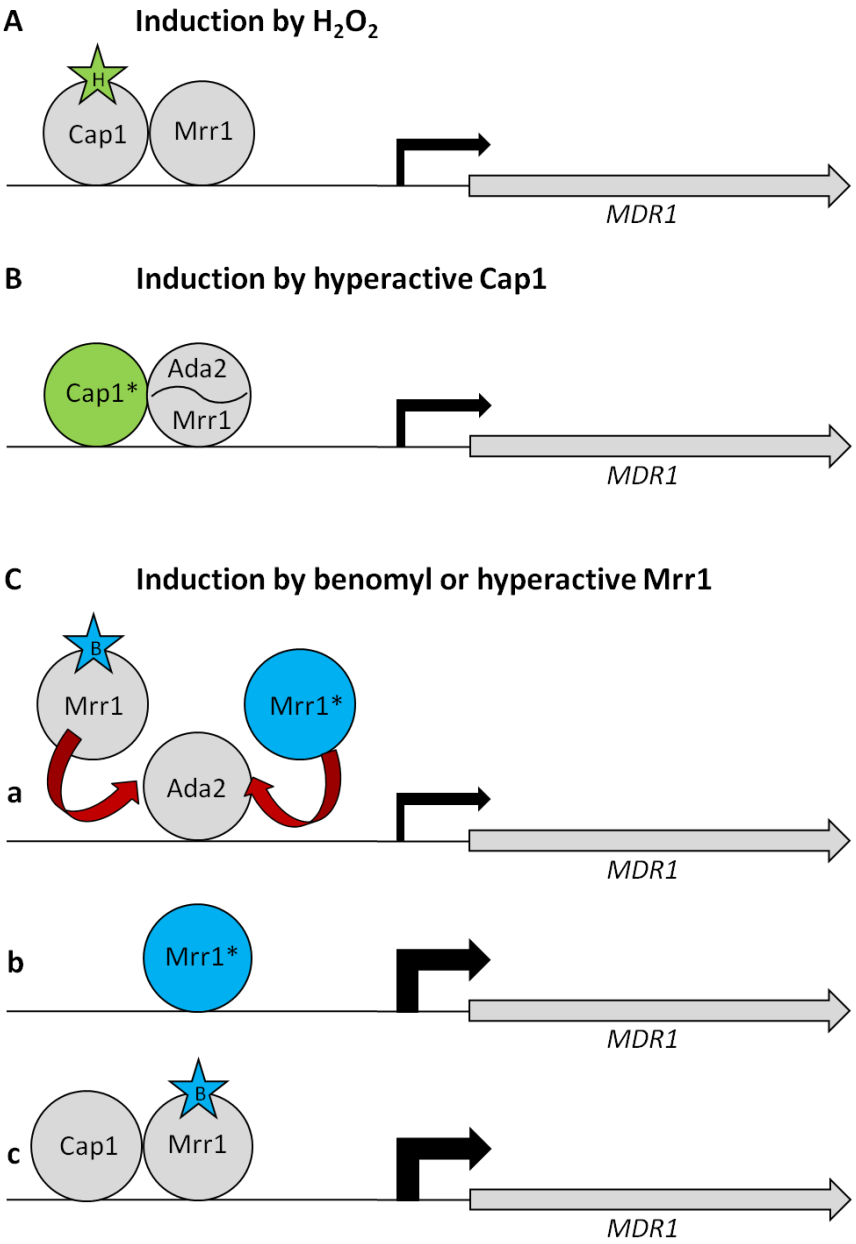

Fig. 25. Model of the role of Cap1, Mrr1 and Ada2 in *MDR1* upregulation.
Role of the transcription factors Cap1, Mrr1 and Ada2 in *MDR1* upregulation by inducing chemicals or gain-of-function mutations. Thicker bent arrows indicate increased *MDR1* promoter activity in the absence of the missing transcription factor. (A) H_2O_2 activates Cap1, resulting in accumulation of the transcription factor in the nucleus, where it can induce *MDR1* expression together with Mrr1 in an Ada2-independent fashion. (B) A hyperactive form of Cap1 (labeled Cap1*) can induce the *MDR1* promoter in the absence of inducing stimuli but needs the presence of Ada2. Mrr1 is dispensable. (C) Ada2 and Mrr1 co-localize on the same region of *MDR1* promoter (a); in the absence of Ada2 both the hyperactive form of Mrr1 (labeled Mrr1*) (b) and the Mrr1 activated by the presence of benomyl (c) can induce an even higher expression of *MDR1*.

Concerning the role of Ada2 in Cap1 dependent *MDR1* expression, results obtained in this study demonstrate that hydrogen peroxide-activated Cap1 allele is able to completely induce the expression of *MDR1* even in the absence of Ada2, while the hyperactive allele of Cap1 (although results refer to one single mutant and thus need further confirmation) seems to need both Mrr1 and Ada2 on the *MDR1* promoter to perform its activity of activator.

4.2.2 Role of Ada2 in the activation of *CDR2* expression

The cis-acting drug-responsive element (DRE), present in the promoters of *CDR1* and *CDR2* genes and necessary for their upregulation, contains 5'-CGG-3' triplets that are often recognized by transcriptional activators with Zn(2)-Cys(6) fingers. Tac1 TF is the main regulator of the expression of those two efflux pumps [**41**], and Ada2 binds to *CDR1* promoter too [**59**], but the relationship between Tac1 and Ada2 is not yet known. The absence of Ada2 impairs *CDR1* inducibility by tunicamycin, therefore in this study it has been evaluated if a similar trend could be observed in *CDR2* inducibility by hyperactive Tac1 or fluphenazine, an inducer of *CDR1* and *CDR2* expression. The results obtained in this study indicate that the resistance regulator Tac1 shares the recruitment of Ada2 on the *CDR2* promoter: indeed, the absence of Ada2 leads to a reduced expression of *CDR2* induced by the hyperactive form of Tac1 (Fig. 26). An opposite behavior can be observed when the expression of *CDR2* is induced by the presence of fluphenazine. In the absence of the Ada2 TF, fluphenazine has a much more powerful effect on *CDR2* expression. Therefore, Ada2 has a modulator role, upregulating or down-regulating gene expression in response to environmental changes. This aspect of Ada2 activity had already been hinted in a previous study published by Jacobson and Pillus [**106**], where Ada2 is shown to participate in both transcriptional repression and activation, in response to nutrient signaling.

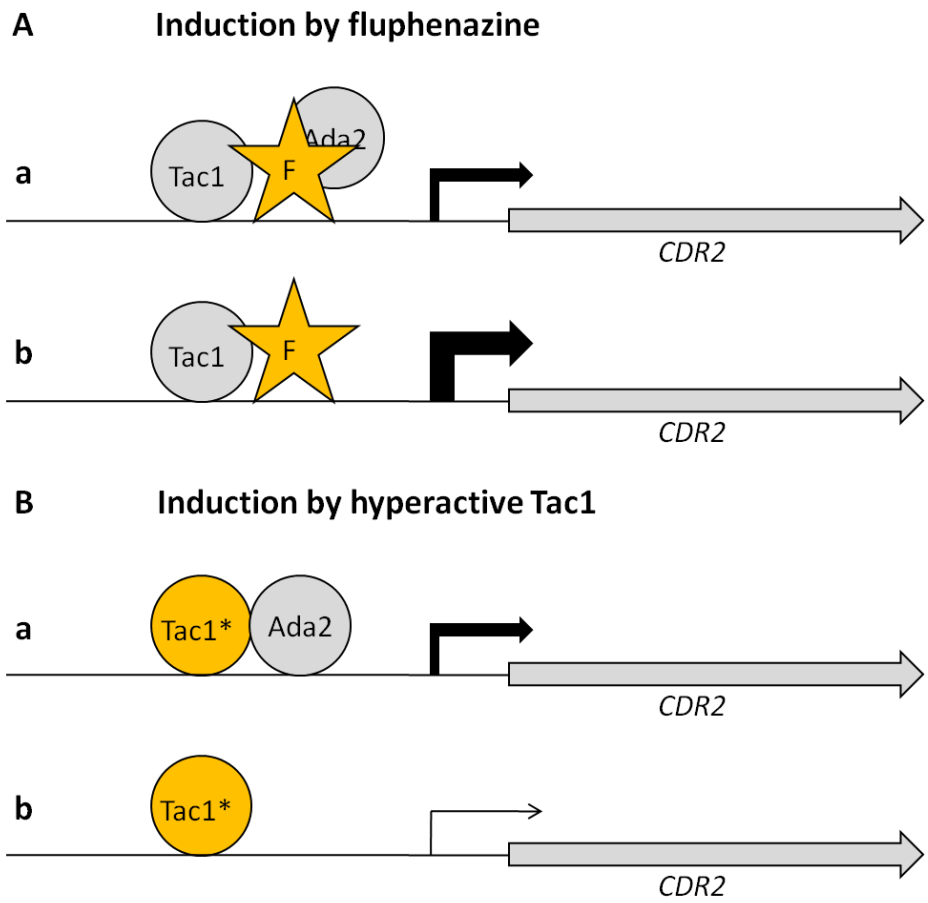

Fig. 26. Model of the role of Tac1 and Ada2 in *CDR2* upregulation.
Role of the transcription factors Tac1 and Ada2 in *CDR2* upregulation by inducing chemicals or gain-of-function mutations. Thicker or thinner bent arrows indicate increased or decreased *CDR2* promoter activity in the absence of the missing transcription factor respectively. (A) Fluphenazine induces *CDR2* expression (a) but in the absence of Ada2 this expression is increased (b). (B) A hyperactive form of Tac1 (labeled Tac1*) can induce the *CDR2* promoter in the absence of inducing stimuli (a) but needs the presence of Ada2 for a full induction (b).

4.2.3 Role of Ada2 in the activation of *ERG11* expression

Despite a very high autofluorescence observed in the FACS analysis experiments with *ΔΔada2* mutants containing a *GFP* tagged *ERG11* promoter, relevant information could be obtained. In fact, subtracting the autofluorescence from the mean fluorescence values of the *GFP* containing mutants, a relatively high *ERG11* promoter activation can be detected, even in the absence of inducing chemicals or hyperactive *UPC2* alleles. This

could suggest that Ada2 has a repressive effect on the promoter of *ERG11*. Interestingly, both ketoconazole and the hyperactive *UPC2* alleles seem, when autofluorescence is subtracted from the mean fluorescence, to induce more efficiently in the *ΔΔada2* mutant than in the wild-type background. These data do not directly reflect the MIC values obtained from fluconazole susceptibility tests. *UPC2* hyperactive alleles confer fluconazole resistance in the wild-type background, but no effect can be detected when the hyperactive *UPC2* TF is cloned in the *ΔΔada2* mutant background. A possible explanation for this apparent discrepancy is that the effect of the missing Ada2 TF on the increased basal *ERG11* expression could be masked by the drastic effects on cell wall integrity which indirectly lead to an increase in susceptibility to fluconazole. Alternatively, the overall increase in fluorescence could be interpreted as fold increase over background. In this case the *ERG11* promoter activation in *ΔΔada2* mutants results lower than in the wild-type in all cases, with or without inducing stimuli. Additional control experiments are therefore required to unequivocally confirm either option. In this regard, quantitative qRT-PCR on *ERG11* transcripts, or the measurement of *GFP* protein with a specific antibody, although semi-quantitative, would bypass auto fluorescence problems.

4.2.4 Effect of Ada2 absence on fluconazole and cerulenin resistance

In agreement with previous studies [**59**] these data confirm that Ada2 removal renders *Candida albicans* more susceptible to fluconazole. In addition, these results demonstrate that the removal of Ada2 leads to activation or repression of a certain promoter depending on the environmental conditions. This explains why FACS analysis does not always strictly correlate with MIC assay results, confirming that fluconazole resistance is a multi-factorial event and cannot be explained by the analysis of a single gene expression.

Since the depletion of Ada2 does not seem to have any detectable effect on cerulenin susceptibility, the results obtained from the MIC assays performed with this drug are easier to interpret. Cerulenin is a substrate for both the ABC transporters Cdr1 and Cdr2 and the major facilitator Mdr1. Hyperactive Cap1 and Tac1 alleles need Ada2 to fully activate *MDR1* and *CDR2* expression respectively and this is reflected in a higher susceptibility towards cerulenin when *ΔΔada2* mutants containing hyperactive Cap1 or Tac1 are analyzed. On the contrary, Mrr1 does not need Ada2 to induce *MDR1* expression, since Ada2 represses the full activation of *MDR1* promoter in the presence of hyperactive Mrr1. *ΔΔada2* mutants containing hyperactive *MRR1* alleles could be expected to have a higher cerulenin MIC value. Instead the results obtained showed that the difference in MIC value in the SC5314 background and in the *ΔΔada2* mutant background is only one dilution step. It could therefore be hypothesized that Ada2 depletion has some other unknown effect, which hampers Mrr1 contribution to cerulenin resistance. As expected, *UPC2* hyperactive allele does not have a significant effect on cerulenin resistance, and this is consistent with the fact that Upc2 does not have Mdr1, Cdr1 or Cdr2 as targets.

4.3 Concluding remarks

Fluconazole resistance in *Candida albicans* is a multi-factorial event. Depending on the inducing conditions and the combination of transcription factors involved, *C. albicans* uses different mechanisms to upregulate expression of the various genes involved in drug resistance.

The present study provides new insights into the functional role of transcription factors known to be involved in regulation of core drug resistance genes.

Future research will be aimed to unravel how each considered transcription factor activates its target promoter in order to identify new potential targets for the development of novel antifungal strategies.

5 REFERENCES

1. **Schauer, F., Hanschke, R.:** Taxonomy an ecology of the genus Candida. Mycoses (1999) 42 Suppl 1:12-21. Review. German.
2. **Lewis, R.:** Overview of the changing epidemiology of candidemia. Curr Med Res Opin (2009) Jul; 25(7):1732-40. Review.
3. **Ghannoum, M., Rice, L.:** Antifungal agents: mode of action, mechanisms of resistance, and correlation of these mechanisms with bacterial resistance. Clin Microbiol Rev (1999) Oct;12(4):501-17. Review.
4. **Sanglard, D., Odds, F.:** Resistance of Candida species to antifungal agents: molecular mechanisms and clinical consequences. Lancet Infect Dis (2002) Feb;2(2):73-85.
5. **White, T., Marr, K., Bowden, R.:** Clinical, cellular, and molecular factors that contribute to antifungal drug resistance. Clin Microbiol Rev (1998) Apr;11(2):382-402. Review.
6. **Polak, A., Scholer, H.:** Mode of action of 5-fluorocytosine and mechanisms of resistance. Chemotherapy (1975) 21(3-4):113-30.
7. **Morschhäuser, J.:** Regulation of multidrug resistance in pathogenic fungi. Fungal Genet Biol (2010) Feb;47(2):94-106. Epub 2009 Aug 7. Review.
8. **Defever, K., Whelan, W., Rogers, A., Beneke, E., Veselenak, J., Soll, D.:** Candida albicans resistance to 5-fluorocytosine: frequency of partially resistant strains among clinical isolates. Antimicrob Agents Chemother (1982) Nov;22(5):810-5.
9. **Bolard, J.:** How do the polyene macrolide antibiotics affect the cellular membrane

properties? Biochim Biophys Acta (1986) Dec 22;864(3-4):257-304.

10. **Pasko, M., Piscitelli, S., Van Slooten, A.:** Fluconazole: a new triazole antifungal agent. DICP (1990) Sep;24(9):860-7.

11. **Perlin, D.:** Resistance to echinocandin-class antifungal drugs. Drug Resist Updat (2007) Jun;10(3):121-30. Epub 2007 Jun 13.

12. **Sanglard, D., White, T.:** Molecular principles of antifungal drug resistance. Molecular principles of fungal pathogenesis; ASM Press; Joseph Heitman (2006) Chapter 14.

13. **Hope, W., Tabernero, L., Denning, D., Anderson, M.:** Molecular mechanisms of primary resistance to flucytosine in Candida albicans. Antimicrob Agents Chemother (2004) Nov;48(11):4377-86.

14. **Noël, T., François, F., Paumard, P., Chastin, C., Brèthes, D., Villard, J.:** Flucytosine-fluconazole cross-resistance in purine-cytosine permease-deficient Candida lusitaniae clinical isolates: indirect evidence of a fluconazole uptake transporter. Antimicrob Agents Chemother (2003) Apr;47(4):1275-84.

15. **Sanglard, D., Ischer, F., Parkinson, T., Falconer, D., Bille, J.:** Candida albicans mutations in the ergosterol biosynthetic pathway and resistance to several antifungal agents. Antimicrob Agents Chemother (2003) Aug;47(8):2404-12.

16. **Kurtz, M., Douglas, C.:** Lipopeptide inhibitors of fungal glucan synthase. J Med Vet Mycol (1997) Mar-Apr;35(2):79-86.

17. **Inoue, S., Qadota, H., Arisawa, M., Anraku, Y., Watanabe, T., Ohya, Y.:** Signaling toward yeast 1,3-beta-glucan synthesis. Cell Struct Funct (1996) Oct;21(5):395-402.

18. **Qadota, H., Python, C., Inoue, S., Arisawa, M., Anraku, Y., Zheng, Y.,**

Watanabe, T., Levin, D., Ohya, Y.: Identification of yeast Rho1p GTPase as a regulatory subunit of 1,3-beta-glucan synthase. Science (1996) Apr 12;272(5259):279-81.

19. **Morris, M., Villmann, M.:** Echinocandins in the management of invasive fungal infections. Am J Health Syst Pharm (2006) Oct 1;63(19):1813-20. Review.
20. **Kartsonis, N., Killar, J., Mixson, L., Hoe, C., Sable, C., Bartizal, K., Motyl, M.:** Caspofungin susceptibility testing of isolates from patients with esophageal candidiasis or invasive candidiasis: relationship of MIC to treatment outcome. Antimicrob Agents Chemother (2005) Sep;49(9):3616-23.
21. **Douglas, C., Foor, F., Marrinan, J., Morin, N., Nielsen, J., Dahl, A., Mazur, P., Baginsky, W., Li, W., el-Sherbeini, M.:** The Saccharomyces cerevisiae FKS1 (ETG1) gene encodes an integral membrane protein which is a subunit of 1,3-beta-D-glucan synthase. Proc Natl Acad Sci U S A (1994) Dec 20;91(26):12907-11.
22. **Douglas, C., Marrinan, J., Li, W., Kurtz, M.:** A Saccharomyces cerevisiae mutant with echinocandin-resistant 1,3-beta-D-glucan synthase. J Bacteriol (1994) Sep;176(18):5686-96.
23. **Douglas, C., D'Ippolito, J., Shei, G., Meinz, M., Onishi, J., Marrinan, J., Li, W., Abruzzo, G., Flattery, A., Bartizal, K., Mitchell, A., Kurtz, M.:** Identification of the FKS1 gene of Candida albicans as the essential target of 1,3-beta-D-glucan synthase inhibitors. Antimicrob Agents Chemother (1997) Nov;41(11):2471-9.
24. **Kurtz, M., Abruzzo, G., Flattery, A., Bartizal, K., Marrinan, J., Li, W., Milligan, J., Nollstadt, K., Douglas, C.:** Characterization of echinocandin-resistant mutants of Candida albicans: genetic, biochemical, and virulence studies. Infect Immun (1996) Aug;64(8):3244-51.

25. **Park, S., Kelly, R., Kahn, J., Robles, J., Hsu, M., Register, E., Li, W., Vyas, V., Fan, H., Abruzzo, G., Flattery, A., Gill, C., Chrebet, G., Parent, S., Kurtz, M., Teppler, H., Douglas, C., Perlin, D.:** Specific substitutions in the echinocandin target Fks1p account for reduced susceptibility of rare laboratory and clinical Candida sp. Isolates. Antimicrob Agents Chemother (2005) Aug;49(8):3264-73.

26. **Hitchcock, C.:** Cytochrome P-450-dependent 14 alpha-sterol demethylase of Candida albicans and its interaction with azole antifungals. Biochem Soc Trans (1991) Aug;19(3):782-7.

27. **Sanglard, D., Ischer, F., Monod, M., Bille, J.:** Cloning of Candida albicans genes conferring resistance to azole antifungal agents: characterization of CDR2, a new multidrug ABC transporter gene. Microbiology (1997) Feb;143 (Pt 2):405-16.

28. **Sanglard, D., Bille, J.:** Current understanding of the mode of action and of resistance mechanisms to conventional and emerging antifungal agents for treatment of Candida infections. Candida and Candidiasis; ASM press; Washington, D.C. (2002) In Calderone (ed.); p.349-383.

29. **Wirsching, S., Michel, S., Köhler, G., Morschhäuser, J.:** Activation of the multiple drug resistance gene MDR1 in fluconazole-resistant, clinical Candida albicans strains is caused by mutations in a trans-regulatory factor. J Bacteriol (2000) Jan;182(2):400-4.

30. **Wirsching, S., Michel, S., Morschhäuser, J.:** Targeted gene disruption in Candida albicans wild-type strains: the role of the MDR1 gene in fluconazole resistance of clinical Candida albicans isolates. Mol Microbiol (2000) May;36(4):856-65.

31. **Sanglard, D., Ischer, F., Monod, M., Bille, J.:** Susceptibilities of Candida albicans multidrug transporter mutants to various antifungal agents and other metabolic

inhibitors. Antimicrob Agents Chemother (1996) Oct;40(10):2300-5.

32. **Perea, S., López-Ribot, J., Kirkpatrick, W., McAtee, R., Santillán, R., Martínez, M., Calabrese, D., Sanglard, D., Patterson, T.:** Prevalence of molecular mechanisms of resistance to azole antifungal agents in Candida albicans strains displaying high-level fluconazole resistance isolated from human immunodeficiency virus-infected patients. Antimicrob Agents Chemother (2001) Oct;45(10):2676-84.

33. **Marichal, P., Koymans, L., Willemsens, S., Bellens, D., Verhasselt, P., Luyten, W., Borgers, M., Ramaekers, F., Odds, F., Bossche, H.:** Contribution of mutations in the cytochrome P450 14alpha-demethylase (Erg11p, Cyp51p) to azole resistance in Candida albicans. Microbiology (1999) Oct;145 (Pt 10):2701-13.

34. **Franz, R., Kelly, S., Lamb, D., Kelly, D., Ruhnke, M., Morschhäuser, J.:** Multiple molecular mechanisms contribute to a stepwise development of fluconazole resistance in clinical Candida albicans strains. Antimicrob Agents Chemother (1998) Dec;42(12):3065-72.

35. **White, T.:** The presence of an R467K amino acid substitution and loss of allelic variation correlate with an azole-resistant lanosterol 14alpha demethylase in Candida albicans. Antimicrob Agents Chemother (1997) Jul;41(7):1488-94.

36. **Nolte, F., Parkinson, T., Falconer, D., Dix, S., Williams, J., Gilmore, C., Geller, R., Wingard, J.:** Isolation and characterization of fluconazole- and amphotericin B-resistant Candida albicans from blood of two patients with leukemia. Antimicrob Agents Chemother (1997) Jan;41(1):196-9.

37. **Miyazaki, Y., Geber, A., Miyazaki, H., Falconer, D., Parkinson, T., Hitchcock, C., Grimberg, B., Nyswaner, K., Bennett, J.:** Cloning, sequencing, expression and allelic sequence diversity of ERG3 (C-5 sterol desaturase gene) in Candida albicans.

Gene (1999) Aug 5;236(1):43-51.

38. **Lopez-Ribot JL, M., Lee, L., Kirkpatrick, W., White, T., Sanglard, D., Patterson, T.:** Distinct patterns of gene expression associated with development of fluconazole resistance in serial candida albicans isolates from human immunodeficiency virus-infected patients with oropharyngeal candidiasis. Antimicrob Agents Chemother (1998) Nov;42(11):2932-7.

39. **Sanglard, D., Kuchler, K., Ischer, F., Pagani, J., Monod, M., Bille, J.:** Mechanisms of resistance to azole antifungal agents in Candida albicans isolates from AIDS patients involve specific multidrug transporters. Antimicrob Agents Chemother (1995) Nov;39(11):2378-86.

40. **Sanglard, D., Ischer, F., Koymans, L., Bille, J.:** Amino acid substitutions in the cytochrome P-450 lanosterol 14alpha-demethylase (CYP51A1) from azole-resistant Candida albicans clinical isolates contribute to resistance to azole antifungal agents. Antimicrob Agents Chemother (1998) Feb;42(2):241-53.

41. **Coste, A., Karababa, M., Ischer, F., Bille, J., Sanglard, D.:** TAC1, transcriptional activator of CDR genes, is a new transcription factor involved in the regulation of Candida albicans ABC transporters CDR1 and CDR2. Eukaryot Cell (2004) Dec;3(6):1639-52.

42. **De Micheli, M., Bille, J., Schueller, C., Sanglard, D.:** A common drug-responsive element mediates the upregulation of the Candida albicans ABC transporters CDR1 and CDR2, two genes involved in antifungal drug resistance. Mol Microbiol (2002) Mar;43(5):1197-214.

43. **White, T.:** Increased mRNA levels of ERG16, CDR, and MDR1 correlate with increases in azole resistance in Candida albicans isolates from a patient infected with

human immunodeficiency virus. Antimicrob Agents Chemother (1997) Jul;41(7):1482-7.

44. **Franz, R., Ruhnke, M., Morschhäuser, J.:** Molecular aspects of fluconazole resistance development in Candida albicans. Mycoses (1999) 42(7-8):453-8.

45. **Dunkel, N., Liu, T., Barker, K., Homayouni, R., Morschhäuser, J., Rogers, P.:** A gain-of-function mutation in the transcription factor Upc2p causes upregulation of ergosterol biosynthesis genes and increased fluconazole resistance in a clinical Candida albicans isolate. Eukaryot Cell (2008) Jul;7(7):1180-90. Epub 2008 May 16.

46. **Morschhäuser, J., Barker, K., Liu, T., BlaB-Warmuth, J., Homayouni, R., Rogers, P.:** The transcription factor Mrr1p controls expression of the MDR1 efflux pump and mediates multidrug resistance in Candida albicans. PLoS Pathog (2007) Nov;3(11):e164.

47. **Dunkel, N., Blass, J., Rogers, P., Morschhäuser, J.:** Mutations in the multi-drug resistance regulator MRR1, followed by loss of heterozygosity, are the main cause of MDR1 overexpression in fluconazole-resistant Candida albicans strains. Mol Microbiol (2008) Aug;69(4):827-40. Epub 2008 May 27.

48. **Alarco, A., Balan, I., Talibi, D., Mainville, N., Raymond, M.:** AP1-mediated multidrug resistance in Saccharomyces cerevisiae requires FLR1 encoding a transporter of the major facilitator superfamily. J Biol Chem (1997) Aug 1;272(31):19304-13.

49. **Harry, J., Oliver, B., Song, J., Silver, P., Little, J., Choiniere, J., White, T.:** Drug-induced regulation of the MDR1 promoter in Candida albicans. Antimicrob Agents Chemother (2005) Jul;49(7):2785-92.

50. **Rognon, B., Kozovska, Z., Coste, A., Pardini, G., Sanglard, D.:** Identification of

promoter elements responsible for the regulation of MDR1 from Candida albicans, a major facilitator transporter involved in azole resistance. Microbiology (2006) Dec;152(Pt 12):3701-22.

51. **Hiller, D., Stahl, S., Morschhäuser, J.:** Multiple cis-acting sequences mediate upregulation of the MDR1 efflux pump in a fluconazole-resistant clinical Candida albicans isolate. Antimicrob Agents Chemother (2006) Jul;50(7):2300-8.

52. **Silver PM, O.:** Role of Candida albicans transcription factor Upc2p in drug resistance and sterol metabolism. Eukaryot Cell (2004) Dec;3(6):1391-7.

53. **Henry, K., Nickels, J., Edlind, T.:** Upregulation of ERG genes in Candida species by azoles and other sterol biosynthesis inhibitors. Antimicrob Agents Chemother (2000) Oct;44(10):2693-700.

54. **Shore, P., Sharrocks, A.:** The MADS-box family of transcription factors. Eur J Biochem (1995) Apr 1;229(1):1-13. Review.

55. **Mai, B., Miles, S., Breeden, L.:** Characterization of the ECB binding complex responsible for the M/G(1)-specific transcription of CLN3 and SWI4. Mol Cell Biol (2002) Jan;22(2):430-41.

56. **Riggle, P., Kumamoto, C.:** Transcriptional regulation of MDR1, encoding a drug efflux determinant, in fluconazole-resistant Candida albicans strains through an Mcm1p binding site. Eukaryot Cell (2006) Dec;5(12):1957-68. Epub 2006 Oct 13.

57. **Rottmann, M., Dieter, S., Brunner, H., Rupp, S.:** A screen in Saccharomyces cerevisiae identified CaMCM1, an essential gene in Candida albicans crucial for morphogenesis. Mol Microbiol (2003) Feb;47(4):943-59.

58. **Baker, S., Grant, P.:** The SAGA continues: expanding the cellular role of a transcriptional co-activator complex. Oncogene (2007) Aug 13;26(37):5329-40.

59. **Sellam, A., Askew, C., Epp, E., Lavoie, H., Whiteway, M., Nantel, A.:** Genome-wide mapping of the coactivator Ada2p yields insight into the functional roles of SAGA/ADA complex in Candida albicans. Mol Biol Cell (2009) May;20(9):2389-400. Epub 2009 Mar 11.

60. **Theiss, S., Köhler, G., Kretschmar, M., Nichterlein, T., Hacker, J.:** New molecular methods to study gene functions in Candida infections. Mycoses (2002) Nov;45(9-10):345-50. Review.

61. **Berman, J., Sudbery, P.:** Candida Albicans: a molecular revolution built on lessons from budding yeast. Nat Rev Genet (2002) Dec;3(12):918-30. Review.

62. **Reuss, O., Vik, A., Kolter, R., Morschhäuser, J.:** The SAT1 flipper, an optimized tool for gene disruption in Candida albicans. Gene (2004) Oct 27;341:119-27.

63. **Ernst, J., Bockmühl, D.:** Gene expression and genetic techniques. Candida and Candidiasis. Calderone, R.A. (Ed). ASM Press, Washington, DC (2002) pp 267-278.

64. **Swoboda, R., Broadbent, I., Bertram, G., Budge, S., Gooday, G., Gow, N., Brown, A.:** Structure and regulation of a Candida albicans RP10 gene which encodes an immunogenic protein homologous to Saccharomyces cerevisiae ribosomal protein 10. J Bacteriol (1995) Mar;177(5):1239-46.

65. **Murad AM, L.:** CIp10, an efficient and convenient integrating vector for Candida albicans. Yeast (2000) Mar 15;16(4):325-7.

66. **Dennison, P., Ramsdale, M., Manson, C., Brown, A.:** Gene disruption in Candida albicans using a synthetic, codon-optimised Cre-loxP system. Fungal Genet Biol (2005) Sep;42(9):737-48.

67. **Fonzi, W., Irwin, M.:** Isogenic strain construction and gene mapping in Candida albicans. Genetics (1993) Jul;134(3):717-28.

68. De Backer, M., Magee, P., Pla, J.: Recent developments in molecular genetics of Candida albicans. Annu Rev Microbiol (2000) 54:463-98. Review.

69. Lay, J., Henry, L., Clifford, J., Koltin, Y., Bulawa, C., Becker, J.: Altered expression of selectable marker URA3 in gene-disrupted Candida albicans strains complicates interpretation of virulence studies. Infect Immun (1998) Nov;66(11):5301-6.

70. Bain, J., Stubberfield, C., Gow, N.: Ura-status-dependent adhesion of Candida albicans mutants. FEMS Microbiol Lett (2001) Nov 13;204(2):323-8.

71. Sundstrom, P., Cutler, J., Staab, J.: Reevaluation of the role of HWP1 in systemic candidiasis by use of Candida albicans strains with selectable marker URA3 targeted to the ENO1 locus. Infect Immun (2002) Jun;70(6):3281-3.

72. Brand, A., MacCallum, D., Brown, A., Gow, N., Odds, F.: Ectopic expression of URA3 can influence the virulence phenotypes and proteome of Candida albicans but can be overcome by targeted reintegration of URA3 at the RPS10 locus. Eukaryot Cell (2004) Aug;3(4):900-9.

73. Wilson, R., Davis, D., Mitchell, A.: Rapid hypothesis testing with Candida albicans through gene disruption with short homology regions. J Bacteriol (1999) Mar;181(6):1868-74.

74. Wilson, R., Davis, D., Enloe, B., Mitchell, A.: A recyclable Candida albicans URA3 cassette for PCR product-directed gene disruptions. Yeast (2000) Jan 15;16(1):65-70.

75. Wellington, M., Rustchenko, E.: 5-Fluoro-orotic acid induces chromosome alterations in Candida albicans. Yeast (2005) Jan 15;22(1):57-70.

76. Morschhäuser, J., Michel, S., Staib, P.: Sequential gene disruption in Candida albicans by FLP-mediated site-specific recombination. Mol Microbiol (1999)

May;32(3):547-56.

77. **Dennison, P., Ramsdale, M., Manson, C., Brown, A.:** Gene disruption in Candida albicans using a synthetic, codon-optimised Cre-loxP system. Fungal Genet Biol (2005) Sep;42(9):737-48.

78. **Leuker, C., Hahn, A., Ernst, J.:** beta-Galactosidase of Kluyveromyces lactis (Lac4p) as reporter of gene expression in Candida albicans and C. tropicalis. Mol Gen Genet (1992) Nov;235(2-3):235-41.

79. **Leuker, C., Sonneborn, A., Delbrück, S., Ernst, J.:** Sequence and promoter regulation of the PCK1 gene encoding phosphoenolpyruvate carboxykinase of the fungal pathogen Candida albicans. Gene (1997) Jun 19;192(2):235-40.

80. **Uhl, M., Johnson, A.:** Development of Streptococcus thermophilus lacZ as a reporter gene for Candida albicans. Microbiology (2001) May;147(Pt 5):1189-95.

81. **Srikantha, T., Klapach, A., Lorenz, W., Tsai, L., Laughlin, L., Gorman, J., Sol, DR:** The sea pansy Renilla reniformis luciferase serves as a sensitive bioluminescent reporter for differential gene expression in Candida albicans. J Bacteriol (1996) Jan;178(1):121-9.

82. **Cormack BP, B.:** Yeast-enhanced green fluorescent protein (yEGFP)a reporter of gene expression in Candida albicans. Microbiology (1997) Feb;143 (Pt 2):303-11.

83. **Morschhäuser, J., Michel, S., Hacker, J.:** Expression of a chromosomally integrated, single-copy GFP gene in Candida albicans, and its use as a reporter of gene regulation. Mol Gen Genet (1998) Feb;257(4):412-20.

84. **Barelle, C., Manson, C., MacCallum, D., Odds, F., Gow, N., Brown, A.:** GFP as a quantitative reporter of gene regulation in Candida albicans. Yeast (2004) Mar;21(4):333-40.

85. **Barelle, C., Priest, C., Maccallum, D., Gow, N., Odds, F., Brown, A.:** Niche-specific regulation of central metabolic pathways in a fungal pathogen. Cell Microbiol (2006) Jun;8(6):961-71.

86. **Gerami-Nejad, M., Berman, J., Gale, C.:** Cassettes for PCR-mediated construction of green, yellow, and cyan fluorescent protein fusions in Candida albicans. Yeast (2001) Jun 30;18(9):859-64.

87. **Sambrook, J., Fritsch, E., Maniatis, T.:** Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1989)

88. **Ausubel, F., Brent, R., Kingston, R., Smith, J., Struhl, K.:** Current protocols in molecular biology. New York: John Wiley&Sons (1989)

89. **Heilmann, C., Schneider, S., Barker, K., Rogers, P., Morschhäuser, J.:** An A643T mutation in the transcription factor Upc2p causes constitutive ERG11 upregulation and increased fluconazole resistance in Candida albicans. Antimicrob Agents Chemother (2009) Jan;54(1):353-9. Epub 2009 Nov 2.

90. **Gillum, A., Tsay, E., Kirsch, D.:** Isolation of the Candida albicans gene for orotidine-5'-phosphate decarboxylase by complementation of S. cerevisiae ura3 and E. coli pyrF mutations. Mol Gen Genet (1984) 198(1):179-82.

91. **Köhler, G., White, T., Agabian, N.:** Overexpression of a cloned IMP dehydrogenase gene of Candida albicans confers resistance to the specific inhibitor mycophenolic acid. J Bacteriol (1997) April; 179(7): 2331–2338.

92. **Alarco, A., Raymond, M.:** The bZip transcription factor Cap1p is involved in multidrug resistance and oxidative stress response in Candida albicans. J Bacteriol (1999) Feb;181(3):700-8.

93. **Znaidi S, B.:** Identification of the Candida albicans Cap1p regulon. Eukaryot Cell

(2009) Jun;8(6):806-20. Epub 2009 Apr 24.

94. **Lavoie, H., Sellam, A., Askew, C., Nantel, A., Whiteway, M.:** A toolbox for epitope-tagging and genome-wide location analysis in Candida albicans. BMC Genomics (2008) Dec 2;9:578.

95. **Tuch, B., Li, H., Johnson, A.:** Evolution of eukaryotic transcription circuits. Science (2008) Mar 28;319(5871):1797-9.

96. **Lucau-Danila, A., Lelandais, G., Kozovska, Z., Tanty, V., Delaveau, T., Devaux, F., Jacq, C.:** Early expression of yeast genes affected by chemical stress. Mol Cell Biol (2005) Mar;25(5):1860-8.

97. **Yan, C., Lee, L., Davis, L.:** Crm1p mediates regulated nuclear export of a yeast AP-1-like transcription factor. EMBO J (1998) Dec 15;17(24):7416-29.

98. **Zhang, X., De Micheli, M., Coleman, S., Sanglard, D., Moye-Rowley, W.:** Analysis of the oxidative stress regulation of the Candida albicans transcription factor, Cap1p. Mol Microbiol (2000) May;36(3):618-29.

99. **Thakur, J., Arthanari, H., Yang, F., Pan, S., Fan, X., Breger, J., Frueh, D., Gulshan, K., Li, D., Mylonakis, E., Struhl, K., Moye-Rowley, W., Cormack, B., Wagner, G., Näär, A.:** A nuclear receptor-like pathway regulating multidrug resistance in fungi. Nature (2008) Apr 3;452(7187):604-9.

100. **Askew, C., Sellam, A., Epp, E., Mallick, J., Hogues, H., Mullick, A., Nantel, A., Whiteway, M.:** The zinc cluster transcription factor Ahr1p directs Mcm1p regulation of Candida albicans adhesion. Mol Microbiol (2011) Feb;79(4):940-53. Epub 2010 Dec 30.

101. **Bruno, V., Kalachikov, S., Subaran, R., Nobile, C., Kyratsous, C., Mitchell, A.:** Control of the C. albicans cell wall damage response by transcriptional regulator

Cas5. PLoS Pathog (2006) Mar;2(3):e21. Epub 2006 Mar 17.

102. Monici, M.: Cell and tissue autofluorescence research and diagnostic applications. Biotechnol Annu Rev (2005) 11:227-56.

103. Hennings, L., Kaufmann, Y., Griffin, R., Siegel, E., Novak, P., Corry, P., Moros, E., Shafirstein, G.: Dead or alive? Autofluorescence distinguishes heat-fixed from viable cells. Int J Hyperthermia (2009) Aug;25(5):355-63.

104. Coste, A., Selmecki, A., Forche, A., Diogo, D., Bougnoux, M., d'Enfert, C., Berman, J., Sanglard, D.: Genotypic evolution of azole resistance mechanisms in sequential Candida albicans isolates. Eukaryot Cell (2007) ct;6(10):1889-904. Epub 2007 Aug 10.

105. Znaidi, S., Weber, S., Al-Abdin, O., Bomme, P., Saidane, S., Drouin, S., Lemieux, S., De Deken, X., Robert, F., Raymond, M.: Genomewide location analysis of Candida albicans Upc2p, a regulator of sterol metabolism and azole drug resistance. Eukaryot Cell (2008) May;7(5):836-47. Epub 2008 Apr 4.

106. Jacobson, S., Pillus, L.: The SAGA subunit Ada2 functions in transcriptional silencing. Mol Cell Biol (2009) Nov;29(22):6033-45. Epub 2009 Sep 8.

107. Shahi, P., Gulshan, K., Näär, A., Moye-Rowley, W.: Differential roles of transcriptional mediator subunits in regulation of multidrug resistance gene expression in Saccharomyces cerevisiae. Mol Biol Cell (2010) Jul 15;21(14):2469-82. Epub 2010 May 26.

108. Pitarch, A., Sánchez, M., Nombela, C., Gil, C.: Analysis of the Candida albicans proteome. I. Strategies and applications. J Chromatogr B Analyt Technol Biomed Life Sci (2003) Apr 5;787(1):101-28.

109. Pitarch, A., Sánchez, M., Nombela, C., Gil, C.: Analysis of the Candida albicans

proteome. II. Protein information technology on the Net (update 2002). J Chromatogr B Analyt Technol Biomed Life Sci Apr 5;787(1):129-48. Review.

110. **Yin, Z., Stead, D., Selway, L., Walker, J., Riba-Garcia, I., McLnerney, T., Gaskell, S., Oliver, S., Cash, P., Brown, A.:** Proteomic response to amino acid starvation in Candida albicans and Saccharomyces cerevisiae. Proteomics (2004) Aug;4(8):2425-36.

111. **Odds, F., Brown, A., Gow, N.:** Candida albicans genome sequence: a platform for genomics in the absence of genetics. Genome Biol (2004) 5(7):230. Epub 2004 Jun 11. Review.

112. **Gerami-Nejad, M., Hausauer, D., McClellan, M., Berman, J., Gale, C.:** Cassettes for the PCR-mediated construction of regulatable alleles in Candida albicans. Yeast (2004) Apr 15;21(5):429-36.

113. **Liu, T., Znaidi, S., Barker, K., Xu, L., Homayouni, R., Saidane, S., Morschhäuser, J., Nantel, A., Raymond, M., Rogers, P.:** Genome-wide expression and location analyses of the Candida albicans Tac1p regulon. Eukaryot Cell (2007) Nov;6(11):2122-38. Epub 2007 Sep 28.

114. **Hoehamer, C., Cummings, E., Hilliard, G., Morschhäuser, J., Rogers, P.:** Proteomic analysis of Mrr1p- and Tac1p-associated differential protein expression in azole-resistant clinical isolates of Candida albicans. Proteomics Clin Appl (2009) Aug;3(8):968-78. doi: 10.1002/prca.200800252.