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Rep1p negatively regulating *MDR1* efflux pump involved in drug resistance in *Candida albicans*

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ABSTRACT

Overexpression of *MDR1* efflux pump is a major mechanism contributing to drug resistance in *Candida albicans*, the most common human fungal pathogen. To elucidate the regulatory pathway of drug resistance, we have identified a negative regulator of *MDR1* and named it Regulator of Efflux Pump 1 (*REP1*). Overexpression of *REP1* in *Saccharomyces cerevisiae* increased susceptibility to fluconazole. Furthermore, null mutations on *REP1* decreased the susceptibility to antifungal drugs in *C. albicans* resulting from increased expression of *MDR1* mRNA. Hence, Rep1p is involved in drug resistance by negatively regulating *MDR1* in *C. albicans*.

1. Introduction

In the past decade, the prevalence of yeast infections has increased dramatically. Among the fungal pathogens infecting human, *Candida albicans* is the most frequently isolated one and has caused mortality in immunocompromised hosts (Hung et al., 2005; Pfaller et al., 2001; Yang et al., 2003, 2005). The increased use of antifungal agents has led to an increase in incidences of drug resistance (White et al., 1998; Yang and Lo, 2001).

The predominant target enzyme of the azole drugs is lanosterol demethylase, a product of the *ERG11* gene (Joseph-Horne and Hollomon, 1997). Numerous publications have shown that mutations on (Franz et al., 1998; Kelly et al., 1999; Lamb et al., 2000; Lee et al., 2004; Marichal et al., 1999; Perea et al., 2001; Sanglard et al., 1998; Xu et al., 2008) or overexpression of (Franz et al., 1998; Lamb et al., 1997; Perea et al., 2001; White, 1997) Erg11p contributing to drug resistance. Recently, a positive regulator of *ERG11*, Upc2p, has been reported (Dunkel et al., 2008b; Oliver et al., 2007; Znaidi et al., 2008). Treatment of azoles results in accumulating 14α-methylergosta-8,24-dien3,6 diol, the toxic product from the sterol Δ5,6-desaturase activity, encoded by the *ERG3* gene (Sanglard et al., 1998). Mutations on *ERG3* can suppress the toxicity by blocking the production of 14α-methylergosta-8, 24-dien3,6 diol and cause resistance to azole (Lupetti et al., 2002; Sanglard et al., 2003; Yan et al., 2008). Furthermore, in addition to virulence (Lo et al., 1997), Efg1p is also involved in drug resistance in *C. albicans* by negatively regulating *ERG3* (Lo et al., 2005).

Overexpression of efflux pumps is another major mechanism contributing to drug resistance in Candida clinical isolates. At least two types of efflux pumps are involved in drug resistance in *C. albicans* (Yang and Lo, 2001). One consists of *CDR1* and *CDR2*, belonging to the ATP binding cassette family and the other is *MDR1* of the major facilitator family. Recently, two transcription factors, CaNdt80p (Chen et al., 2004; Wang et al., 2006) and Tac1p (Coste et al., 2004), have been identified as positive regulators of *CDR1*. Overexpression of *CDR2* was observed in the clinical resistant isolates from matched sets of susceptible and resistant ones (Morschhäuser et al., 2007). Nevertheless, Cdr1p is the major determinant of azole resistance, while Cdr2p plays a complementary role (Holmes et al., 2008; Sanglard et al., 1997; Tsao et al., 2009).

MDR1 was originally identified by its ability to confer both benomyl and methotrexate resistance when transformed into *Saccharomyces cerevisiae* (Fling et al., 1991). The expression of *MDR1* is not activated by stresses such as pH and temperature. Instead, it is induced by drugs, such as benomyl, methotrexate, 4-nitroquinoline-N-oxide (4-NQO), o-phenanthroline and sufomethuron methyl (Gupta et al., 1998). Recently, Mrr1p has been identified as an activator of *MDR1* and null mutations on *MRR1* increased susceptibilities to several drugs, including fluconazole (Morschhäuser et al., 2007). Furthermore, gain-of-function mutations in Mrr1p followed

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by loss of heterozygosity are the main mechanism resulting in *MDR1* overexpression in clinical isolates (Dunkel et al., 2008a). Cap1p has been reported as a negative regulator of *MDR1* in *C. albicans* (Alarco and Raymond, 1999). Interestingly, Cap1p has also been described as a positive regulator of *MDR1* expression in response to hydrogen peroxide (Rognon et al., 2006). Thus, the existence of negative regulators of multidrug resistance genes in *C. albicans* has not been yet reported. In this study, we have identified an open reading frame (orf), orf19.7521, encoding negative regulator of *MDR1* in *C. albicans*. This gene is named *REP1*, standing for Regulator of Efflux Pump 1.

2. Materials and methods

2.1. Strains and media

Strains of *S. cerevisiae* were all generated from 10560-2B, *MATa, his3Δ::hisG leu2Δ::hisG ura3-52* (Chen et al., 2004). SLO2 and SLO112 were *S. cerevisiae* containing the pRS426 vector and the LOB77 plasmid, respectively. The *C. albicans* strains used in this study were SC5314, the wild-type strain (Gillum et al., 1984); BWP17, *ura3Δ::λimm434/ura3Δ::λimm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG* (Wilson et al., 1999) was kindly provided by Dr. A. Mitchell (Department of Biological Sciences, Carnegie Mellon University, USA); DSY448, *ura3Δ::λimm434/ura3Δ::λimm434 cdr1Δ::hisG/cdr1Δ::hisG-URA3-hisG* (Sanglard et al., 1996) was kindly provided by Dr. D. Sanglard (Institute of Microbiology, University of Lausanne and University Hospital Center, Switzerland); and the SCMRR1M4A, *mrr1Δ/mrr1Δ* mutant (Morschhäuser et al., 2007) was kindly provided by Dr. J. Morschhäuser (Institut für Molekulare Infektionsbiologie, Universität Würzburg, Germany). Yeast Peptone Dextrose (YPD, 1% yeast extract, 2% peptone, and 2% dextrose) and Synthetic Dextrose (SD, 0.67% yeast nitrogen base without amino acids and 2% dextrose) were prepared as described (Sherman, 2002). The compounds for adding to media were from Difco unless otherwise noted.

2.2. Molecular cloning of REP1

Primers HJL74, 5′TTTCCCGGGTGCCTGAGTTCAAGATGG3′ and HJL75, 5′TTTCCCGGGTGACTGTGGATGACGTTG3′ were used to amplify the genomic DNA containing the *REP1* gene of *C. albicans* from the SC5314 strain. The amplified DNA fragment starts 900 base-pair (bp) upstream of the predicted start codon of the *REP1* gene to 427 bp downstream of the predicted stop codon (TGA) of *REP1*. After being digested with *Xma*I, the 3.4 kb DNA fragment was introduced into the pRS426 vector to generate the LOB78 plasmid.

2.3. Construction of rep1Δ/rep1Δ and rep1Δ/rep1Δ mrr1Δ/mrr1Δ C. albicans cells

A homozygous *rep1Δ/rep1Δ* null mutant was constructed based on the gene disruption method previously described (Wilson et al., 1999). The disruption primers HJL104 5′aattccagaccaaacccataagaaa aaaaaaaaggaatcattcgagccacacccacaatggattca GGTGGTGGTTCAAAG GTGAAGAATTATT3′ and HJL105 5′ggcgtcttatgaatcaaaaataaatgtgtg tattgtatagcaagtaatgaacatacaaatgtaacagac GCATACATAAGGACCTAA TTTATCA3′ were used to amplify the *YFP-CaARG4* fragment with the *REP1* short homology regions at the two extremities. The region starting 9 bp downstream of the translation initiation site to 99 bp downstream of the stop codon of the *REP1* gene was replaced by *YFP-CaARG4* to generate YLO140. The primers HJL108 5′cgcccttcaac agtcaattcgtcaccttcaaatgctactagtccccatgcatctaaatctgaagactac GGTTT TCCCAGTCACGACGTT3′ and HJL109 5′gtgattgatgagctctgtggggaaaa tataccacgtttatcggtgggaggtaatatacgctgttgatggg TGTGGAATTGTGAG CGGATA3′ were used to amplify the *CaURA3-dpl200*-based cassette, which replaced the sequence of the second copy of *REP1* from 130 bp downstream of the translation initiation site to 138 bp upstream of the stop codon, to produce strain YLO143 (*ura3-Δ::λimm434/ura3Δ::λimm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG rep1Δ::ARG4/rep1Δ::URA3-dpl200*). The pT7tetR-*CaHIS1* digested with *Acc*I was integrated into the *ENO1* promoter of YLO143 to create strain YLO141 (*ura3Δ::λimm434/ura3Δ::λimm434 his1Δ::-hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG rep1Δ::ARG4/rep1Δ::URA3-dpl200 ENO1/eno1::ENO1-tetR-ScHAP4-3xHA-HIS*). Real-time PCR were used to confirm the *REP1* homozygous mutant. Plasmid LOB79, pGEM-*REP1-CaHIS1*, was constructed by integrating the *Sma*I DNA fragment of LOB78 containing full-length *REP1* into the pGEM-*HIS1* plasmid at *Bam*HI site blunted by Klenow enzymes. To restore the *REP1* function in the *rep1Δ/rep1Δ* mutant strain, we have digested LOB79 with *Eco*RI at 248 bp upstream of the translation initiation site of the *REP1* and transformed it into YLO143 to generate YLO142 (*ura3Δ::λimm434/ura3Δ::λimm434 his1Δ::hisG/his1Δ::hisG arg4Δ::hisG/arg4Δ::hisG rep1Δ::ARG4/rep1Δ::URA3-dpl200-REP1-HIS1*). Two-step method for knocking out *MRR1* in the *rep1Δ/rep1Δ* cells was performed as previously described (Morschhäuser et al., 2007). The inserts from plasmids pZCF36M2, a gift from Dr. J. Morschhäuser (Institut für Molekulare Infektionsbiologie, Universität Würzburg, Germany), were excised as *Apa*I–*Sac*I fragments and transformed into the *rep1Δ/rep1Δ* cells by electroporation. The transformants were selected for nourseothricin resistant colonies by plating cells on YPD containing 200 μg/ml of nourseothricin. YLO381, the *rep1Δ/rep1Δ mrr1Δ/mrr1Δ* double mutant, sensitive to nourseothricin, was selected after the *SAT1* flipper cassette (*SAT1–FLIP*) was removed by growing the transformants in YCB-BSA (23.4 g of yeast carbon base, 4 g of BSA, pH 4.0 per liter) for two days. Different constructed strains were verified by southern blot assays.

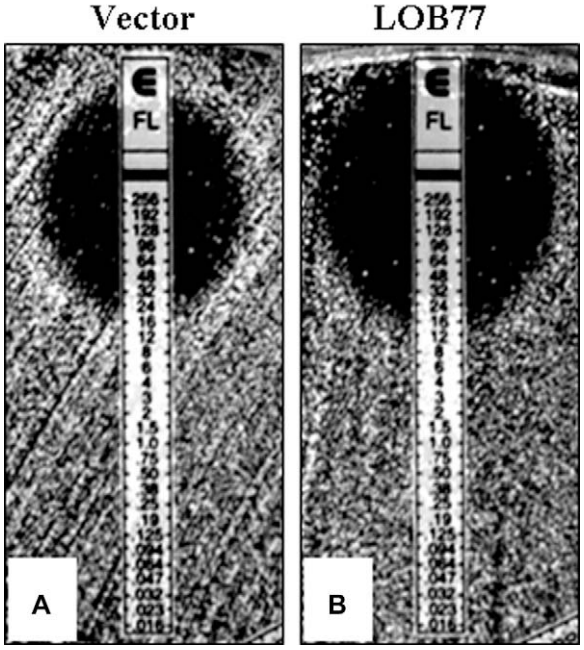

Fig. 1. The susceptibility to fluconazole of *S. cerevisiae* transformants. The susceptibilities to fluconazole of strains containing either vector alone (SLO2) or LOB77 (SLO112) were determined by the E test assay. The results were photographed after 48 h of growth on SD at 30 °C.

2.4. Antifungal susceptibility tests

The E test assay (Pfaller et al., 2003; Tapia et al., 2003) was used to determine the susceptibilities to antifungal agents for *S. cerevisiae* containing either a high-copy-number vector (pRS426) or a high-copy-number vector with *REP1* plasmid (LOB77). To prepare the inoculum suspension, homogenized colonies from an overnight SD plus adenine and histidine agar medium were transferred into 0.85% NaCl to achieve a density of 5×10^6 cells/ml. A sterile cotton swab was dipped into the inoculum suspension and set to swab the entire agar surface of SD medium evenly. The fluconazole (FL, 0.016–256 μg/ml) drug strip (AB BIODISK, Solna, Sweden) was then applied onto the plate when the excess moisture was absorbed completely.

The broth microdilution method was used to determine the susceptibility of *C. albicans* to fluconazole according to the published standard protocol (Clinical Laboratory Standards Institute, 1997). The range of fluconazole concentrations was from 0.0625 to 1 mg/l in SD broth. The growth of cells was determined by Biotrak II plate reader (Amersham Biosciences, Biochrom Ltd., Cambridge England) after incubation at 30 °C for 48 h.

The agar dilution method was also applied to determine the susceptibilities of *C. albicans* to antifungal agents. Fluconazole, miconazole, and voriconazole were prepared to the final concentrations of 25 mg/l, 1.2 mg/l, and 2 mg/l in Dimethyl Sulfoxide (DMSO), respectively. Cells grown on YPD containing equal amount of DMSO in the absence of drug was the control. Cells were diluted to an OD_{600} of 2 (approximately 2×10^7 ml^{-1}) for each strain. Approximately 0.5 μl per spot was spotted onto agar media containing different drugs with a replica device (Oxoid Inc., Canada) along with 10-fold serial dilution. Cells were photographed after incubation at 30 °C for 48 h.

2.5. Quantitative analysis of the mRNA level by real-time PCR

Cells of *C. albicans* were harvested after being grown at 35 °C for 1 h in 20 ml of SD broth containing either 100 mg/l miconazole or 0.5 mg/l 4-NQO (OD_{600} = 0.7–1.0). Real-time PCR was performed according to the instruction from the manufacturer with the LC FastStartDNA Master SYBR Green I kit in a LightCycler (Cat. No. 2239264, Roche, Germany) to determine the level of mRNA. The expressions of *ACT1* and/or *SNF3* in each strain were used as loading controls.

3. Results and discussion

3.1. Identification of a C. albicans gene altering drug susceptibility of S. cerevisiae

A high-copy-number *S. cerevisiae* plasmid LOB77 containing a 2.7 kb *C. albicans* genomic DNA fragment increased the susceptibil-

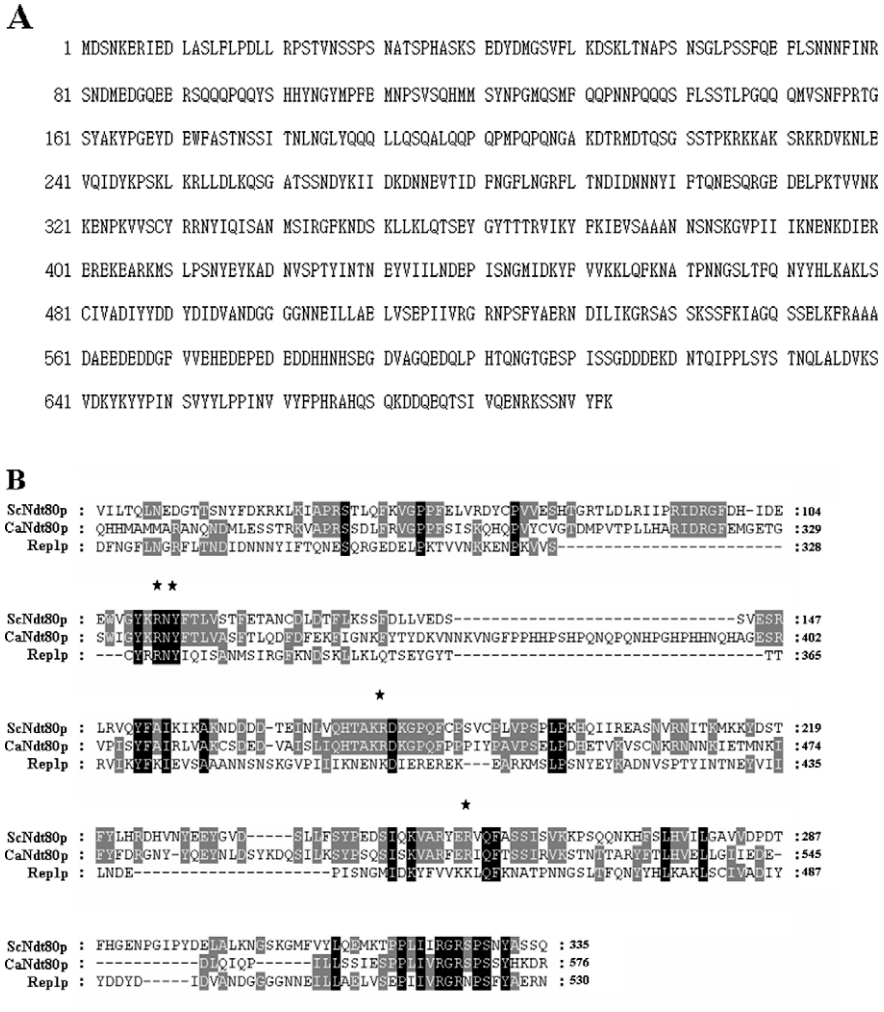

Fig. 2. Analysis of the sequence of the Rep1 protein: (A) the sequence of the Rep1 protein, (B) sequence comparison of Rep1p, CaNdt80p, and ScNdt80p. The regions shared among these three proteins are shaded in black and those between two of them are in gray. Numbers indicate the positions of residues in the proteins. Stars indicate the four conserved residues of this novel type of DNA binding domain.

ity to fluconazole of *S. cerevisiae* transformants (Fig. 1) from minimum inhibitory concentration of 24 mg/l to 12 mg/l. This 2.7 kb genomic fragment contained only one full-length orf orf19.7521, along with 185 bp upstream region. This orf is named *REP1*, standing for Regulator of Efflux Pump 1. It encodes a hypothetical protein with 693 amino acids (aa) in length (Fig. 2A) and shares homology with the DNA binding domain of CaNdt80p, a known activator of *CDR1* in *C. albicans* (Chen et al., 2004; Wang et al., 2006), and ScNdt80p, a meiosis specific transcription factor in *S. cerevisiae* (Chu et al., 1998; Chu and Herskowitz, 1998) (Fig. 2B). This type of DNA binding domain recognizes its targets through the mid-sporulation element (MSE) consensus site (gNCRCAAAA/T) (Chu and Herskowitz, 1998). Within the DNA binding domain, Rep1p (from 280th to 530th aa) shared 14% and 13% identity and 28% and 29% similarity with CaNdt80p (from 257th to 576th aa) and ScNdt80p (from 33rd to 335th aa), respectively. There are four residues reported to be important for the activity of this type of DNA binding domain (Montano et al., 2002). They are identical in both ScNdt80p and CaNdt80p and indicated by stars in Fig. 2B. Rep1p shares consensus with two of the four residues. Overexpression of *REP1* in *S. cerevisiae* increases the susceptibility to antifungal agents, suggesting that Rep1p, in *S. cerevisiae*, may negatively regulate the genes involved in drug susceptibility, especially on *ERG11*, *FLR1*, *PDR1*, and *TOP1* whose promoters have either perfect or potential MSEs.

3.2. Null mutations on REP1 decrease the susceptibility to antifungal drugs in C. albicans

To investigate the function(s) of *REP1*, we constructed homozygous *rep1Δ/rep1Δ* null mutant and *rep1Δ/rep1Δ::REP1* rescued strains (detail in materials and methods). To investigate whether Rep1p is involved in fluconazole susceptibility, we determined the susceptibility to fluconazole of different strains by the broth microdilution method. The *cdr1Δ/cdr1Δ* null mutant cells (open circles) were the most sensitive one among the four strains. The *rep1Δ/rep1Δ* mutant cells (open triangles) were more resistant to fluconazole than the wild-type (solid circles) and the *rep1Δ/rep1Δ::REP1* cells (solid triangles). This result is consistent with that in *S. cerevisiae*: overexpressing *REP1* increases drug susceptibility

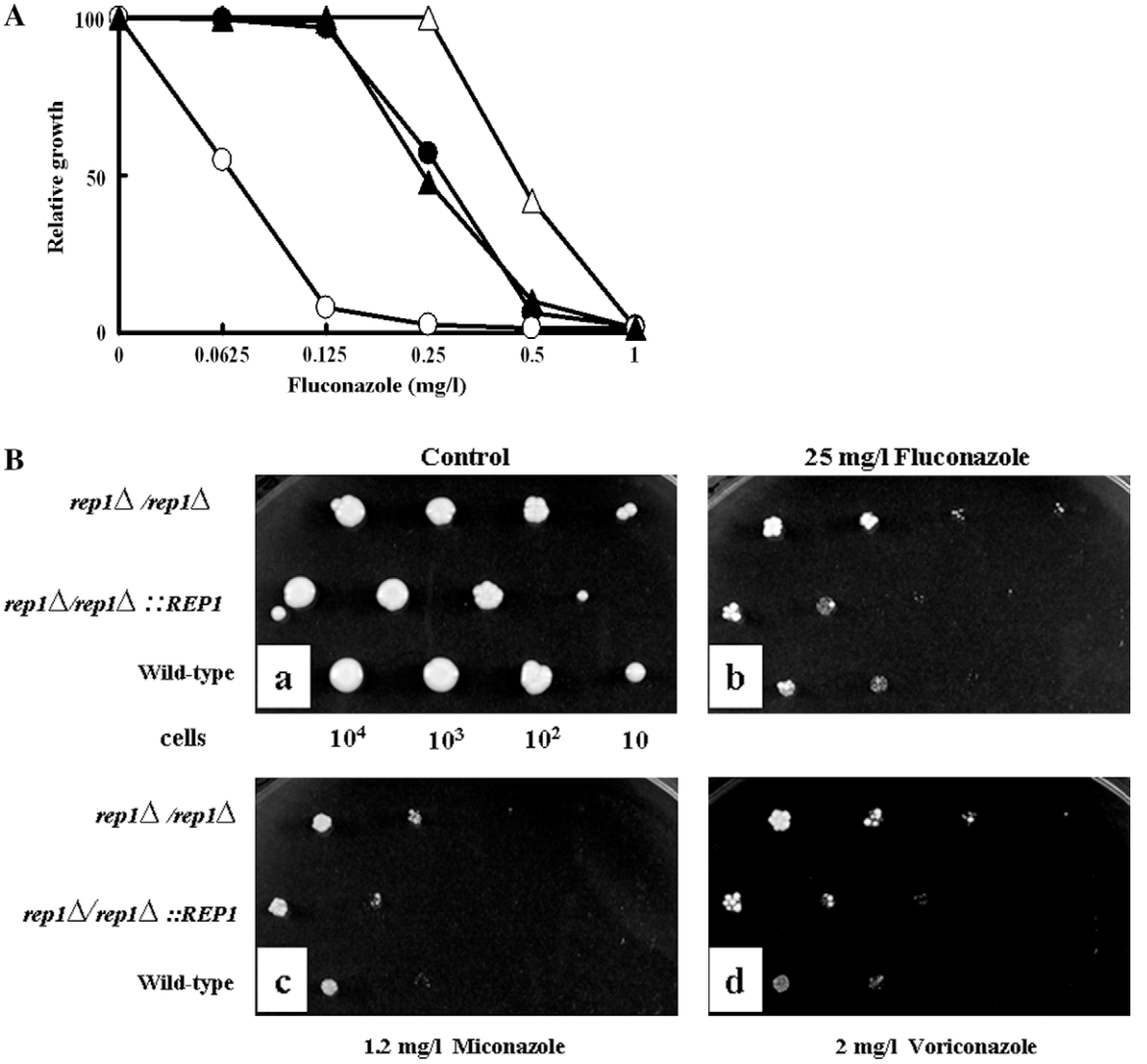

Fig. 3. The susceptibilities to antifungal drugs of different *C. albicans* strains: (A) The susceptibilities of four strains, wild-type (SC5314, solid circles), *cdr1Δ/cdr1Δ* (DSY448, open circles), *rep1Δ/rep1Δ* (YLO141, open triangles), and *rep1Δ/rep1Δ::REP1* (YLO142, solid triangles) were determined by the broth microdilution assay. The growth of each strain in the absence of fluconazole was defined as 100 and those in the presence of various concentrations of drugs were normalized accordingly, (B) the susceptibilities of three strains, wild-type (SC5314), *rep1Δ/rep1Δ* (YLO141), and *rep1Δ/rep1Δ::REP1* (YLO142) were determined by the agar dilution method. Concentrations of drugs are as indicated. The results were photographed after 48 h of growth at 30 °C.

(Fig. 1). To further determine whether Rep1p is also involved in the susceptibilities to other azole drugs in addition to fluconazole, we applied various azoles to the *rep1Δ/rep1Δ* cells by the agar dilution method. As expected, cells grew in all spots in the absence of drugs (Fig. 3B, a). The *rep1Δ/rep1Δ* mutant cells were more resistant to azole drugs, especially fluconazole (Fig. 3B, b) and voriconazole (Fig. 3B, d), than the wild-type and the *rep1Δ/rep1Δ::REP1* rescued cells.

3.3. Null mutations on REP1 increase the expression of MDR1 in C. albicans

To elucidate the mechanism of drug resistance, we investigated whether Rep1p regulate the expression of efflux pumps, such as *CDR1* and *MDR1*, in *C. albicans*. The expressions of *CDR1* and *MDR1* are induced by different compounds. For examples, the expression of *CDR1* can be induced by miconazole (Chen et al., 2004; Puri et al., 1999) and that of *MDR1* by 4-NQO (Gupta et al., 1998). To determine whether Rep1p regulates the expression of *CDR1*, we compared the levels of *CDR1* mRNA by real-time PCR between the *rep1Δ/rep1Δ* mutant and the wild-type cells in the presence of miconazole. As expected, the expression of *CDR1* was 10-fold higher in the presence of miconazole (Fig. 4A, bar 2) than that in the absence of the inducer (Fig. 4A, bar 1). Null mutations on *REP1* did not affect the expression of *CDR1* significantly in *C. albicans* in the presence of miconazole (Fig. 4A, bar 2 vs. bar 4).

To determine the effect of Rep1p on *MDR1* expression, we have compared the levels of *MDR1* mRNA induced by 4-NQO. The basal level of *MDR1* was barely detectable in all strains (Fig. 4B, lanes 1, 3, and 5 on *MDR1* Short exp.). Nevertheless, after longer exposure, a slight *MDR1* signal was observed in the *rep1Δ/rep1Δ* mutant cells in the absence of drug (Fig. 4B, lane 3 on *MDR1* Long exp.). As expected, the expression of *MDR1* in the wild-type cells was induced by 4-NQO (Fig. 4B, lane 1 vs. lane 2, on *MDR1* Long exp.). Interestingly, in the presence of 4-NQO, null mutations on *REP1* increased the expression of *MDR1* significantly (Fig. 4B, lane 2 vs. lane 4). This result was further assessed by real-time PCR. First of all, the expression of *MDR1* in the wild-type cells was induced by 4-NQO approximately 40-fold (Fig. 4C, bar 1 vs. bar 2). Mutations on *REP1* de-repressing the expression of *MDR1* in the absence (Fig. 4C, bar 1 vs. bar 3) or presence (Fig. 4C, bar 2 vs. bar 4) of 4-NQO, which is consistent with the result of northern blot assay (Fig. 4B, lane 2 vs. lane 4). These data suggest that Rep1p acts as a negative regulator on *MDR1*.

Interestingly, null mutations on *REP1* decreased susceptibilities to other azole drugs, such as voriconazole, in addition to fluconazole (Fig. 3B, d) even though *MDR1* has been reported to be the efflux pump specifically contributing to fluconazole resistance

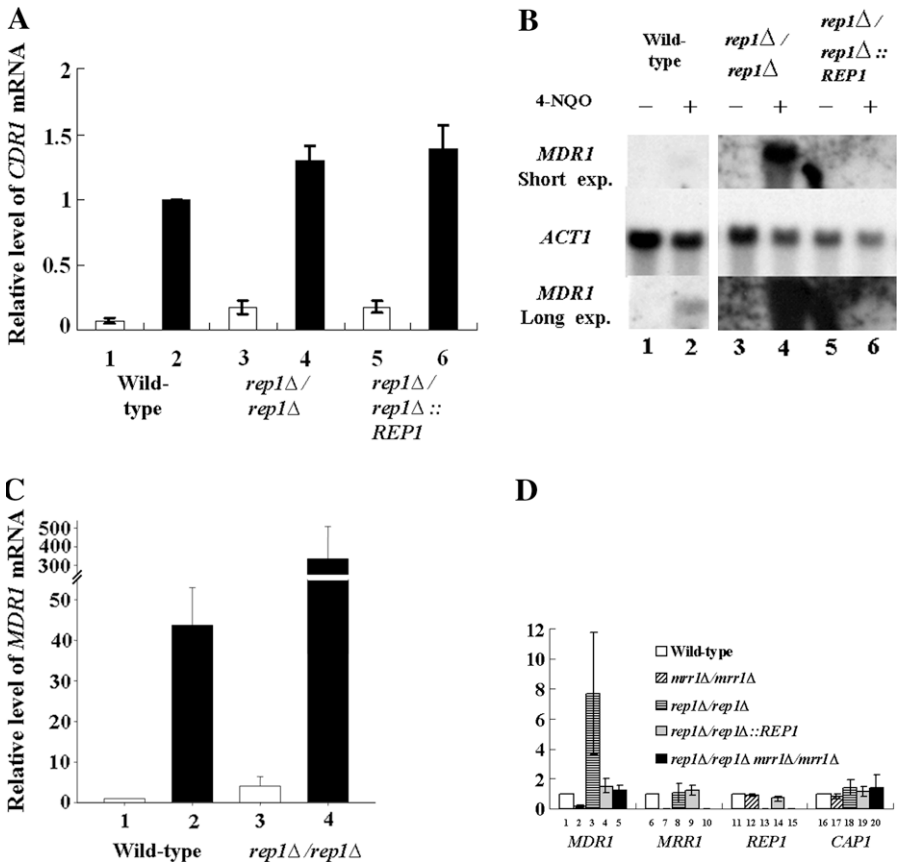

Fig. 4. The levels of *CDR1* and *MDR1* mRNA in different *Candida albicans* strains. The mRNA levels in the wild-type (SC5314), *rep1Δ/rep1Δ* mutant (YLO41), *rep1Δ/rep1Δ::REP1* (YLO142), *mrr1Δ/mrr1Δ* (SCMRR1M4A), and *rep1Δ/rep1Δ mrr1Δ/mrr1Δ* (YLO381) cells were determined: (A) Real-time PCR quantitation of the *CDR1* mRNA in the absence (bars 1, 3, and 5) or the presence (bars 2, 4, and 6) of miconazole in the wild-type (bars 1 and 2), *rep1Δ/rep1Δ* (bars 3 and 4), and *rep1Δ/rep1Δ::REP1* (bars 5 and 6) cells. The level of mRNA isolated from the wild-type cells in the presence of miconazole was defined as one and the relative level of the *CDR1* mRNA from different strains was normalized accordingly. (B) Northern blot determination of *MDR1* mRNA. The upper panel represents the northern blot of *MDR1* mRNA in a short exposure (*MDR1* Short exp.) and the bottom panel is the same northern with longer exposure (*MDR1* Long exp.). The middle panel represents *ACT1* mRNA, as a loading control. The cells were grown in the absence (lanes 1, 3, and 5) or the presence (lanes 2, 4, and 6) of 4-NQO, (C) Real-time PCR quantitation of the *MDR1* mRNA in the absence (bars 1 and 3) or presence (bars 2 and 4) of 4-NQO from the wild-type (bars 1 and 2) and *rep1Δ/rep1Δ* (bars 3 and 4) cells, and (D) Real-time PCR quantitation of the *MDR1* (bars 1–5), *MRR1* (bars 6–10), *REP1* (bars 11–15), and *CAP1* (bars 16–20) mRNAs in the presence of 4-NQO. The level of RNA of different genes isolated from the wild-type cells was defined as one and the relative level of the mRNA from the same gene isolated from different strains was normalized accordingly.

(White et al., 1998; Yang and Lo, 2001). One explanation for the results obtained here is that voriconazole, a molecule structurally related to fluconazole, can actually be a substrate of Mdr1p. This hypothesis can also explain the development of cross-resistance to fluconazole and voriconazole in some clinical isolates (Muller et al., 2000; Yang et al., 2004). Otherwise, it is also a possibility that Rep1p regulates other genes in addition to *MDR1* involved in susceptibility to antifungal drugs, especially voriconazole.

To elucidate the regulatory mechanism, we then investigated whether Rep1p and Mrr1p, a positive regulator of *MDR1*, affect the expression on each other. Our results (Fig. 4D, bar 11 vs. bar 12) as well as previous microarray data (Morschhäuser et al., 2007) suggest that the expression of *REP1* is not altered in the *mrr1Δ/mrr1Δ* mutant cells. Furthermore, our results also indicate that null mutations on *REP1* do not significantly affect the expression of *MRR1* (Fig. 4D, bar 6 vs. bar 8) or *CAP1* (Fig. 4D, bar 16 vs. bar 18).

Next, we investigated the interaction between Rep1p and Mrr1p acting upon the same target. In the presence of 4-NQO, null mutations on *MRR1* decreased the expression of *MDR1* approximately 5-fold (Fig. 4D, bar 1 vs. bar 2), consistent with the previous report (Morschhäuser et al., 2007). The activity of Mrr1p is important for activating the expression of *MDR1* in the *rep1Δ/rep1Δ* cells since the expression of *MDR1* was significantly reduced in the *rep1Δ/rep1Δ mrr1Δ/mrr1Δ* double mutant cells (7.7- vs. 1.2-fold in Fig. 4D, bar 3 vs. bar 5). Thus, in the case of the *rep1Δ/rep1Δ* null mutant cells, as the repression activity becomes invalid, the expression of *MDR1* is under the control of activator Mrr1p. There are two potential MSEs located at 335 and 773 bp upstream of the translation initiation site of *MDR1*. As a repressor, Rep1p may intrinsically occupy the promoter region of *MDR1* and inhibit Mrr1p activation by competing for the same binding region. In the presence of drug, *MDR1* promoter could be released from Rep1p. Alternatively, in the absence of inducer, Rep1p may block the activity of Mrr1p by binding directly or indirectly to this transcription factor. In the presence of drug, Rep1p then releases Mrr1p from this inhibition. Although there are some low level expressions of *MDR1* in the *rep1Δ/rep1Δ* cells, the inducer increases the expression of *MDR1* dramatically (Fig. 4B and C). This observation indicates that either an inducer is required for the full activity of Mrr1p or there is another activator whose activity is required for the expression of *MDR1* in the presence of an inducer.

Interestingly, the expression of *MDR1* in the *rep1Δ/rep1Δ mrr1Δ/mrr1Δ* double mutant cells is higher than that in the *mrr1Δ/mrr1Δ* single mutant cells (1.2 vs. 0.2, Fig. 4D, bar 5 vs. bar 2). There is at least one other protein, Ndt802p, in *C. albicans* genome containing the novel type of DNA binding domain. Hence, it is a possibility that Ndt802p and/or CaNdt80p could be recruited in the *rep1Δ/rep1Δ mrr1Δ/mrr1Δ* double mutant cells and respond by *MDR1* upregulation to drug treatment.

Due to the current technical limitations of performing molecular manipulation on *C. albicans*, we have used *S. cerevisiae* as the tool to screen genes involved in drug resistance in *C. albicans* and have successfully identified CaNdt80p as a positive regulator of *CDR1* in a previous study (Chen et al., 2004). In this study, we successfully used *S. cerevisiae* to identify Rep1p as a repressor of *MDR1* in *C. albicans*. Hence, *S. cerevisiae* is an excellent model for screening genes involved in interested pathways in *C. albicans*. But more importantly, the biological functions of those candidate genes have to be demonstrated in *C. albicans* since the divergence of function for the same gene between these two organisms has also been demonstrated (Coste et al., 2008).

Acknowledgments

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