Results and Discussion
SECTION - 1
3.1 Squalene epoxidase encoded by ERG1 affects Morphogenesis and Drug Susceptibilities of Candida albicans.

3.1.1 Introduction.
The incidence of C. albicans cells acquiring resistance to azoles has increased considerably in the recent years, which has posed serious problems in successful chemotherapy of candidiasis. Current evidence suggests that multidrug resistance (MDR) is a multifactorial phenomenon comprising multiple mechanisms: including failure in drug export by extrusion pumps such as Cdr1p, Cdr2p (ABC family) and Mdr1p (major facilitator family), alterations in theazole-target Erg11p, as well upregulation of the encoding ERG11 gene (Lopez-Ribot et al., 1999; Prasad et al., 2002; Sanglard and Odds, 2002). A combination of different resistance mechanisms has been reported to be responsible for fluconazole resistance in clinical isolates of C. albicans (Sanglard and Odds, 2002).

The important role of lipids in drug susceptibilities of Candida cells has become apparent from recent studies (Kaur and Bachhawat, 1999; Kohli et al., 2002; Mukhopadhyay et al., 2004). Azole resistant C. albicans isolates exhibit altered membrane phospholipid and sterol composition (Kohli et al., 2002; Loffler et al., 2000; Hitchcock et al., 1986; Hitchcock et al., 1987a). We and others have observed that ABC transporters Cdr1p of C. albicans and Pdr5p of Saccharomyces cerevisiae are particularly sensitive to changes in lipid composition where functions mediated by these drug extrusion pump proteins are affected (Kaur and Bachhawat, 1999; Kelly et al., 1996; Smriti et al., 1999). Taken together, it appears that the associated changes in membrane lipid composition (phospholipid/ergosterol), its order (fluidity), and asymmetry could be important determinants of drug susceptibilities of yeast cells (Kohli et al., 2002; Mukhopadhyay et al., 2004).

Among various classes of lipids in C. albicans, membrane ergosterol is an important constituent, which is also the target of common antifungals like polyenes and azoles (Klobucnikova et al., 2003; Sanglard et al., 2003; Barrett-Bee and Dixon, 2005). Ergosterol is also responsible for membrane rigidity, stability and resistance to physical stresses. In addition, ergosterol modulates membrane fluidity, permeability and the activities of membrane-
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bound enzymes (Parks and Casey, 1995). Interestingly, the action of antifungals is affected by the changes in membrane lipid composition in general and by ergosterol in particular (Smriti et al., 1999; Parks and Casey, 1995). Because of the relationship between drug resistance and membrane ergosterol composition, many genes of the ergosterol biosynthetic pathway of C. albicans such as ERG3, ERG6, ERG11, ERG24 and ERG26 etc. have been analysed (Sanglard et al., 2003; Pergakes et al., 1998; Jia et al., 2002; Aaron et al., 2001).

ERG1 (GenBank accession no. U69674) encodes for Squalene epoxidase (EC 1.14.99.7), which is a FAD-containing monooxygenase that converts squalene into 2, 3-oxidosqualene (Favre and Ryder, 1997; Leber et al., 2003). Squalene epoxidase plays a key role in the synthesis of essential sterol compounds, hence homozygous disruption of ERG1 was found to have deleterious effects in yeast cells (Tsai et al., 2004; Leber et al., 1998). In order to explore the direct involvement of ergosterol in morphogenesis and drug susceptibility of C. albicans, in this study, we disrupted one allele of ERG1 and expressed the second allele under the control of the regulatable MET3 promoter (Care et al., 1999; Krishnamurthy et al., 2004). We show that conditional suppression of ERG1 of C. albicans increases its sensitivity to drugs and leads to defects in hyphal formation. The enhanced sensitivity of the conditional erg1 mutant to various drugs is linked to increased passive diffusion and reduced efflux of drugs mediated by ABC transporter Cdr1p, which as a result of imbalance in sterol composition was poorly localised to the plasma membrane.

3.1.2 Results and Discussion.

3.1.2.1 ERG1 is an essential gene.

For the disruption of ERG1, which encodes for Squalene epoxidase, a key enzyme of ergosterol biosynthesis, C. albicans sequence data were obtained from the Stanford Genome Technology Centre (http://www.sequence.stanford.edu/group/candida). The deduced C. albicans Erg1 protein contains 496 residues, with a predicted molecular mass of 55.3 kDa (Favre and Ryder, 1997). The genomic region of ERG1 was isolated by PCR using genomic DNA of CA14 strain. A Sad-SphI fragment from plasmid pSKM54 containing a ERG1 disruption cassette was used to
transform CAI4 strain to get heterozygous strain ΔE4 (ERG1/Δerg1::hisG-URA3-hisG). Heterozygous Erg1/erg1 strains were generated without any difficulty but construction of the homozygous erg1/erg1 strain using the same hisG-URA3-hisG blaster cassette failed repeatedly. This result suggested that ERG1 is an essential gene in Candida albicans similar to the ScERG1 gene of Saccharomyces cerevisiae (Leber et al., 1998). To reconfirm this, we put the second allele under the control of regulatable promoter, which is repressed by methionine and cysteine (Care et al., 1999; Krishnamurthy et al., 2004). The cells with one ERG1 allele under the control of the regulatable promoter were designated as ΔE4.2.7 (MET3p-ERG1/Δerg1::hisG) (fig. 9). Correct integrations in heterozygous and conditional strains were checked by Southern blot, as shown in figure 10. Two independent clones were picked and analyzed simultaneously for the phenotypes described below. Pairs of isogenic mutant strains were identical in all phenotypes tested.

3.1.2.2 Growth of the conditional mutant strain is suppressed.

The conditional mutant strain ΔE4.2.7 (MET3p-ERG1/Δerg1::hisG) was grown in liquid SD medium with or without M/C. We observed that 0.5mM of M/C was enough to considerably suppress the growth of the conditional mutant ΔE4.2.7, while growth of the wild-type CAF2-1 and heterozygous strain ΔE4 remained almost unaffected under similar repressing conditions (figure 11). However, growth suppression ceased after 24 h and conditional mutants with suppressor were found to regain their growth. Of note, strain ΔE4.2.7, when streaked on SD plates containing 0.5mM M/C, continued to show suppression of growth and no regrowth was observed. The regrowth of conditional strain at 24 h could be because of the leakiness of the MET3 promoter as was reported previously (Mao et al., 2002), or partly could be due to exhaustion of M/C.

3.1.2.3 ERG1 conditional mutant strain lacks ergosterol.

To confirm the status of ergosterol levels in the erg1 mutant strain, spectrophotometer and reverse phase HPLC was employed (figure 12 and 13...
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Figure 9. Genomic configuration of the C. albicans ERG1 wild-type locus and its deleted derivatives.

Figure 10. Southern blot to confirm the disruption of the first ERG1 allele and orientation of MET3 promoter upstream of second allele. Genomic DNA from each strain was extracted and digested with HindIII, blotted and probed with the 1 kb fragment indicated by asterisks in the figure. Strains tested were: lane 1, CAF2-1 (ERG1/ERG1); lane 2, ΔE4 (ERG1/erg1Δ::hisG-URA3-hisG); lane 3, ΔE4.2 (ERG1/erg1Δ::hisG); and lanes 4 and 5, ΔE4.2.5/ΔE4.2.7 (MET3p::ERG1/erg1Δ::hisG).
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Figure 11. Growth curve of the *C. albicans* and its *erg1* mutant. Filled symbols represent growth of CAF2-1 without M/C (green filled circles), CAF2-1 with M/C (blue diamonds), ΔE4.2.7 without M/C (pink filled triangles), and ΔE4.2.7 with M/C (red filled squares).

Figure 12. Sterol profiling of the wild-type strain and *ERG1/erg1* conditional mutants by spectrophotometry. Sterol extracted were analysed by taking absorption spectra between wavelength 200-320 nm.
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Figure 13. Sterol profiling of the wild-type strain and ERG1/erg1 conditional mutants by reverse phase HPLC. Sterols were analysed by reverse phase HPLC. Elution of compounds was automatically monitored by absorption at 214, 230, 260 and 282 nm. Ergosterol and squalene were used as standards for comparison of their respective retention times with extracted sterols from Candida cells. Retention time of ergosterol is 40 min and for squalene is around 50 min (shown in parentheses).
respectively) (Bocking et al., 2000; Mukhopadhyay et al., 2004). Mutants of ergosterol biosynthetic pathway do not produce ergosterol and utilize sterol intermediates to compensate for the loss of ergosterol (figure 12 and 13 respectively) (Smriti et al., 1999; Loffler et al., 2000; Kaur and Bachhawat, 1999). The HPLC analyses of extracted sterols revealed that ergosterol levels in the heterozygous strain ΔE4 were lower compared with the wild type CAF2-1. Interestingly, the conditional disruptant strain (ΔE4.2.7), after growth in the presence of M/C, showed no detectable characteristic ergosterol peak with a retention time of 40 min, but instead showed a high peak of squalene at a retention time 50 min (fig. 13). Thus, sterol analysis revealed that ΔE4.2.7 strain, which conditionally lacks functional squalene epoxidase (Erg1p), is unable to synthesize ergosterol and instead accumulates squalene (figure 12 and 13). Both the quantitative methods thus confirmed that the conditional strain in the presence of M/C does not synthesize ergosterol and instead accumulates squalene.

3.1.2.4 ERG1 expression levels affect drug susceptibility.

In view of the fact that alterations in lipid composition affect drug susceptibilities, (Mukhopadhyay et al., 2004; Kohli et al., 2002; Kelly et al., 1996; Kelly et al., 1997) we examined if the depletion of ergosterol and concomitant accumulation of squalene affected drug sensitivities of conditional disruptant cells. Interestingly, under repressing conditions in serial dilution spot assays, the ΔE4.2.7 cells were hypersensitive to all tested drugs like fluconazole, cycloheximide, ketoconazole, amphotericin B, nystatin and terbinafine as compared to CAF2-1 and heterozygous L1E4 (figure 14). The increased sensitivity of ΔE4.2.7 conditional mutant cells to terbinafine needs special mention. Terbinafine is known to target ERG1, therefore, its conditional disruption, as in ΔE4.2.7 cells, should result in resistance rather that supersensitivity. However, our observations support earlier reports in C. glabrata, (Tsai et al., 2004) where an erg1 defect led to increased sensitivity to terbinafine. These results imply that various unidentified factors could also modulate the response of fungal cells to this allylamine (Klobucnikova et al., 2003; Sorger et al., 2004; Leber et al., 1998). Interestingly, the erg1 conditional strain ΔE4.2.7 became sensitive to polyenes- nystatin and amphotericin B, even in the complete absence of detectable ergosterol compared to the wild type CAF2-1 (fig. 14).
Figure 14. Drug susceptibility tests by spot assay. Yeast cells were spotted onto SD plates in the presence of M/C, containing the following drugs: fluconazole (FLC) (10 µg/µl), cycloheximide (CYH) (500 µg/µl), ketoconazole (KTC) (0.05 µg/µl), terbinafine (TRB) (0.1 µg/µl), nystatin (NYT) (1.25µg/µl), amphotericin B (AMB) (0.2 m µg/µl). Growth differences were recorded following incubation of the plates for 2 days at 30°C.
The conditional disruptant strain ($\Delta E4.2.7$), in the presence of M/C, showed no detectable growth (figure 14). This finding was unexpected, because polyenes specifically interact with ergosterol and hence $erg1$ mutants lacking ergosterol are expected to be resistant to these drugs. Our results support increasing evidence suggesting that membrane lipids other than ergosterol could also influence polyene sensitivity (Klobucnikova et al., 2003; Sorger et al., 2004; Leber et al., 1998).

### 3.1.2.5 $erg1$ conditional mutants show reduced passive diffusion and efflux of rhodamine 6G.

Both the entry (passive diffusion) and exit (efflux) of drugs affect the drug susceptibilities of *Candida albicans* cells (Kohli et al., 2002; Mukhopadhyay et al., 2004). We therefore examined this aspect by measuring passive diffusion and energy-dependent efflux of rhodamine 6G, which is a substrate of Cdr1p of *C. albicans* (Mukhopadhyay et al., 2004). The conditional $erg1$ knockout strain $\Delta E4.2.7$, in the presence of M/C, displayed decreased amounts of extracellular R6G in de-energized cells, suggesting increased passive diffusion of the substrate (fig. 15). To determine active drug-efflux, the fluorescent compound R6G was allowed to equilibrate in de-energized *Candida* mutant cells by passive diffusion and the energy-dependent extrusion of R6G was then initiated by the addition of glucose. As depicted in fig. 15, reduction in glucose-mediated efflux, indicated by decrease in extracellular concentration of R6G was seen in conditional mutant strain $\Delta E4.2.7$ (70% reduction in efflux of R6G at 25 minutes after the addition of glucose, as compared to the CAF2-1 cells). Thus, the increase in passive diffusion and reduced ability to efflux, both probably contribute to hypersusceptibility of the conditional $erg1$ mutant.

### 3.1.2.6 Localization of membrane bound transporter Cdr1p is impaired.

We have earlier observed that the ABC transporter Cdr1p is selectively sensitive to changes in lipid composition (Kohli et al., 2002; Mukhopadhyay et al., 2004). It is thus possible that the observed impaired efflux in the conditional mutant strain could be due to poor functionality of Cdr1p. Our immunoblot results revealed that in $\Delta E4.2.7$ cells, which were collected after
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**Figure 15. Transport of Rhodamine 6G.** For passive diffusion, de-energized cells were incubated with 10 μM rhodamine 6G (R6G) at 30 °C, and at different time points, cells were rapidly centrifuged and the extracellular concentration of R6G in the supernatant was determined spectrophotometrically at 527 nm. Active efflux was initiated by the addition of 1 mole of glucose at indicated time to de-energized cells. The filled symbols represent the extracellular amount of R6G in CAF2-1, CAF2-1 (M+C), ΔE4.2.7 (no M/C) and ΔE4.2.7 (M/C).
14 hours of M/C addition in SD media, the expression of Cdr1p in plasma membrane was decreased as compared to plasma membrane isolated from the wild-type strain (fig. 16 A). The heterozygous mutant ΔE4 showed intermediate levels of Cdr1p expression in plasma membrane, which was similar to that in plasma membrane of conditional ΔE4.2.7 grown without M/C (fig. 16, lane 2).

In another set of experiments, we expressed Cdr1p as a GFP-tagged protein by integrating the CDR1-GFP cassette to the CDR1 locus by exploiting SAT1 marker, (Reuss et al., 2004) in both wild type and ΔE4.2.7 cells. Confocal microscopic images confirmed that GFP-tagged Cdr1p was poorly localized on the membrane of the conditional mutant strain, if grown under repressing conditions. Typical rimmed fluorescence appearance of Cdr1p-GFP was seen in CAF2-1, while ΔE4.2.7, under repressing conditions showed more of intracellular fluorescence (fig. 16 B). Localization of Cdr1p-GFP produced in strain CAF2-1 was not affected with the presence or absence of M/C (fig. 16 B, panel (b)).

These results suggest that the absence of ergosterol and/or the accumulation of squalene lead to poor surface localization of Cdr1p in erg1 conditional mutants and consequently, to poor efflux of drugs.

3.1.2.7 Hyphal morphogenesis requires wild-type ERG1 expression levels.

The availability of a conditional ERG1 strain provided an opportunity to examine its role in morphogenesis of C. albicans. The ability of erg1 disruptants to form hyphae was checked on solid Lee’s and Spider media. Additionally, hyphal-inducing liquid media supplemented with 15% serum and N-acetyl glucosamine supplemented with 0.50mM of M/C were also used (Timpel et al., 1998). Results showed that as compared with CAF2-1, the heterozygous strain ΔE4 was found to have reduced formation of hyphae, while the conditional disruptant strain ΔE4.2.7 (erg1/erg1) appeared as colonies lacking filaments and thus had completely lost its ability to form hyphae under the conditions tested (figure 17). The inability of the conditional disruptant strain to form hyphae in all the tested media suggests that wild-type Erg1p-levels are critical for morphogenesis of C. albicans (figure 17).
RESULTS AND DISCUSSION

Figure 16. (A) Expression of Cdr1p. Western blot analysis of the plasma membrane fractions as from CAF2-1, CAF2-1 (M/C), ΔE4.2.7 and ΔE4.2.7 (M/C) Fractions of strain CAF2-1 (lane 1), CAF2-1 with M/C (lane 2), ΔE4.2.7 without M/C (lane 3) and ΔE4.2.7 with M/C (lane 4) were analyzed. (B) Confocal pictures of the strain ΔE4.2.7 cells producing Cdr1p-GFP were grown in SD media with and without M/C and viewed directly under confocal microscope. Left panel shows the fluorescence image and the right panel the corresponding phase contrast image. GFP fluorescence from CAF2-1/CDR1-GFP shows rimmed localization of Cdr1p on the plasma membrane both without and with methionine and cysteine, whereas those of ΔE4.2.7/CDR1-GFP without and with M/C result in a patchy appearance with more fluorescence inside the cells.
Of note, both media used for hyphal induction already contained M/C and complex sources of nitrogen, therefore, no additional repressor to the hyphal media was necessary to partially suppress \textit{ERG1} expression, while still permitting growth (Krishnamurthy \textit{et al.}, 2004).

In conclusion, any imbalance in sterol composition severely increases drug susceptibility of \textit{C. albicans} cells. The complete lack of ergosterol in the conditional \textit{erg1} mutant and/or accumulation of squalene therein affect functioning of the ABC drug extrusion pump protein Cdr1p mainly because of its poor surface localization. There have been previous reports describing the role of ergosterol in the morphogenesis of \textit{Candida}, (Lees \textit{et al.}, 1990) but in this study, we show a direct link between sterol composition and the ability of \textit{Candida} cells to form hyphae. In this context, it is important to refer to a recent report, where the presence of polarized membrane domains, rich in ergosterol and sphingolipid in \textit{C. albicans}, was demonstrated (Martin and Konopka, 2004). Considering recent reports, it appears that Cdr1p of \textit{C. albicans} may preferentially be associated with raft microdomains (Kohli \textit{et al.}, 2002; Mukhopadhyay \textit{et al.}, 2004).
Figure 17. Filamentation of erg1 mutants. Strains tested are designated as CAF2-1 (ERG1/ERG1), the heterozygous strain ΔE4 (ERG1/erg1Δ::hisG-URA3-hisG) and conditional mutant ΔE4.2.7(MET3p::ERG1/Δerg1::hisG).
SECTION- 2
3.2 Structure and Function analysis of CaMdr1p, a MFS antifungal efflux transporter protein of Candida albicans: Identification of amino acid residues critical for Drug/H+ transport.

3.2.1 Introduction-
In recent years, incidences of acquired resistance to azoles, in the fungal pathogen C. albicans, have increased considerably, which poses serious problems in successful chemotherapy of candidiasis. Current evidence suggests that Candida acquires azole resistance by employing multiple mechanisms and that includes- (a) failure of drug accumulation mediated by extrusion pump proteins such as Cdr1p and Cdr2p belonging to ATP Binding Cassette (ABC), and CaMdr1p belonging to Major Facilitator Superfamilies (MFS); (b) alterations in the azole-target protein Erg11p; as well as (c) up regulation of the ERG11 gene (Mukhopadhyay et al., 2004; Prasad et al., 2005).

The multidrug transporters of Candida belonging to ABC superfamily have been studied extensively. Typically, the predicted topology of Cdr1p and Cdr2p exhibits characteristic features of an ABC transporter; it contains two highly hydrophobic transmembrane domains (TMD) and two cytoplasm localized nucleotide-binding domains (NBD). Each TMD comprises of six transmembrane segments (TMS), which are envisaged to confer substrate specificity (Shukla et al., 2003). The nature of Cdr1p and Cdr2p substrates varies enormously as it includes structurally unrelated compounds such as azoles, lipids and steroids (Krishnamurthy et al., 1998; Smriti et al., 2002). The structure and function of MFS multidrug transporter CaMdr1p of C. albicans, is poorly understood than ABC drug exporters.

The MFS superfamily consists of membrane transport proteins from bacteria to higher eukaryotes, involved in- symport, antiport or uniport of various substrates (De et al., 2002; Ginn et al., 2000; Sharoni et al., 2005; Sigal et al., 2005; Varela et al., 1995). One major cluster of this superfamily consists of proton motif force (PMF)-dependent drug efflux proteins (Paulsen et al., 1996). Bacterial MFS drug transporters are antiporters, which have unique ‘antiporter motif’ also called as ‘motif C’ [G(X)₅G(X)₃GP(X)₂GG], necessary for the drug/H+ antiport activity (Ginn et al., 2000; Polgar et al.,
Independent of antiporter's substrate specificities, the 'antiporter motif' in the predicted TMS 5, is conserved in all the functionally related subgroups in bacteria and plants (Paulsen et al., 1996; Simmons et al., 2003; Varela et al., 1995). Although MFS drug exporters in yeast also possess 'antiporter motif' but its relevance remains to be established. This 'motif C' also has repetitive GXXXG stretch, which is thought to be important in proper helix packing and dimerization in ABCG2 transporter of humans (Polgar et al., 2004). Multiple-sequence analysis of the MFS transporters reveal that proteins within this family share greater similarity between their N-terminal halves than in their C-terminal halves and it is assumed that the later half is responsible for substrate recognition (Paulsen et al., 1996). Additionally, the MFS drug antiporter proteins possess many other conserved residues scattered throughout the length of the protein, like motifs A and B are conserved throughout the MFS, while motif 'C' is conserved in only 12- and 14- TMS subfamilies (Paulsen et al., 1996).

The CaMdr1p (Accession No. X53823) of C. albicans (formerly BENp, benomyl resistance) is a 564 amino acid protein with 12 TMS's (Ben-Yaacov et al., 1994; Fling et al., 1991; Goldway et al., 1995; Kohli et al., 2001). Based on sequence homology, CaMdr1p is a putative antiporter, with an 'antiporter motif' in the TMS 5 \([G(X)6G(X)3GP(X)2GP(X)2G]\) (Paulsen et al., 1996). Keeping in view, the relevance of the MFS multidrug transporter CaMdr1p inazole resistance in Candida, in this study, we have examined this protein in terms of its structure and function. For this, we have overexpressed CaMdr1p as GFP tagged protein in a heterologous system. To evaluate the residues of TMS 5 that potentially contributes to drug/H+ transport, we did alanine scanning of all the 21 amino acids of the TMS 5 by site directed mutagenesis. Our results highlighted the importance of the mutant variants of TMS 5, which became hypersensitive to different class of drugs, with severely impaired efflux activity. Our results for the first time supports the prediction that MFS CaMdr1p of C. albicans functions as drug/H+ antiporter, wherein amino acid residues within conserved motifs as well as outside are crucial for its functioning.

3.2.2 Results-

3.2.2.1 Overexpressed CaMdr1p-GFP is properly surface localized.
CaMdr1p has recently been overexpressed in *C. albicans* cells (Hiller *et al.*, 2006). However, in order to functionally characterize CaMdr1p, in this study, we have overexpressed it in a heterologous system. For this, we have cloned CaMdr1p-GFP ORF in the plasmid pSK-PDR5PPUS (Nakamura *et al.*, 2001), and overexpressed it by integrating at the *PDR5* locus downstream from the *PDR5* promoter in the *S. cerevisiae* strain AD1-8u· (Nakamura *et al.*, 2001). The host AD1-8u· was derived from a *Pdrl-3* mutant strain with a gain of function mutation in the transcription factor Pdr1p, resulting in constitutive hyper-induction of the *PDR5* promoter (fig. 18 A) (Nakamura *et al.*, 2001). Single copy integration at the *PDR5* promoter was confirmed by Southern hybridization. The wild type CaMdr1p-GFP was transcribed (fig. 18 B), expressed and properly targeted to the plasma membrane (PM) as evident from the Western blot analysis of the PM fraction of cells (fig. 18 C). Expression and localization was also confirmed by confocal microscopy and FACS analysis (fig. 18 D and 23).

### 3.2.2.2 Overexpressed CaMdr1p-GFP confers drug resistance.

We examined the drug sensitivities of the cells overexpressing wild type CaMdr1p-GFP (RPCaMDR1-GFP) by two independent drug susceptibility methods viz., microdilution and spot assays. The microdilution assay revealed that the host strain (AD1-8u-) as expected, was hypersensitive to all the tested drugs when compared to the growth control (without drug) (Table 11). On the contrary, cells expressing the wild type CaMdr1p-GFP (RPCaMDR1-GFP) were able to tolerate the same concentrations of the drugs. As compared to the host strain (AD1-8u·), MIC$_{80}$ values (minimum inhibitory concentration for 80% inhibition in growth) for RPCaMDR1-GFP strain was considerably higher (MIC$_{80}$ 16 μg/ml for fluconazole (FLC), 0.5 μg/ml for cycloheximide (CYH), 8 μg/ml for cerulenin (CER), 1 μg/ml for 4-nitroquinoline (4-NQO) and 128 μg/ml for methotrexate (MTX)) (fig. 18 D). The spot assays confirmed the microdilution results (fig. 18 D and Table 11). The CaMdr1p-GFP chimeric protein was able to confer drug resistance similar to the untagged protein CaMdr1p, indicating that GFP tagging does not affect its ability to function (fig. 18 D and Table 11). In the following experiments, we examined the functions of the RPCaMdr1p-GFP.
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A Mutated *Pdr1* & *Pdr3*

Hyperinduction

![Diagram showing the cloning and integration of CaMDR1 as CaMDR1-GFP at the PDR5 locus in the overexpression strain of *S. cerevisiae* AD1-8u, derived from a *Pdr1-3* mutant strain with a gain of function mutation in the transcription factor *Pdr1p*, resulting in constitutive hyper-induction of the PDR5 promoter.](image)

B Expression of the *C. albicans* CaMDR1 mRNA in *S. cerevisiae* strain AD1-8u and RPCaMDR1-GFP. Total RNA (30 µg) from parental strain, AD1-8u, and RPCaMDR1-GFP, was hybridized with a mixture of [α-32P]dATP-labeled *C. albicans* CaMDR1 probe. The lower part of panel shows loading control to indicate equal loading. (C) Immunodetection of CaMdr1p in the PM of strains- AD1-8u(control) and RPCaMDR1-GFP. The Western blot analyses were done with anti-GFP monoclonal antibody. The purity of the plasma membrane fraction was assessed by using anti-Pma1p polyclonal antibody. For Confocal pictures of the *S. cerevisiae* cells expressing GFP-tagged wild type CaMDR1, cells were viewed directly on a glass slide with a 100X oil immersion objective on a Bio-Rad confocal microscope. (D) Spot test showing CaMDR1 (untagged; without GFP) and CaMDR1-GFP, mediated drug resistance for various drugs. Cells were spotted on YEPD plates in the absence of drug (control) and presence of the following drugs, FLC (0.17 µg/ml), CYH (0.2 µg/ml), CER (3 µg/ml), 4-NQO (0.2 µg/ml) and MTX (65 µg/ml).
3.2.2.3 CaMdr1p effluxes methotrexate and fluconazole.

For checking the ability of drug extrusion by the overexpressed CaMdr1p, we measured the net intracellular accumulated levels of two drug substrates, 3H-MTX and 3H-FLC in RPCaMDR1-GFP cells (figs. 19 A and B). An increased or decreased level of accumulation of the drug, at a given point in time, implies its reduced or enhanced efflux, respectively. It is apparent from figs. 19 A and B that as compared to the host (AD1-8u-) cells, the accumulation of 3H-MTX and 3H-FLC, was considerably reduced (more efflux) in cells expressing CaMdr1p-GFP tagged protein.

3.2.2.4 Efflux is sensitive to different inhibitors.

We observed that the accumulation of 3H-MTX and 3H-FLC was sensitive to different energy inhibitors such as sodium azide, sodium orthovanadate, Carbonyl cyanide m-chloro phenyl hydrazone [CCCP] and sodium arsenate (figs. 19 A and B). The accumulation of 3H-MTX and 3H-FLC was increased in presence of these inhibitors, implying reduction in efflux. The sensitivity of 3H-MTX and 3H-FLC accumulation, particularly to proton conductor CCCP suggests that CaMdr1p function is sensitive to pH and may functions as H+-antiporter. Unlike the efflux of drugs mediated by ABC transporter Cdr1p (Jha et al., 2003), the efflux mediated by MFS CaMdr1p was insensitive to -SH blocker, N-ethylmaleimide (NEM).

3.2.2.5 Efflux of MTX and FLC is pH dependent.

To confirm that CaMdr1p is a putative drug/H+ transporter, we monitored the efflux of 3H-MTX and 3H-FLC by exposing cells to buffers with different pH values (fig. 20 A and B respectively). In a typical experiment, cells expressing wild-type CaMdr1p-GFP were treated with proton conductor CCCP (100μM) and were allowed to accumulate 3H-MTX and 3H-FLC up to 30 min. These cells were then rapidly pelleted and resuspended in buffers of different pH values to initiate the efflux of accumulated drug against pH gradient. The efflux of the drug was measured in rapid succession as detailed in Materials and Methods. Our results revealed that MTX efflux is very sensitive to pH (fig. 20 A), for example; RPCaMDR1-GFP cells exposed to acidic pH (3.5-5.5) showed maximum efflux (lower accumulation) of the drug.
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Figure 19. Functional characterization of the RPCaMdr1p-GFP and effect of different inhibitors on accumulation of radiolabeled drugs. (A) 3H-MTX accumulation and (B) 3H-FLC accumulation in RPCaMDR1-GFP cells and host strain AD1-8u- (control). Effect of different inhibitors on accumulation of 3H-MTX (panel A) and 3H-FLC (panel B), in the RPCaMdr1p-GFP strain was monitored as described in Materials and Methods. Different inhibitors were added to various cells ten minutes prior to the commencement of transport. Different inhibitors used included sodium azide (10mM), sodium orthovanadate (1mM), CCCP (Carbonyl cyanide m-chloro phenyl hydrazone) (100μM), sodium arsenate (1mM) and NEM (N-ethymaleimide) (1mM). AD1-8u- is shown as the control. The values plotted are 10 min after commencement of transport. The results are the mean ± SD of three independent experiments.

Figure 20. Effect of pH on 3H-MTX and 3H-FLC efflux. The efflux of 3H-MTX (panel A) and 3H-FLC (panel B) was monitored by exposing cells to different pH buffers. In a typical experiment, RPCaMdr1p-GFP cells were treated with CCCP (100μM) for 10 min and were then allowed to accumulate 3H-MTX for 30 min. These cells were then rapidly pelleted and resuspended in buffers of different pH values. The accumulation of 3H-MTX and 3H-FLC was measured at rapid succession as detailed in Materials and Methods. The values plotted are 10 min after commencement of efflux. The results are the mean ± SD of three independent experiments.
No such pH effect was observed with the host strain AD1-8u· that did not show any significant efflux of the drugs (fig. 20 A and B). The efflux of 3H-FLC mediated by CaMdr1p was also pH sensitive, which was similar to 3H-MTX and maximum at acidic pH (3.5-5.5) (fig. 20 B). It should be pointed out that ABC drug transporter Cdr1p of C. albicans, when expressed in S. cerevisiae strain AD1-8u· could also extrude FLC but at a pH optimum of 7.5.

3.2.2.6 CaMdr1p harbors conserved drug/H⁺ antiporter domain.  
The alignment of TMS 5 sequence of CaMdr1p with other MFS transporters revealed extensive conservation of the amino acids (fig. 21 C). Notably, all the five glycines of CaMdr1p are conserved in other MFS transporters (fig. 21 C). Considering the fact that irrespective of the differences in substrate specificities among MFS antiporters, a great level of conservation of amino acid exists in ‘motif C’, we analysed the functional significance of this motif in drug transport by alanine scanning of entire TMS 5 of CaMdr1p. TMS 5 sequence was deduced by taking the consensus sequence from the various bioinformatic programs including HMMTOP, SOSUI, TMHMM and TopPred programmes (Rost and Sander, 1993; Rost and Sander, 1994; Saini et al., 2005; Tusnady and Simon, 2001; Tusnady and Simon, 1998).

For alanine scanning of TMS 5, we employed site directed mutagenesis approach employing mutagenic oligonucleotides, where all the amino acids of the TMS 5 were substituted to neutral alanine. To avoid the introduction of new side chains, the three existing alanine in TMS 5 (A246, A247 and A252) were replaced by glycine, while 5 glycines (G244, G251, G255, G259 and G263) were substituted with alanine as well as with long side chain amino acid leucine. All the 26 mutant variants of CaMdr1p-GFP were stably overexpressed as GFP tagged variants in a heterologous system as described in Materials and Methods. Single copy integration was confirmed with Southern blot analysis.

3.2.2.7 Conserved TMS 5 residues substitution results in hypersensitive cells.  
Confirmed positive mutants were screened for their sensitivity to different substrates by two independent methods: microdilution assay, as well as spot assay.
RESULTS AND DISCUSSION

Figure 21 (A). Predicted topology of the CaMDR1 with twelve transmembrane segments. The TMS 5 is encircled, for showing location of 'antiporter motif' in the protein. (B) The putative TMS 5 is magnified to show the amino acid residues of TMS 5. (C) Alignment of the protein sequences of the C. albicans antiporter CaMDR1 TMS 5 with the other fungal and bacterial drug antiporters, showing the presence of the unique and conserved 'antiporter motif'. The amino-acid sequences of TMS 5 of CaMDR1p between positions 243 and 263 (boxed). The sequence of the 'antiporter motif' or 'motif C' is written for comparison, where 'X' can be any amino acid. Residues conserved in all the MFS transporters and are part of the motif are highlighted in grey, whereas residues conserved only in fungal MFS and were found critical for the activity are highlighted in black.
Based on the drug sensitivity results of these two methods and location of the residues inside or outside the conserved motif, mutant variants could be placed into four categories. The first category had four residues G244, G251, G255, and G259, which are also part of the ‘motif C’ and their substitution with alanine, (G244A, G251A, G255A, and G259A), led to variable enhanced sensitivity to tested drugs (fig. 22 A and table 12). For example, variants G244A, G251A, and G255A were only partly sensitive to FLC, CER and MTX, but were in comparison more sensitive towards CYH and 4-NQO. The variant G259A, however, showed only slight change in sensitivity towards all the tested drugs (fig. 22). Interestingly, all four glycines of first category when substituted with leucine (G244L, G251L, G255L and G259L) showed more drastic results, as can be seen by complete abrogation of growth in the presence of the same concentration of the drugs (fig. 22 B and table 12). The residues, which are conserved, and part of motif such as P260 and those which were not part of motif such as L245, W248, P256 and F262 were included in second category. The substitution of these residues with alanine only, resulted in a more dramatic increase in drug susceptibility and showed almost no growth in the presence of tested drugs (fig. 22 C). The third category which has one residue, G263 and is a part of the conserved motif, showed no major change in drug susceptibility levels when replaced with alanine (G263A) or leucine (G263L) (fig. 22 D). All the eleven residues of TMS 5 belonging to the fourth category, when replaced with alanine, (V243A, A246G, A247G, S249A, L250A, A252G, V253A, C254A, S257A, F258A, and F261A), did not affect the drug resistance profile (table 11 and 12). Considering that the residues of fourth category were non-conserved and also not part of antiporter motif, these residues were not replaced with other amino acids such as leucine. Thus, out of the 21 residues of TMS 5, substitution of only nine residues, namely G244, L245, W248, G251, G255, P256 G259, P260, and F262, affected drug susceptibilities to varying degrees (fig. 22 A, B, C and table 11 and 12). The drug sensitivities revealed by spot assays matched well with the microdilution results (table 11).

In order to exclude the possibility that the observed hyper susceptibility of the mutant variants was not due to poor expression or impaired surface localization, we compared the protein localization of RPCaMdr1p-GFP and mutant variants by Western blot analysis, FACS and by confocal microscopy. Western blot analysis with anti-GFP antibody confirmed similar expression...
levels of the mutants and the wild type (fig. 23 A). FACS and confocal images also confirmed that there was no difference in cell surface localization of CaMdr1-GFP between the cells expressing wild type and mutant variants (fig. 23 B).

3.2.2.8 Mutant variants display different levels of accumulation (efflux) of radiolabeled drugs.
All the mutants were further analyzed for their functionality by measuring the intracellular accumulation of $^3$H-MTX and $^3$H-FLC. First category of mutant variants, when substituted with alanine, G244A G251A, G255A and G259A, showed increased accumulation of $^3$H-MTX and $^3$H-FLC (impaired efflux) (fig. 24 A, B and table 12). Similar to drug sensitivity results, the replacement of glycines of first category, when substituted with leucine, (G244L, G251L, G255L and G259L), resulted in further increase in accumulation of $^3$H-MTX and $^3$H-FLC (fig. 24 A, B and table 12). The mutant variants of second category including, L245A, W248A, P256A, P260A and F262A, also showed increase in their ability to accumulate $^3$H-MTX and $^3$H-FLC, thus implying decreased efflux of drugs (fig. 24 A, B and table 12). The third category mutant variant G263, whether substituted with alanine or leucine, had no major impact on drug resistance profile, displayed unaltered accumulation of $^3$H-MTX and $^3$H-FLC, comparable to cells expressing wild type protein (fig. 24 A, B and table 12).

The mutant variants of fourth category (V243A, A246G, A247G, S249A, L250A, A252G, V253A, C254A, S257A, F258A, and F261A), that showed no change in the drug resistance profile, did also not display any change in the level of accumulation of the drugs. Of note, the reduced ability to efflux drug matched well with the drug susceptibility data for all the categories (Figs. 22 and 24).

3.2.2.9 TMS 5 and substrate binding and transport.
The mutant variants which displayed sensitivity in the drug susceptibility assays and were defective in transport were also checked for substrate binding. To examine the effect of TMS 5 mutations on the drug binding sites or ion coupling and transport, $^3$H-MTX accumulation was competed with 5-fold molar excess of different drugs.
**RESULTS AND DISCUSSION**

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Figure 22. Drug resistance profile of wild type and mutant *CaMDR1* strains determined by the spot assay. Cells were spotted on to YEPD plates in the absence (control) and presence of the following drugs used- FLC (0.17 mg/ml), CYH (0.2 µg/ml), 4-NQO (0.2 µg/ml), MTX (65 µg/ml). Growth differences were recorded following incubation of the plates for 48 h at 30°C. Growth was not affected by the presence of the solvents used for the drugs (data not shown). (A) and (B) shows the mutant variant of first category of mutant variants substituted with alanine and leucine respectively. (C) Second category of mutant variants substituted with alanine only. (D) Third category of mutant variants substituted with both alanine and leucine.
## RESULTS AND DISCUSSION

<table>
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<tr>
<th>Strain</th>
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**Table 11.** Minimum inhibitory concentration (MIC<sub>80</sub> µg/ml) of the host strain AD1-8u<sup>+</sup>, RPCaMDR1 (cells expressing the untagged wild-type CaMdr1p (without GFP)), RPCaMDR1-GFP strains, and the various mutant variants for the different drugs tested.
Figure 23. Comparison of expression of RPCaMDR1p-GFP and different mutant variants localization. (A) Western blots analyses of the PM fractions of mutant variants, with anti-GFP antibody, to confirm their proper targeting. (B) Confocal images and FACS analyses of the mutants of first, second and third category, to check their expression, proper folding and localization and their comparison with AD1-8u (negative control) and RPCaMDR1-GFP (positive control).
The accumulation of $^3$H-MTX could only be competed out by molar excess of MTX and 4-NQO, while other tested drugs could not affect the accumulation in wild-type RPCaMDR1-GFP cells (fig. 25 A). It means that MTX and 4-NQO share common substrate binding site(s). R6G is not a substrate of CaMdr1p-GFP and expectedly was unable to affect the accumulation of $^3$H-MTX (fig. 25 A). Similar to native CaMdr1p-GFP, the accumulation of $^3$H-MTX by the first category of mutants: G244A, G251A, G255A and G259A, could be competed out with MTX and 4-NQO, suggesting that these residues are not directly involved in drug transport (fig. 25 B). However, when glycines of first category were replaced with leucine (G244L, G251L, G255L and G259L), both MTX and 4-NQO could not compete with the accumulation of $^3$H-MTX (fig. 25 C). Of note, as a result of substitution of conserved glycines with leucine, the accumulation of $^3$H-MTX was severely reduced in these variants and hence a clear distinction between the inability of MTX and 4-NQO to compete with $^3$H-MTX and the mutant variants inability to participate in drug/H$^+$ transport could not be made. However, based on the fact that the efflux properties do not change after substitution of conserved glycines with alanine, one could cautiously state these residues may not directly be involved in MTX binding and transport.

As shown in fig. 25 D, the accumulation of $^3$H-MTX could not be competed out by any drug in the five mutant variants of second category namely- L245A, W248A, P256A, P260A and F262A. The mutant variant of the third category, G263 behaved as wild type protein whether substituted with alanine (G263A) or with leucine (G263L) (fig. 25 E).

### 3.1.3 Discussion.

CaMdr1p is one of the major efflux pumps of *C. albicans* belonging to the MFS superfamily, which is involved in clinically encounteredazole resistance. CaMdr1p is functionally identical to ABC drug transporters such as Cdr1p and Cdr2p; however, they differ with regard to the mechanisms of drug extrusion. While the MFS CaMdr1p is a putative antiporter that exchanges H$^+$ with antifungal compounds, the ABC transporters such as Cdr1p and Cdr2p accomplish drug efflux by coupling it directly to the hydrolysis of ATP. Our studies confirmed that an overexpression of CaMDR1-GFP in a heterologous system confers resistance to a variety of tested drugs because of its ability to expel these substrates.
Figure 24. $^3$H-MTX and $^3$H-FLC accumulation in the different mutant variants. (A) $^3$H-MTX and (B) $^3$H-FLC accumulation in the first, second and third category of mutant variants. The first and third category of five glycines of 'antiporter motif' substituted with leucine are also shown as black bars for better comparison of glycines substituted with alanine. Controls AD1-8u and RPCaMDR1-GFP are also included for comparison. The results are the mean ± SD of three independent experiments.
Results and Discussion

Figure 25. Competition of various drugs with ³H-MTX for common substrate binding sites. For competition between ³H-MTX and various drugs, ³H-MTX was used at a final concentration of 25µM and; a fivefold concentration of each drug (125µM), MTX, FLC, CYH, 4-NQO and R6G, was used for competition studies. (A) Comparison of the control strains AD1-8u (negative) with RPCaMdrlp-GFP (positive). MTX and 4-NQO compete for the ³H-MTX in RPCaMdrlp-GFP. (B) and (C) shows the first category of the mutant variants, either substituted with alanine or leucine respectively. (D) Second category of the mutant variants, showing drastic increase in accumulation, implying reduced efflux. (E) Third category of mutant which remained unaffected whether substituted with alanine or leucine. The results are the mean of three independent experiments and are represented in pmole/mg dry weight. The values are derived from the accumulation.
Figure 26. Helical wheel projection of the primary amino acid sequence was constructed, using 3.6 amino acids per turn of the helix by the EMBOSS PEPWHEEL program. Mutations that affected the functionality of the CaMdr1p are numbered and highlighted in black.
### Table 12

Summarized representations of the mutant variants. Drug susceptibility column shows the degree of growth on all the tested drugs, where hyper resistant cells are depicted with ++++, moderately resistant with ++, slightly resistant with +, and slight growth is shown by + only. Hypersensitive variants, with no growth at all are shown with - only. Drug accumulation, column shows the relative % accumulation of the two drugs: *H-FLC and *H-MTX. The results are shown as relative % with respect to drug accumulation of host AD1-8u taken as 100%. Position, the last lane depicts the position of the variant if that is inside or outside the motif 'C' or 'antiporter motif'.

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Table 12: Summarized representations of the mutant variants. Drug susceptibility column shows the degree of growth on all the tested drugs, where hyper resistant cells are depicted with ++++, moderately resistant with ++, slightly resistant with +, and slight growth is shown by + only. Hypersensitive variants, with no growth at all are shown with - only. Drug accumulation, column shows the relative % accumulation of the two drugs: *H-FLC and *H-MTX. The results are shown as relative % with respect to drug accumulation of host AD1-8u taken as 100%. Position, the last lane depicts the position of the variant if that is inside or outside the motif 'C' or 'antiporter motif'.

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Results and Discussion
The efflux of drug substrates such as MTX and FLC is sensitive to pH, which could be blocked by proton dissipater like CCCP, confirms that electrochemical gradient of protons is utilized to extrude the drugs.

The characteristic and well conserved 'antiporter motif' in TMS 5 was found to be essential for the functionality of CaMdr1p, which was evident from the fact that not even a single substitution of conserved residue within the motif is functionally compensated. Interestingly, all the amino acid residues of CaMdr1p whose substitution resulted in enhanced drug susceptibility and abrogated efflux are clustered in a helical wheel projection of TMS 5 (fig. 26). The clustering of mutation-sensitive residues on the same face of helix further confirms that these residues are important for the structural and functional role of the transporter protein (fig. 26). Importantly, we observed that the residues such as W248 and F262 of CaMdr1p, typical of fungal MFS transporters, were critical for enzymatic activity. Previous studies have shown that the aromatic amino acids such as Phe, Tyr and Trp are involved in cationic-\(\Lambda\) interaction, where aromatic side chains bind to the cations in the aqueous media to pull it out from the water molecule into a hydrophobic environment, to possibly help in the generation of proton gradient (Dougherty, 1996). This observation is important because generally negatively charged residues are involved in proton-coupled translocation, but recent reports of MdfA, transporter of *E. coli* have shown that different MFS transporters might utilize different proton recognition strategies (Sigal *et al.*, 2006). It is thus probable that W248 and F262 of CaMdr1p and of other fungal MFS transporters may also lie in the glycine rich pocket and help in utilizing proton gradient. The significance of W248 and F262 in CaMdr1p requires further evaluation.

Interestingly, on one hand both FLC and MTX could be extruded by the transporter but were unable to compete for each other, thus implying that these two drug substrates share different binding sites. This selectivity between the two substrates was retained by all the mutant variants, which were capable of extruding MTX. Our competition results further suggest that at least conserved glycines of the core antiporter motif do not appear to affect drug binding to CaMdr1p. Thus observed impairment in efflux by conserved glycines substitution with alanine and the fact that similar to wild type protein, MTX and 4-NQO could compete with \(^{3}\text{H}\)-MTX efflux mediated by these variants, suggest that these conserved motif residue are do not
directly participate in drug binding. The impairment in drug efflux is probably because of the mutant variants inability to participate in drug/H\(^+\) transport. This issue can however, be resolved only by direct drug binding studies by employing radio and photoaffinity labeled drug substrates.

Taken together, our study supports the prediction that MFS CaMdr1p of *C. albicans* functions as drug/H\(^+\) antiporter. The high degree of conservation in TMS 5, along with mutational data strongly confirms that the motif \([G(X)_5G(X)G(X)_3G(X)G]\) is essential for drug/H\(^+\) transport. The placements of critical residues which are typical to fungal transporter do suggest structural and functional differences among MFS drug transporters.
SECTION 3
3.3 Cdr1p and CaMdr1p multidrug transporters of Candida albicans display different lipid specificities: both ergosterol and sphingolipids are required for targeting of Cdr1p to membrane rafts.

3.3.1 INTRODUCTION

In both pathogenic as well as non-pathogenic yeasts, several mechanisms can contribute to the development of multidrug resistance (MDR). Point mutations or overexpression of the drug target, decrease in the import of drugs and an enhanced efflux of drugs are some of the strategies employed by drug resistant yeast to overcome the lethal effects of the drugs (Kaur and Bachhawat, 1999; Mukhopadhyay et al., 2004). However, extrusion of noxious compounds from the cell, mediated by efflux pumps is one of the most frequently used strategies for the development of drug resistance in yeasts, which holds true for several prokaryotic and eukaryotic organisms (Pumbwe et al., 2006; Schinkel and Borst, 1991; Zgurskaya, 2002).

The two major efflux pump proteins involved in MDR belong to ATP-binding cassette (ABC) and Major Facilitator (MFS) super families. Genome analysis of Saccharomyces cerevisiae and of the pathogenic yeast Candida albicans reveal the existence of 30 and 28 putative ABC transporters, respectively, of which only a few function as drug transporters (Decottignies and Goffeau, 1997; Gaur et al., 2005). Similar to ABC protein superfamily, very few members of the MFS family are drug exporters. For example, out of 62 putative transporters in S. cerevisiae, (Braun et al., 2005) only FLR1 (fluconazole resistance) has been shown to confer resistance to drugs (Broco et al., 1999). In pathogenic C. albicans, out of 71 MFS proteins, only CaMDRI is known to extrude drugs, where its overexpression has been linked to azole resistance (Braun et al., 2005).

The efflux pumps Cdr1p and CaMdr1p are both localized on the plasma membrane (PM). Interestingly, Cdr1p is sensitive to changes in the membrane environment and also plays a role in maintaining membrane asymmetry (Mukhopadhyay et al., 2004; Prasad et al., 2005; Smriti et al., 1999). Human Pgp/MDR1, a homologue of the yeast ABC proteins, is predominantly localized in microdomains within the PM. The presence of the microdomains, also called 'Lipid Rafts' in various organisms plays an
important role in cell signaling, protein sorting and virulence (Dieterich et al., 2002; Martin and Konopka, 2004; Moffett et al., 2000; Mongrand et al., 2004; Pike et al., 2005; Wu et al., 2004). Lipid rafts are highly enriched in sphingolipid and ergosterol or cholesterol and are characterized by their insolubility in detergent. Depletion of cholesterol from these domains impairs Pgp-mediated drug transport in a substrate and cell-type specific manner (Demeule et al., 2000). It is also observed that human Pgp/MDR1 contributes in stabilizing the cholesterol-rich microdomains by mediating cholesterol redistribution within the cell membrane (Garrigues et al., 2002). The acquisition of the MDR phenotype in certain mammalian cell lines is not only due to overexpression of the drug efflux pumps but is also accompanied by an upregulation of genes required for normal lipid metabolism that constitute membrane rafts (Lavie and Liscovitch, 2001). In yeasts also, we have previously shown that efflux pump proteins particularly of the ABC super family, are influenced by imbalances in membrane lipid composition (Mukhopadhyay et al., 2004; Prasad et al., 2005; Smriti et al., 1999). The presence of DRMs within the yeast PM has recently been demonstrated (Martin and Konopka, 2004; Wachtler et al., 2003). In order to critically evaluate the role of the DRM lipid constituents in the localization of the efflux pumps, in this study, we have overexpressed GFP tagged Cdr1p and CaMdr1p in different lipid mutant backgrounds of S. cerevisiae. The mutants used were either defective in ergosterol (∆erg24 or ∆erg6 or ∆erg4) or in the sphingolipid (∆sur4 or ∆fen1 or ∆ipt1) biosynthesis pathway.

Here we report that the observed abrogated functioning of Cdr1p in the various mutant backgrounds is mainly due to its missorting, resulting in its poor localization in the PM. CaMdr1p interestingly remains unaffected by the defects in the mutant strains. Our study clearly establishes that out of the two different classes of multidrug transporters, only one (Cdr1p) is exclusively directed to the membrane rafts for proper localization and functioning. Coupled together, it appears that membrane sphingolipid and sterols as individual components as well as their mutual interactions are critical in sorting and functioning of the ABC efflux pump protein of yeasts.

3.3.2 Results

3.3.2.1 Overexpression of GFP tagged Cdr1p and CaMdr1p.
In this study, we have exploited the well established and extensively used expression system of *S. cerevisiae* for the overexpression of Cdr1p and CaMdr1p (Shukla *et al.*, 2006; Shukla *et al.*, 2003). The strategy of GFP-tagging and cloning of Cdr1p and CaMdr1p in the plasmid pSK-PDR5PPUS is shown in figure 27 A. It is clear from the figure that both the strains expressing Cdr1p (PSCDR1-GFP) and CaMdr1p (RPCaMDR1-GFP), show green rimmed appearance typical of PM localized proteins thus confirming their proper expression and surface localization (figure 27 A & 27 B). Spot assays for drug susceptibility revealed that both the proteins are fully functional (figure 27 C).

### 3.3.2.2 Deletion of ergosterol and sphingolipid biosynthetic genes.

PSCDR1-GFP and RPCaMDR1-GFP (AD1-8u derivatives expressing Cdr1p and CaMdr1p, respectively) were tested for their sensitivity to the drug geneticin, which is the selectable marker of the ‘yeast knock out’ (YKO) collection of *S. cerevisiae* (figure 27 D). Both PSCDR1-GFP and RPCaMDR1-GFP strain were sensitive to geneticin, which was used as the selectable marker, for the disruption of ergosterol and sphingolipid biosynthesis genes (figure 27 D). The knock out was done, based on a PCR-generated deletion strategy, which was used to systematically replace yeast ORF from its start-to stop- codon with a KanMX module (Baudin *et al.*, 1993; Wach *et al.*, 1994). The disruption cassette with homology at the flanking region and geneticin as the selection marker was amplified, as shown in figure 27 E. The amplicon was purified and transformed by the lithium acetate transformation protocol (Shukla *et al.*, 2003). The integration of disruption cassette at the right locus was confirmed by PCR as well as Southern blotting. The genes disrupted in the sphingolipid biosynthesis pathway in PSCDR1-GFP and RPCaMDR1-GFP included: **FEN1**, which codes for fatty acid elongase and acts on fatty acids of up to 24 carbons in length (\(\Delta\text{fen1}/\text{CDR1-GFP}\) and \(\Delta\text{fen1}/\text{CaMDR1-GFP}\)); **SUR4** that also codes for an elongase, involved in fatty acid and sphingolipid biosynthesis and synthesizes very long chain 20-26 carbon fatty acids from C18-CoA primers (\(\Delta\text{sur4}/\text{CDR1-GFP}\) and \(\Delta\text{sur4}/\text{CaMDR1-GFP}\)); **IPT1**, codes for inositol
Figure 27. (A) Strategy shows the cloning and integration of CDR1 and CDR3 as C-terminal GFP tagged proteins at the PDR5 locus in the overexpression strain of S. cerevisiae AD1-8u, derived from a Pdr1-3 mutant strain with a gain of function mutation in the transcription factor Pdr1p, resulting in constitutive hyper-induction of the PDR5 promoter. The GFP tagged proteins showed typical rimmed appearance on the periphery of the cell.

(B) Western blot, of the PM fractions of the PSCDR1-GFP and RPCaMDR1-GFP strains with GFP monoclonal antibody showing that the proteins are properly expressed and targeted.

(C) Spot assay, showing the pattern of drug resistance of AD1-8u, PSCDR1-GFP and RPCaMDR1-GFP strains, for different drugs tested in the absence (control) and in the presence of various drugs - FLC (0.17 μg/ml), CYH (0.2 μg/ml), CER (3 μg/ml), 4-NQO (0.2 μg/ml), MTX (65 μg/ml). Growth differences were recorded following incubation of the plates for 48 h at 30°C. (D) Plates showing the sensitivity of the PSCDR1-GFP and RPCaMDR1-GFP strains for geneticin, which is used as the selection marker for transformation, while the lipid knockout mutants from the yeast knock out library are resistant to geneticin. (E) Strategy for the disruption of the genes involved PCR amplification of the disruption cassette as described in Materials and Methods.
Figure. 28 (A) Schematic representation of the ergosterol biosynthetic pathway showing different steps and intermediates, each step being carried out with a specific enzyme. The genes disrupted are shown with a cross in the pathway. The gene disrupted includes: ERG24, ERG6 and ERG4. (B and C) Spot assay of wild type Cdr1p-GFP, CaMdr1p-GFP and ergosterol mutants (Δerg24, Δerg6, Δerg4) expressing Cdr1p or CaMdr1p-GFP, in the absence (control) and presence of the following drugs - FLC (0.17 μg/ml), CYH (0.2 μg/ml), CER (3 μg/ml), 4-NQO (0.2 μg/ml) and MTX (65 μg/ml). Growth differences were recorded following incubation of the plates for 48 h at 30°C.
Figure. 29 (A) Schematic representation of the sphingolipid biosynthetic pathway in fungi. The disrupted genes FEN1, SUR4 and IPT1 are shown with a cross in the pathway. (B and C) Spot assays of wild type Cdr1p-GFP, CaMdr1p-GFP and sphingolipid mutants (Δfen1, Δsur4 or Δipt1) expressing Cdr1p-GFP or CaMdr1p-GFP, in the absence (control) and presence of the following drugs- FLC (0.17 μg/ml), CYH (0.2 μg/ml), CER (3 μg/ml), 4-NQO (0.2 μg/ml) and MTX (65 μg/ml). Growth differences were recorded following incubation of the plates for 48 h at 30°C.
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phospho transferase 1, involved in synthesis of mannose-(inositol-P)$_2$-ceramide (M(IP)$_2$C) ($\Delta$ipt1/CDR1-GFP and $\Delta$ipt1/CaMDR1-GFP). In ergosterol biosynthesis pathway, we disrupted: **ERG24**, which codes for C-14 sterol reductase ($\Delta$erg24/CDR1-GFP and $\Delta$erg24/CaMDR1-GFP); **ERG6** coding for $\Delta$(24)-sterol C-methyltransferase, ($\Delta$erg6/CDR1-GFP and $\Delta$erg6/CaMDR1-GFP); **ERG4** encodes for sterol C-24(2S) reductase, ($\Delta$erg4/CDR1-GFP and $\Delta$erg4/CaMDR1-GFP). The respective position of all these genes in the ergosterol and sphingolipid biosynthetic pathway is shown in figure 28A and 29A, respectively.

3.3.2.3 Cdr1p-mediated resistance depends on the membrane status.

To analyze the activity of Cdr1p-GFP and CaMdr1p-GFP expressing strains in the ergosterol and sphingolipid biosynthetic mutant backgrounds, the mutant strains were tested for their susceptibility to drugs by spot and microdilution assays. In comparison to highly sensitive AD1-8u· cells (figure 27 C), the spot assay revealed that the cells expressing the wild type Cdr1p-GFP (PSCDR1-GFP) (Shukla *et al.*, 2003) and CaMdr1p-GFP (RPCaMDR1-GFP) were able to tolerate all the tested drugs such as FLC, MTX, 4-NQO, CER and AMP B. Overexpression of Cdr1p-GFP in ergosterol ($\Delta$erg24/CDR1-GFP, $\Delta$erg6/CDR1-GFP, $\Delta$erg4/CDR1-GFP) (figure 28 B) or sphingolipid ($\Delta$sur4/CDR1-GFP, $\Delta$fen1/CDR1-GFP, $\Delta$ipt1/CDR1-GFP) (figure 29 B) knockout strains resulted in hypersensitivity to drugs. In contrast, no abrogation of drug resistance was observed in CaMdrlp-GFP when expressed in the above null mutants (figure 28C and 29C). Spot assay results were also confirmed by microdilution method.

3.3.2.4 The efflux of methotrexate and fluconazole mediated by Cdr1p-GFP in lipid mutants was severely hampered.

In order to correlate Cdr1p-GFP-mediated drug sensitivity to the reduced efflux of drugs in the ergosterol and sphingolipid mutant backgrounds, efflux of two radiolabelled drug substrates such as $^3$H-MTX and $^3$H-FLC was measured, while the former is a good substrate of CaMdr1p, the latter is a preferred substrate of Cdr1p (Kohli *et al.*, 2001). An increase or decrease in the level of accumulation of the drug, at a given time point, implies its reduced or enhanced efflux, respectively.
Figure 30. $^3$H-MTX and $^3$H-FLC accumulation in the PSCDR1-GFP and lipid mutants expressing Cdr1p-GFP. (A) $^3$H-FLC and (B) $^3$H-MTX accumulation in sphingolipid mutants- Δsur4, Δfen1 and Δpt1 and ergosterol mutants- Δerg6, Δerg24 and Δerg4 expressing Cdr1p-GFP. AD1-8u- is shown as the control. The values plotted are 10 min after commencement of transport. The results are the mean ± SD of three independent experiments.
Figure 31. $^3$H-MTX and $^3$H-FLC accumulation in RPCaMDR1-GFP and lipid mutants expressing CaMdr1p-GFP. Accumulation of (A) $^3$H-FLC and (B) $^3$H-MTX for sphingolipid mutants- Δsur4, Δfen1 and Δipt1 and ergosterol mutants- Δerg6, Δerg24 and Δerg4 expressing CaMdr1p-GFP. The values plotted are 10 min after commencement of transport. The results are the mean ± SD of three independent experiments.
It is apparent from figures 30 & 31, that as compared to the host (AD1-8u) cells, the accumulation of $^{3}$H-MTX and $^{3}$H-FLC, was considerably reduced (more efflux) in cells expressing Cdr1p-GFP and CaMdr1p-GFP tagged protein.

We examined the efflux activity of Cdr1p-GFP in the lipid null mutant backgrounds by measuring the accumulation of drug substrates in cells expressing Cdr1p-GFP namely: $\Delta$sur4/CDR1-GFP, $\Delta$fen1/CDR1-GFP, $\Delta$erg6/CDR1-GFP, $\Delta$erg24/CDR1-GFP $\Delta$ipt1/CDR1-GFP and $\Delta$erg4/CDR1-GFP. The accumulation was significantly increased (decrease in efflux) for FLC (between 24 and 57%) and for MTX (between 13 and 35%), when compared with the accumulation of these drugs in normal cells expressing Cdr1p-GFP (figures 30A & B respectively). In comparison, CaMdr1p-GFP when expressed in the same backgrounds showed no significant difference in their ability to efflux both the drugs (figure 31A & B).

3.3.2.5 Lipid imbalance caused selective recruitment defect of Cdr1p-GFP.

We also examined the effect of lipid changes on Cdr1p-GFP localization in the PM. Immunoblot results demonstrated that, between CaMdr1p, and Cdr1p, the expression of the latter in the PM was considerably reduced in most of the ergosterol ($\Delta$erg6/CDR1-GFP and $\Delta$erg24/CDR1-GFP, and $\Delta$erg4/CDR1-GFP) and sphingolipid null mutants ($\Delta$sur4/CDR1-GFP, $\Delta$fen1/CDR1-GFP, and $\Delta$ipt1/CDR1-GFP), as compared to control cells expressing Cdr1p-GFP (figure 32 A). Confocal images of Cdr1p-GFP in the null mutants also showed poor surface localization as evident from the lack of total rimmed appearance on the periphery of the cell and trapped GFP fluorescence inside the cells was seen in all of the mutants (figure 32 B, C and D upper panel). Results obtained from FACS analyses also showed reduced total fluorescence, which was consistent with immunoblot and confocal confirming poor expression of Cdr1p in the PM (figure 32 C and D lower panel). Interestingly, such was not the case with the lipid mutants expressing CaMdr1p-GFP. The expression and localization of CaMdr1p was not affected in altered lipid background (figure 33 A- D).
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Figure 32. (A) Immunodetection of Cdr1p in the PM of strains: PSCDR1-GFP and lipid mutants expressing Cdr1p-GFP with α-GFP monoclonal antibody and, (B) Fluorescence imaging (upper panel) by confocal microscope showing membrane localization of Cdr1p-GFP. Flow cytometry (lower panel) of S. cerevisiae expressing Cdr1p-GFP and lipid mutants expressing GFP-tagged CDR1. The histogram derived from the cell quest programme depicts the total fluorescence intensities of AD1-8 (control) (purple filled area), PSCDR1-GFP (solid pink line) for each panel, and the other extra line (solid green) represents that for the respective lipid mutant variant expressing Cdr1p-GFP.
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Figure 33. (A) Immunodetection of CaMdr1p in the PM of strains: RPCaMDR1-GFP and lipid mutants expressing CaMdr1p-GFP with α-GFP monoclonal antibody. (B) Fluorescence imaging (upper panel) by confocal microscope showing membrane localization of CaMdr1p-GFP. Flow cytometry (lower panel) of S. cerevisiae expressing CaMdr1p-GFP and lipid mutants expressing GFP-tagged CaMDR1. The histogram derived from the cell quest programme depicts the total fluorescence intensities of AD1-8u (control) (purple filled area), RPCaMDR1-GFP (solid green line) for each panel, and the other extra line (solid pink) represents that for the respective lipid mutant variant expressing CaMdr1p-GFP.
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Figure 34. Isolation of DRM’s from yeast. (A) Schematic of gradient, illustrating the concentration of OPTIPREP® in each step. (B) Proteins isolated from six gradient fractions, separated by SDS-PAGE. (C) Immunoblot of Pma1p with α-Pma1p polyclonal antibody to confirm raft preparation. (D) Immunoblot of gradient fractions of Cdr1p with α-GFP monoclonal antibody in the wild-type (upper panel), sphingolipid mutants- Δsur4, Δfen1 and Δipt1 and ergosterol mutants-Δerg6, Δerg24 or Δerg4. (E) Immunoblot of gradient fractions of CaMdr1p with α-GFP monoclonal antibody in the wild-type (upper panel), sphingolipid mutants- Δsur4, Δfen1 and Δipt1 and ergosterol mutants- Δerg6, Δerg24 and Δerg4.
Figure 35. Immunoblot of gradient fractions of Pma1p with α-PMA1 polyclonal antibody in the wild-type (upper panel), sphingolipid mutants- Δsur4, Δfen1 and Δipt1 and ergosterol mutants- Δerg6, Δerg24 and Δerg4.
3.3.2.6 Cdr1p is localized in membrane rafts.
Ergosterol and sphingolipids are major constituents of lipid rafts, also called DRMs for their property of being resistant to solublization when treated with non-ionic detergents at low temperature (Bagnat et al., 2001; Bagnat et al., 2000). Since we observed that Cdr1p is mislocalized following imbalances in raft lipid constituents, we checked if Cdr1p is associated with these discrete DRM. For this, we isolated membrane rafts by the detergent insolubility method using non-ionic detergent Triton X-100, as described in Materials and Methods (Bagnat et al., 2000; Denny et al., 2001; Malinska et al., 2003; Mongrand et al., 2004). We used OPTIPREP, for gradient formations wherein DRM generally constitute upper two fractions, when six equal fractions from top to bottom are taken (Denny et al., 2001; Malinska et al., 2003) (figure 34 A). Immunoblot analysis with α-GFP antibody of the DRM fraction clearly showed the presence of Cdr1p in the top two floating raft fractions (figure 34 D, first panel). The rafts preparation was verified by re-probing the immunoblot with the α-Pma1p antibody (figure 34 C), which is a positive marker for raft proteins (Bagnat et al., 2000; Gong and Chang, 2001). Interestingly, the characteristic distribution of Cdr1p in DRMs was lost in all the lipid mutants (figure 34 D). The MFS transporter, CaMdr1p, on the other hand was not exclusively present in raft fractions but rather was evenly distributed in all the fractions. This distribution pattern of CaMdr1p remained unaffected by the lipid perturbations, (figure 34 E).

3.3.3 Discussion
Cdr1p, an ABC and CaMdr1p, a MFS multidrug transporter, have been implicated in acquired multidrug resistance encountered in clinical C. albicans isolates. Interestingly, both the pump proteins have identical functions (drug extrusion), but they mechanistically differ from each other. While Cdr1p couples direct hydrolysis of ATP to drug efflux, CaMdr1p, accomplishes it by employing proton gradient of the cell. However, our recent work suggests that the two drug efflux proteins also have another level of difference. We observed that Cdr1p functioning is sensitive to membrane lipid imbalances (Kohli et al., 2002; Mukhopadhyay et al., 2002; Mukhopadhyay et al., 2004, Smriti et al., 1999).
In this study, we have systemically analyzed and compared the lipid dependency of the two multidrug transporter proteins. For this, we overexpressed both the proteins in a common heterologous host *S. cerevisiae* as GFP-tagged versions, where expression of *CDR1* and *CaMDR1* was driven by the *PDR5* promoter, in a *pdr1-3* (hyperactive allele of Pdr1p) background. In this background, three set of genes of ergosterol (Δ*erg6*, Δ*erg24* or Δ*erg4*) and sphingolipid (Δ*sur4*, Δ*fen1* or Δ*ipt1*) biosynthesis pathways were disrupted. Our results showed that the cells expressing Cdr1p, in lipid mutants (either defective in sphingolipid or ergosterol biosynthesis) turned sensitive to tested drugs, which was also evident from their abrogated efflux of drug substrates. Apparently, lipid imbalance led to trafficking disorder and mislocalization of Cdr1p, since confocal images revealed that the fluorescent protein was trapped inside the cell, and as a result, the normal rimmed appearance of the Cdr1-GFP was lost, in both ergosterol and sphingolipid mutants background. In comparison, cells expressing CaMdr1p in the same lipid mutant background remained properly surface localized, displayed resistant to drugs and showed unaltered efflux of drug substrates. Thus, both ergosterol and sphingolipids, being principle constituents of membrane lateral microdomains were found to be important for Cdr1p localization and functioning. Our study for the first time demonstrates that Cdr1p is exclusively localized within ergosterol and sphingolipid rich DRMs. Any imbalance in either of the two raft lipid constituents resulted in dissociation of Cdr1p from these microdomains. Interestingly MFS transporter, CaMdr1p was not exclusively found within DRM domains. Moreover, any lipid imbalance also did not affect its surface localization and function.

A recent proteomic analysis of DRMs from *C. albicans* identified 29 proteins to be localized within membrane rafts (Insenser et al., 2006). In that study Cdr1p was not detected in DRM’s, which could have been due to the poor level of expression of Cdr1p in the laboratory isolate SC5314. Some other known raft proteins including amino acid permease Fur4p, Tat2p and transporters Can1p, Nce2p were also not detected in that study (Insenser et al., 2006). The expression of the protein in heterologous background probably does not affect the raft associations, as Hup1p of *Chlorella kessleri*, which exclusively exist in raft, when expressed in *S. cerevisiae*, still retained
its property of being localized within the DRM microdomains (Grossman et al., 2006).

Interestingly, only certain proteins are localized within DRMs. Yeast PM-ATPase is one such example, which is exclusively found within rafts. The oligomerization of Pma1p has been linked to membrane lipid composition, since in ceramide depleted cells, Pma1p remains monomeric (Lee et al., 2002). Our present study also confirmed that similar to Cdr1p, the PM-ATPase protein is localized within rafts and it's mistargeted in lipid mutant backgrounds (figure 35). Although, it seems logical that proton generating (Pma1p) and utilizing proteins (CaMdrlp) reside within the same membrane lateral domains, but we demonstrate that this is not the case and apparently tight coupling between PMF-generating Pma1p and PMF dissipating CaMdrlp is not obligatory.

The association of certain proteins within raft domains has emerged as an important regulator of signal transduction, protein and membrane polarized intracellular sorting, cytoskeletal reorganization and entry of infectious organism in living cells (Brooker and Goswitz, 1993). In C. albicans GPI-anchored proteins Eap1p, Dfg5p and Phrlp are present in DRM's and are known to be involved in adhesion to epithelial cells, virulence, and proper hyphal growth (Martin and Konopka, 2004). Gas1p from S. cerevisiae involved in cell-wall biogenesis is a known lipid raft protein (Bagnat et al., 2000; Bagnat and Simons, 2002; Martin and Konopka, 2004). The functional significance of the PM compartmentalization is evident by the protein distribution in polarized, mating induced Schizosaccharomyces pombe cells and S. cerevisiae, which harbours only those proteins in the shmoo tip that are required for mating (Bagnat and Simons, 2002; Wachtler et al., 2003). The hyphal tips of C. albicans also show ergosterol-enriched domains, which may be indicative of clustering of DRMs in its growing tip (Martin and Konopka, 2004). We had earlier observed that CDR1 is highly expressed during hyphal development in C. albicans cells (Dogra et al., 1999). With this background, it is tempting to speculate that if Cdr1p is also localized in growing hyphal tips then it may also have a role in the morphogenesis of Candida. It may not be out of context to mention that most of the transcription factors regulating C. albicans morphogenesis also regulate CDR1 expression (Murad et al., 2001).
The importance of rafts in yeast cellular functions is beginning to emerge. How *Candida* cells uses rafts as hub of signaling remains to be examined.