C. albicans is the most prevalent human fungal pathogen and is especially problematic in immune-compromised individuals. Azoles are antifungal drugs used extensively in the therapy of *C. albicans* infections because they cause few side effects. Resistance to azoles arises during long-term, low-level prophylactic treatment regimes which make it more complicated to treat Candidiasis. The evolution of azole resistance can occur via different pathways, e.g., 1) increased activity of transcription factors that regulate drug pumps, which in turn result in overexpression of the drug efflux pumps. 2) Mutations in the ergosterol biosynthetic pathway which facilitates in target alteration and overexpression of the target. 3) Highly flexible genome facilitates overproduction of the factors that is important to develop resistance (Selmecki *et al*., 2006).

Among mechanisms identified, the overexpression of the drug efflux pumps is a major concern because this is the major mechanism for developing resistance identified so far. Based on several studies, a close interaction between membrane lipids and drug extrusion pump protein has been realized (Mukhopadhay *et al*., 2004; Prasad *et al*., 2005; Kaur and Bacchawat, 1999). Different studies have shown that subtle modification of the membrane lipid composition can modulate the action of antifungals (Hitchcock, 1993; Kohli *et al*., 2002; Loffler *et al*., 2000; Mukhopadhay *et al*., 2002). Clinical as well as adapted azole resistant isolates of *C. albicans* also exhibit altered membrane phospholipid and sterol compositions (Kohli *et al* 2002; Loffler *et al*., 2000).

Biological membranes are organized assemblies of lipids and proteins which regulate the composition of the intracellular medium by controlling the flow of nutrients, waste products, ions etc. The lipid compositions in biological membranes in eukaryotes mainly consist of phospholipids, sphingolipids and sterols but their composition differs in the two leaflets of the membrane, resulting in asymmetry. The glycerophospholipids and sphingolipids are the major contributors for generation of membrane asymmetry. The distribution of the lipids in outer and inner leaflet mainly depends on the overall charge of their head groups. Among the different lipid species, Phosphotidylcholine (PC) and complex sphingolipids, including sphingomyelin (SM) and glycosphingolipids in mammals and myo-inositol containing sphingolipids in yeast are located mainly in the outer leaflet. On the contrary, Phosphotidylethanolamine (PE) and phosphotidylinositol

(PI) are confined to the inner leaflet (Pomorski *et al*., 2004; Ikeda *et al*., 2006).Once the asymmetry is formed, the spontaneous flip-flop between the two leaflets is very slow because of the hydrophilic nature of the head groups of these amphiphilic lipids which hinders their ability to traverse the hydrophobic membrane interior (Bai and Pagano, 1997).

Maintenance of proper membrane asymmetry is important for several cellular functions. For instance, in yeast when certain glycerophospholipid translocase genes (*DNF1, DNF2, DNF3* and *DRS2*) are deleted, intracellular trafficking and maintenance of organelle structure are impaired (Chen *et al*., 1999; Gall *et al*., 2002; Hua *et al*., 2002; Pomoroski *et al.*, 2003; Furuta *et al*., 2007). In mammals, skeletal proteins like spectrin improve the mechanical stability of red blood cells by interacting with PS in the inner leaflet (Manno *et al*., 2002). PS exposed to outer leaflet of apoptotic cells, as a result of membrane collapse, is used as a recognition signal by phagocytes (Fadok *et al*., 1992). PS exposure on activated platelets is also essential for blood coagulation (Zwaal *et al*., 1998; Lentz, 2003). Additionally, transient PE exposure and loss of membrane SM have been observed at cleavage furrows during cytokinesis, and the interaction of exposed PE with PE-binding compounds results in cell cycle arrest (Emoto *et al*., 1996).

Sphingolipids also contribute in membrane asymmetry formation, but the knowledge about their translocases is really limited. In *S. cerevisiae RSB1* was identified as a sphingoid base translocator which contributes in membrane asymmetry formation and any alteration in membrane asymmetry can induce *RSB1* expression is reported (Ikeda *et al.*, 2008). *RSB1* is a member of the Rta-1 like family in *S. cerevisiae*, the other members of this family named as *RTA1* which confers resistance to 7-Aminocholesterol, an inhibitor of ergosterol biosynthesis, *PUG1* involved in hypoxia induced protoporphyrin and hemin uptake, *RTM1* confers resistance to molasses (Soustre *et al*., 1996 ; Protchenko *et al*., 2008 ; Ness *et al*., 1995).

C. albicans genome has three Rta1-like genes namely *RTA2, RTA3* and *RTA4*. Among them, *RTA2* and *RTA4* are regulated by the transcription factor *CRZ1* which is again regulated by the calcium calmodulin activated calcineurin. The other member of this family named as *RTA3* is co-regulated with *CDR1* by the transcription factor *TAC1*. All

of these Rta-1 family proteins have seven transmembrane spans, an extracellular long stretch and a C-terminal cytoplasmic tail as predicted by the TMHMM program.

In *C. albicans* the development of multi drug resistance has been associated with the overexpression of drug efflux pumps like *CDR1*and *CDR2*, which are regulated by *TAC1,* where *RTA3* is a coregulated gene. The calcineurin pathway has also been held responsible for the tolerance of antifungals (Cannon *et al*.,2007 ; Karababa *et al*., 2006 ; Bader *et al*., 2003 ; Sanglard *et al*., 2003 ; Cruz *et al*., 2002., Steinbach *et al*., 2007), cells survival during stress and overall virulence of this pathogen. So, to examine the role of Rta-1 family members in *C. albicans* in pathogenicity, membrane homeostasis and multidrug resistance, we chose *RTA2* and *RTA3* for analysis of their contribution to this phenomenon.

To characterize the function of any gene of interest, either overexpression or deletion of the same from the genome would be required. Due to lack of powerful overexpressing tools in *Candida*, a common approach to assess the function of a gene is to inactivate the gene from its genome by targeted mutagenesis. In case of *C. albicans* this situation is largely mystical due to its diploid genome and due to absence of a complete sexual cycle. So, both of the alleles should be targeted to get the knockout strain in this fungus for a particular locus. Secondly as auxotrophic mutations affect the virulence of the pathogen in animal hosts, so there has been an effort to use strains with less auxotrophies (Negredo *et al*., 1997; Pla *et al*., 1996). Among the techniques for targeted gene deletion the *URA*-blaster strategy is the most conventional one. The cassette consists of *URA3* gene of *C. albicans* flanked by *Salmonella hisG* sequence and then portions of the target gene to be mutated, the cassette then be used for disruption by integrative transformation using the *URA3* gene as selection marker. But recent studies shown that *URA3* expression levels itself can influence *C. albicans* virulence as well as morphogenesis and adhesion, because *URA3* is a virulence factor of this pathogen. We have used the *URA* blaster cassette because our result aimed at the role of *RTA2* in *C. albicans* drug resistance.

1. In this study, we first have compared the auxotrophic variants of the wild type strains in terms of their drug susceptibility, membrane permeability and total lipid content. We mainly have focused on the effects of *URA3*, because this is the most

conventional auxotrophic marker for molecular studies in *C. albicans.* Our observation leads to conclusion that wild type strain RM1000 (*ura* / *ura*) differs from another wild type strain CAF2-1(*URA/ura*).The membrane of CAF2-1 was around four-fold more permeable to a peptide namely MG132 and was more susceptible to azoles when compared to RM1000. Besides, RM1000 has two-fold high total lipid content.

2. We used the most conventional *URA* blaster strategy for chromosomal deletion of *RTA2* in *C. albicans.* We observed differential behaviour of *RTA2* deletants, namely SM1 (*rta2Δ/Δ URA+*) and SM2 (*rta2Δ/Δ ura-*) in respect to their drug susceptibility, membrane permeability, total lipid content, expression of the major efflux pump *CDR1* and in terms of their whole transcriptome. These phenotypes were dependent on the *URA3* status of the strains. After neutralization of the *URA3* status, we have concluded that the differential behaviour was dependent simply on the presence or absence of *URA3,* and was not due to the chromosomal location of *URA3* that it occupied. From this study for the first time we show that *URA3*-blaster strategy is not reliable for gene deletion to study drug resistance of this pathogen.

3. The overexpression of *RTA2* in a heterologous overexpression system in *S. cerevisiae* leads to resistance to cationic stress and detergents but not to azoles tested. We observed increased transcriptional response of *RTA2*, to membrane stress conferred by azoles, allylamines and morpholines and to cell wall stress by detergents. We found that *RTA2* is responsive to cellular iron status also. But the deletion of *RTA2* in a wild type prototroph of *C. albicans* does not alter the susceptibility towards azoles and allylamines although deletion mutants were more susceptible to 7-Aminocholesterol. *RTA2* homologue in *S. cerevisiae* namely *RSB1* maintains membrane asymmetry by transporting LCB's out of the cell. In a heterologous system, *RTA2* does confer resistance to PHS when overexpressed, but it does not participate in LCB transport in *C. albicans*, as we found no difference in accumulation and release of NBD-PHS in absence of *RTA2*. The maintenance of membrane asymmetry is important for various cellular functions (Ikeda *et al*., 2008).

The absence of *RTA2* leads to an increased ergosterol content and decrease in total lipid content. The phospholipid species which are the precursors were accumulated and the end products of this pathway were less in absence of *RTA2*. Lastly we found a significant down regulation of *RTA2* in hyphal inducing media and we also found that *RTA2* can reduce hyphae formation to a significant extent when overexpressed

4. *RTA3*, the other member of Rta-1 family does not respond to cell wall and to cell membrane stress but is significantly upregulated in response to alteration in membrane order, as in presence of membrane fluidizer, benzyl alcohol. In absence of *CDR1*, *RTA3* expression is highly upregulated. In *S. cerevisiae*, the expression of *RSB1* goes up in absence of *PDR5* (Kihara and Igarashi. 2004) due to alteration in membrane asymmetry. In *C. albicans RTA3* resembles *RSB1* of *S. cerevisiae* and transports long chain bases. So, it is probable that the absence of *CDR1* causes changes in membrane asymmetry, in response of which *RTA3* comes up to maintain the same. We propose a role for *RTA3* in maintaining membrane asymmetry like *RSB1* of *S. cerevisiae.*

5. The deletion of *RTA3* in a wild type prototroph of *C. albicans* does not alter the drug susceptibility and 7-Aminocholesterol resistance. An altered surface structure has been detected in absence of this gene as compared to the respective wild type, when treated with fluconazole. Although unaltered membrane structure and membrane permeability has been detected in untreated mutant cells, appearance of small vesicular structures was detected in between the cell wall and plasma membrane when treated with fluconazole. Taken together these results indicate that the presence of *RTA3* is required to maintain the proper surface structure and membrane in presence of fluconazole.

6. Around 60-fold downregulation of *RTA2* was detected in a *rta3*Δ/Δ background. On the contrary in absence of *RTA2*, around 2.5 fold upregulation of *RTA3* has been detected. The upregulation of *RTA3* has also been observed in conditions which

downregulates the expression of *RTA2*, like in presence of BPS, serum and in spider media. So hereby we are showing that these Rta-1 members cross talk with each despite their regulation by the two different pathways.

Taken together, the result presented in the thesis establishes the role of two Rta-1 family genes in *C. albicans* membrane homeostasis and morphogenesis for the first time. These genes maintain membrane homeostasis and its asymmetry which are important determinants of multidrug resistance in this pathogen. This study is important from clinical point of view as Rta-1 family is absent in higher eukaryotes, so they can be used as potential drug targets in this pathogen.