Mutational studies in Als5 protein from *Candida albicans*

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DOCTOR OF PHILOSOPHY

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CERTIFICATE

This is to certify that the research work presented in this thesis entitled "**Mutational studies in Als5 protein from *Candida albicans***" has been carried out in the School of Life Sciences, Jawaharlal Nehru University, New Delhi. This work is original and has not been previously submitted, either fully or in part, for the award of any other degree or diploma in Jawaharlal Nehru University or elsewhere.

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Dedicated to my parents

my husband and to my loving daughter

Contents

Abbreviations

°C	Degree centigrade
%	Percent
B-ME	Beta mercaptoethanol
µg	Microgram
µl	Microlitre
µM	Micromolar
mm	Millimeter
ml	Milliliter
α	Alpha
β	Beta
ω	Omega
AGα	α-agglutinin
Ala	Alanine
ALS	Agglutinin like sequence
AFR	Amyloid forming region
ANS	8-anilino-1-naphthalene-sulfonic acid
Asp	Aspartate
ATP	Adenosine triphosphate
Asn	Asparagine
BEC	Buccal epithelial cells
C. albicans	*Candida albicans*
$CaCl_2$	Calcium chloride
CGD	*Candida* genome database
CD	Circular Dichroism
cDNA	Complementary DNA
CFW	Calcofluor white

C-terminus	Carboxyl terminus
CWPs	Cell wall proteins
Cys	Cystine
dNTP	Deoxyribonuleoside tri-phosphate
DNA	Deoxyribonucleic acid
DAB	Diaminobenzidene
Dol-P	Dolicol-phosphate
DMSO	*Dimethyl sulfoxide*
Dol-P-Man	Dolicol-phosphate-mannose
DPM	Dolichol phosphate mannose
E.coli	*Escherichia coli*
EAP1	Enhanced Adherence to Polystyrene1
ECM33	Extracellular matrix proteins
EDTA	Ethylene diamne tetra-acetate
ER	Endoplasmic reticulum
EtBr	Ethedium Bromide
EtNP	Ethanolamine phosphate
FP	Forward primer
GlcNAc	N-acetylglucosamine
GlcNAc-PI	N-acetylglucosaminephosphatidylinositol
GlcN-PI	Glucosaminephosphatidylinositol
Gly	Glycine
GPI	Glycosilphosphatidylinositol
GPI-CWPs	GPI-cell wall proteins
GPI-GnT	GPI-N-acetylglucosamine transferase
GPI-MT-I/II/III	GPI mannosyltransferase-I/II/III
GPIT	GPI transamidase

GST	Glutathione
hrs	Hours
HIV	Human immunodeficiency virus
H_2O_2	Hydrogen peroxide
HRP	Horseradish peroxidase
HWP	Hyphal wall protein
HYR family	Hyphally regulated proteins family
I	Isoleucine
Ig domain	Immunoglobulin like domain
IPTG	Isopropyl β-D-thiogalactopyranoside
L	Leucine
LiAc	Lithium acetate
L. mexicana	*Leishmania maxicana*
Lys	Lysine
LB	Luria broth
M	Molar
$MgCl_2$	Magnesium chloride
Min	Minute
Man	Mannose
NaCl	Sodium chloride
NCAM	Neural cell adhesion molecule
N-terminus	Amino terminus
OD	Optical density
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBC	Peptide binding cavity
PBS	Phosphate buffer saline

PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PI	Phosphatidilinositol
PIG	Phosphatidylinisitol glycan
PI-PLC	Phosphatidilinositol specific phospholipase-C
PMSF	Phenylmethane sulfonyl fluoride
PNH	Paroxysmal nocturnal hemoglobinuria
PRP	Proline rich protein
PrPC	Prion protein
Ptd-Etn	phosphotidylethanolamine
PVDF	Polyvinylidene difluoride
R	Arginine
RHE cells	Reconstituted human buccal epithelium cells
RNA	Ribonucleic acid
RNase	Ribonuclease
RP	Reverse primer
RPM	Rotation per minute
RT	Room temperature
S. cerevisiae	*Saccharomyces cerevisiae*
SD Ura	Synthetic defined media
Sec	Second
S. gordonii	*Streptococcus gordonii*
SAP	Secreted aspartyl proteinases
SDS	Sodium dodecyl sulphate
Ser/Thr	Serine/Threonine
SS	Signal sequence
T. brucei	*Trypanosoma brucei*

T domain	Threonine rich domain
TAE	Tris-acetate-EDTA
TE	Tris EDTA
TEM	Transmission electron microscopy
TGase	Transglutaminase
TMB	Tetramethylbenzidine
TR domain	Tandem repeat domain
Tyr	Tyrosine
YEPD	Yeast peptone and dextrose
YWP1	Yeast form Wall Protein
UDP-GlcNAc	Uridine diphospho-N-acetylglucosamine
UV light	Ultraviolet light
V	Valine
VB1 and VB2	Variable block 1 and Variable block 2
VSG	Variant surface glycoprotein

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Chapter I

Introduction and Review of Literature

I.1 *Candida albicans*

Among all fungal species, around 600 species are pathogenic for humans and *C. albicans* is the most pathogenic amongst these (Brown et al., 2012; Mayer et al., 2013). *C. albicans* is an opportunistic pathogenic fungus. It resides in the gastrointestinal (GI) and genitourinary (GU) tract of healthy people, but causes severe infections in immune-compromised people (Hostetter, 1994). It causes superficial infections (oral-pharyngeal, esophageal, gastrointestinal and vaginal candidiasis) as well as systemic infections (bloodstream candidiasis). Superficial infections are mucosal infections, like valvovasinal candidiasis (very common in women during pregnancy period), oral-pharyngeal, oesophageal, gastrointestinal infections in HIV patients (Fidel and Sobel, 1996; Odds et al., 1988; Powderly et al., 1999).

I.1.1 *Candida albicans* morphology and morphogenetic transformations

C. albicans is unicellular polymorphic fungus which exists in three forms i.e. yeast, psuedohyphal and hyphal forms. Pseudohyphal form is an elongated bud like structure which is not separated from the mother cell (Bailey et al., 1996; Kim and Sudbery, 2011). Both pseudohyphal and hyphal forms are filamentous but they can be distinguished easily on the basis of width of filament and junction of two filaments. The pseudohyphal form of cells shows a constriction at the junction of the bud and the mother cell whereas hyphal form of cells don't have constriction at the junction of two filaments. It is also known that the width of psuedohyphal cells is greater than that of hyphal cells (Sudbery et al., 2004).

Yeast to hyphae switching is very crucial for *Candida* infection. There are two main pathways which control morphology switching in yeast; MAPK (Mitogen-activated protein kinase) pathway and cAMP Protein Kinase A pathway. It has been reported that MAP kinases regulate the activation of transcription factors in *S. cerevisiae* (Ste12p and Phd1p) and in *C. albicans* (Cph1p and efg1) they govern the morphology switching (Liu et al., 1994;

Lo et al., 1997). The double mutant of both the transcription factors in *S. cerevisiae* leads to blocking the cells in yeast form (Lo et al., 1997). Similarly in *C. albicans* the *cph1* and *efg1* null mutant showed filamentation defects (Lo et al., 1997). While the single mutations showed several defects in filamentation in both of the organisms (Csank et al., 1998; Liu et al., 1994; Lo et al., 1997). Overexpression of Ste12p leads to enhanced psuedohyphal forms indicating that Ste12p is involved in the morphology switching of *S. cerevisiae* (Liu et al., 1993). In *C. albicans* the cAMP-PKA pathway also regulates the transcription factor Efg1 and is thus involved in morphology switching (Whiteway, 2000). Overexpression of Efg1p leads to enhancement in hyphae forms and the *egf1* null mutant is defective in hyphal formation in serum indicating that Efg1p is involved in the morphology switching in *C. albicans* (Stoldt et al., 1997).

Morphology switching of *C. albicans* cells is affected by several environmental factors such as temperature and pH. It has been reported that pH 7.0 and temperature 37 °C is the most suitable condition for yeast to hyphae transition (Nadeem et al., 2013). It has been observed that the temperature less than 37 °C leads to less filamentation whereas higher temperature leads to early filamentation. Hsp90 controls the temperature dependant morphological switching in *C. albicans* through Ras1-PKA signaling pathway (Shapiro et al., 2009).

Acidic pH (<6.5) was found to be suitable for yeast form and the alkaline pH (>6.5) for hyphal form of cells (Nadeem et al., 2013). The pH mediated yeast to hyphae transition is controlled by Rim101 signal transduction pathway (Davis, 2009). It has been identified that Rim13 (calpain-like protease) leads to proteolytic cleavage of C-terminal glutamate-aspartate-rich domain of Rim101, which responds to the expression of pH responsive genes. Several intermediates govern this proteolytic cleavage such as Rim8p, Rim9p, Rim20p, and Rim21p (Li et al., 2004). There are some genes of *C. albicans*, which are involved in the pH

dependent morphogenesis (dimorphism). *PHR1* encodes for Phr1p, a GPI anchored protein, which is a homolog of Gas1p of *S. cerevisiae* and shows the pH dependent expression and morphogenesis (Saporito-Irwin et al., 1995). *PHR1* shows high expression at alkaline pH (>6.0), but is suppressed at pH below 5.5. The *phr1* null mutant showed morphological defects at alkaline pH (pH from 6.0 to 8.0), but grew well at acidic pH. These defects were restored in the revertant strain of *PHR1*, indicating that *PHR1* has a role in cellular morphogenesis at alkaline pH (De Bernardis et al., 1998; Saporito-Irwin et al., 1995). Later on *PHR2* gene was identified which was 54 % identical and inversely related to *PHR1*. *PHR2* shows higher expression at acidic pH (<5.0) and is suppressed at basic pH (>6.0). The *phr2* null mutant showed morphological defects at acidic pH (pH from 4.0 to 6.0), but grew well at alkaline pH. These defects were restored in the revertant strain of *PHR2*, indicating that *PHR2* has a role in cellular morphogenesis at acidic pH (Mühlschlegel and Fonzi, 1997). A complementation assay was also done to show the functional homology of *PHR1* and *PHR2*. It was observed that *PHR1* complemented the function of *PHR2* in a *phr2* mutant in acidic pH and *PHR2* complemented the function of *PHR1* in a *phr1* mutant in alkaline pH. This indicated that both *PHR1* and *PHR2* are functional homologs in *C. albicans* which help the organism to adapt in diverse pH range (Mühlschlegel and Fonzi, 1997).

I.1.2 Cell wall architecture of *Candida albicans*

C. albicans, like all yeast and fungi, possesses a cell wall which maintains the cell shape and cell rigidity of the organism. The *C. albicans* cell wall is a complex structure and primarily consists of polysaccharides. The main components of the cell wall include β-1,3 glucan, β-1,6 glucan, chitin and mannoproteins. Glucans are polymers of glucose and contribute 50 % - 60 % of total cell wall weight, whereas chitin is a polymer of N-acetylglucosamine which contributes about 0.6 % - 9 % of total cell wall weight (Shepherd,

1987; Chaffin *et al.*, 1998). Mannoproteins present on the cell surface contribute to 30 % - 40 % of the cell wall and these mannoproteins are involved in host-pathogen interactions (Chaffin et al., 1998; Kapteyn et al., 2000).

In *C. albicans*, cell wall proteins are linked to the membrane through covalent linkage. The GPI linked cell wall proteins (CWPs) are linked to β-1,6 glucans. These glucans are further linked to the non reducing end of β-1,3 glucans in the cell wall (Chaffin et al., 1998; Kapteyn et al., 2000). These interconnections of β-1,6 glucans to β-1,3 glucans, chitin and mannoproteins in yeast cell wall are critical for the wall integrity of the organism (Kollar et al., 1997; Kollár et al., 1995).

I.1.3 Pathogenicity and drug resistance of *Candida albicans*

Candida species acts as a major cause of pathogenicity in hospitalized persons. Among all *Candida* species, *C. albicans* is a major cause of hospital acquired bloodstream infections which resides in the gastrointestinal tract of humans (Kabir et al., 2012). In women, vulvovaginal candidiasis is a major *Candida* infection in most of the countries (Prasad et al., 2005; Sobel, 2007; Sobel et al., 1998). Studies have shown that untreated vulvovaginal candidiasis could cause infertility in non-pregnant women and abortion in pregnant women (Gugnani et al., 2009; Omar, 2001). *C. albicans* has been identified as a predominant species in many countries. *C. albicans* is the fourth major cause of hospital acquired infections and the second most common cause of death in United States (Pfaller and Diekema, 2010). In India, the weather is favorable for fungal infections and candidiasis is a very serious health problem. A report showed 1 to 12 cases per thousand cases suffering from candidiasis across the country (Chakrabarti et al., 2008). In recent studies, it has been seen that the *Candida* infections shifted towards non *C. albicans Candida* species rather than *C. albicans*. This has been proposed that the extensive use of antifungal drugs could be the

reason for this shifting the predominancy of *C. albicans* towards non *C. albicans Candida* species. A record of susceptibility profile of antifungal drugs and the usage of these drugs of some past years (2000-2008) in Sir Ganga Ram Hospital, New Delhi was carried out (Oberoi et al., 2012; Prasad et al., 2016). It was observed that about 30 % - 90 % cases of candidiasis were due to non *C. albicans Candida* species (*C. tropicalis*, *C. glabrata* and *C. parapsilosis*) where *C. tropicalis* is the major cause of candidiasis (Chakrabarti et al., 2008). In another recent survey carried out during 2010-2013, the occurrence of non *C. albicans Candida* species was highlighted and they were observed to be more sensitive to antifungal drugs (Deorukhkar et al., 2014). Several risk factors have been observed which lead to candidiasis, including prolonged hospitalization, long-term intravenous drug use, HIV infection, major abdominal surgery, uptake of immunosuppressant and antibiotics for long time and premature birth with low birth weight (Verma et al., 2003).

Host-pathogen interaction is a very crucial step for *C. albicans* infection. The first step in this process involves colonization on the host epithelial surface after adhesion of the organism to the host followed by yeast to hyphae transitions and biofilm formation (Finkel and Mitchell, 2011). After colonization, it starts to penetrate epithelial cells and degrade host proteins thus causing superficial infections. It can cause deep infections in immunologically weak individuals, including vascular invasion and tissue penetration (liver, kidney and spleen) (Klotz et al., 1992; Naglik et al., 2003). Once it enters the tissues, it starts entering the endothelial cells and blood stream and causes vascular infections (Kett et al., 2011).

C. albicans has several strategies to adapt to and bypass the host defense system.

- *C. albicans* secretes some hydrolytic enzymes such as proteases, lipases and phospholipases which help the pathogen to invade into host (Naglik et al., 2003).

- Switching of yeast to hyphae forms helps avoid phagocytosis of pathogen by host (Liu et al., 1994; Whiteway, 2000).
- *C. albicans* has flexible metabolic adaptations. It can use host glucose, glycogen, lipids, amino acids and proteins to survive in different organs (Fleck et al., 2011; Lorenz et al., 2004).
- Phr1 and Phr2 are involved in pH dependent morphogenesis of *C. albicans* and help *C. albicans* to survive in an environment with broad range of pH (Mühlschlegel and Fonzi, 1997).
- Iron acquisition from the host by Als3, a GPI anchored protein, helps the organism grow in the host (Almeida et al., 2008).
- Overexpression of multidrug-resistance pumps aid resistance to antifungal drugs (Cannon et al., 2009; Sun et al., 2013).

Eradication of *C. albicans* is a very big challenge because of the presence of very few effective antifungal drugs. Some drugs currently in use to clear the *C. albicans* infections are given below:

Azoles: Azole family includes econazole, miconazole, ketoconazole, fluconazole, itraconazole, voriconazole (derivative of fluconazole) and posaconazole (analogue of itraconazole) (Hof, 2006; Spampinato and Leonardi, 2013). Azoles target lanosterol demethylase enzyme (encoded by *ERG11*) and disrupt the egrosterol biosynthesis which results in structural alterations in plasma membrane (Spampinato and Leonardi, 2013). But studies have shown that *Candida* species develop resistance against these azoles. Two of the major factors contributing to resistance against azoles are mentioned below:

- Overexpression of multidrug resistant genes, which help in the efflux of the antifungal drugs, thus decreased the intracellular accumulation (Cannon et al., 2009).

- Point mutations in *ERG11* (target of azoles) results in reduced inhibition by the drugs (Noel, 2012).

Echinocandins: This class of antifungal drugs includes caspofungin, micafunfin and anidulafungin. These antifungal drugs inhibits beta-1,3-D-Glucan synthase enzyme, thus disrupting the cell wall integrity of fungal cells (Grover, 2010; Spampinato and Leonardi, 2013).

Polyenes: Polyenes include nystatin and amphotericin B. These polyenes bind to the ergosterol and forms aqueous pores in the fungal cell membrane. These aqueous pores result in leakage of the cytosolic matter and ultimately cause death of fungal cells (Spampinato and Leonardi, 2013).

Nucleoside analogues: These analogues act as inhibitors for DNA and RNA synthesis. Flucytosine is an analogue of pyrimidine. It can enter into fungal cells via cytosine permeases, and is converted into 5-fluorodeoxyuridine monophosphate by deamination and phosphorylation reactions (Spampinato and Leonardi 2013). The 5-fluorodeoxyuridine monophosphate interacts with thymidylate synthase and inhibits DNA synthesis. It is also shown that 5-fluorodeoxyuridine monophosphate further phosphorylates and can affect RNA synthesis as well (Spampinato and Leonardi, 2013; Vermes, 2000).

Most of the antifungal drugs mentioned above do not cure *Candida* infections completely though they can reduce the fungal burden for a time (Johnson et al., 1995; Odds, 1993). Hence, prolonged therapeutic usage of these antifungal drugs results in development of drug resistant strains. In recent studies in India, antifungal drugs susceptibility tests were done on *C. albicans* (Prasad et al., 2016). High resistance was observed against commonly used antifungal drug suggesting that there is a need of new antifungal drugs. To eradicate the

C. albicans related infections (candidiasis), we need to explore the characteristic features of *Candida* genes and their role in virulence so that we can work in the direction of identifying novel drugs targets.

Several virulence factors are found to be involved in *C. albicans* pathogenicity, for example, Als family proteins, Hwp1 protein, Eap1 proteins, Ecm33 and Sap proteins (Cormack and Zordan, 2012; Staab et al., 1999). Most of the virulence factors are the cell surface glycoproteins which are anchored in the cell membrane *via* GPI anchors or attached to the cell surface via β-glucan linkages and are involved in the adhesion and invasion of fungal species into the host cells. Therefore study of the GPI anchor biosynthesis pathway is very important to identify effective drug targets.

I.2 Brief introduction about GPI anchors

The concept about the attachment of proteins to membranes came in mid 1970s. Phospholipase C purified from *Bacillus cereus* was found to hydrolyze phosphatidylinositol into diacylglycerols and a mixture of myo-inositol 1- and 1,2-cyclic phosphates (Ikezawa et al., 1976). It was also found that phospholipase C releases alkaline phosphatase from rat kidney slices indicating that it can particularly act on plasma membrane of rat kidney cells (Ikezawa et al., 1976). In later studies, *Staphylococcus aureus* phospholipase C was used to release alkaline phosphatase from tissues slices of several mammalian species (Low and Finean, 1977). It was identified that the release of alkaline phosphatase by the action of phospholipase C from *Staphylococcus aureus* was due to the disruption of phosphatidylinositol specific interactions (Low and Finean, 1977). This idea received greater support when the GPI anchored proteins of human bovine erythrocyte acetylcholinesterase, rat Thy-1, and parasite *Trypanosoma brucei* variant surface glycoprotein (VSG) were identified (Ferguson et al., 1985; Roberts and Rosenberry, 1985; Tse et al., 1985). The first

complete GPI anchor structure of variant surface glycoprotein (VSG) was resolved by Ferguson and group in 1988 (Ferguson et al., 1988). Later Homans and group identified the complete GPI anchor structure of rat brain Thy-1 protein (Homans *et al*., 1988). Masterson *et al*., were the first to elucidate the pathway of GPI anchor biosynthesis for variant surface glycoprotein (VSG) in *Trypanosoma brucei*. They developed a cell free system and identified all the intermediates with the help of [^{3}H]-labelled sugar nucleotides (Masterson et al., 1989).

The core structure of GPI anchor is highly conserved in all eukaryotes. It consists of phosphatidylinositol (PI), glucosamine, three or four mannose residues and phosphoethanolamine as shown in Figure I.3 (Orlean and Menon, 2007). Details of these steps are described later. The precursor GPI anchor is attached to protein carrying the appropriate signal sequence and it then entered to the secretory process through COPII coated vesicles. It has been reported that the GPI anchor undergoes several species specific modifications during its transport through Golgi to the cell surface, which is also discussed later in the text (Doering et al., 1990; Kinoshita and Takeda, 1994).

GPI anchors target a wide variety of eukaryotic proteins on the cell surface as post translational protein modification and are critical for cell viability and embryonic development in yeast and mammals, respectively (Leidich et al., 1995; Maeda and Kinoshita, 2011; Paulick and Bertozzi, 2008). Defects in GPI anchor biosynthesis lead to embryonic lethality in mammals and inviability in yeast (Kawagoe et al., 1996; Leidich et al., 1994). In *C. albicans,* studies from our lab have been shown that GPI anchor biosynthesis is associated with hyphal morphogenesis and ergosterol biosynthesis (Yadav et al., 2014). It has been observed that the CaGpi2 subunit of GPI-GnT complex regulates the hyphal morphogenesis via Ras signaling, whereas CaGpi19 subunit of GPI-GnT complex interacts with CaErg11 and regulates ergosterol biosynthesis in *C. albicans* (Victoria et al., 2012; Yadav et al., 2014). It was observed that there is a negative co-regulation between both subunits, where *CaGPI2*

was up-regulated in *Cagpi19* mutant and vice versa (Yadav et al., 2014). A recent study from our lab has shown that CaGpi14 (first mannosyltransferase) is involved in growth, cell wall integrity and hyphal morphogenesis of *C. albicans* (Singh et al., 2016).

I.2.1 Biosynthesis of GPI anchors

GPI anchor biosynthesis is a highly conserved multistep pathway requiring more than 20 gene products present in the ER membrane. Biosynthesis of GPI anchor starts on the cytosolic side and completes in the ER lumen. The first two steps occur in the cytosolic side of ER and rest of the steps takes place in the ER lumen (Orlean and Menon, 2007).

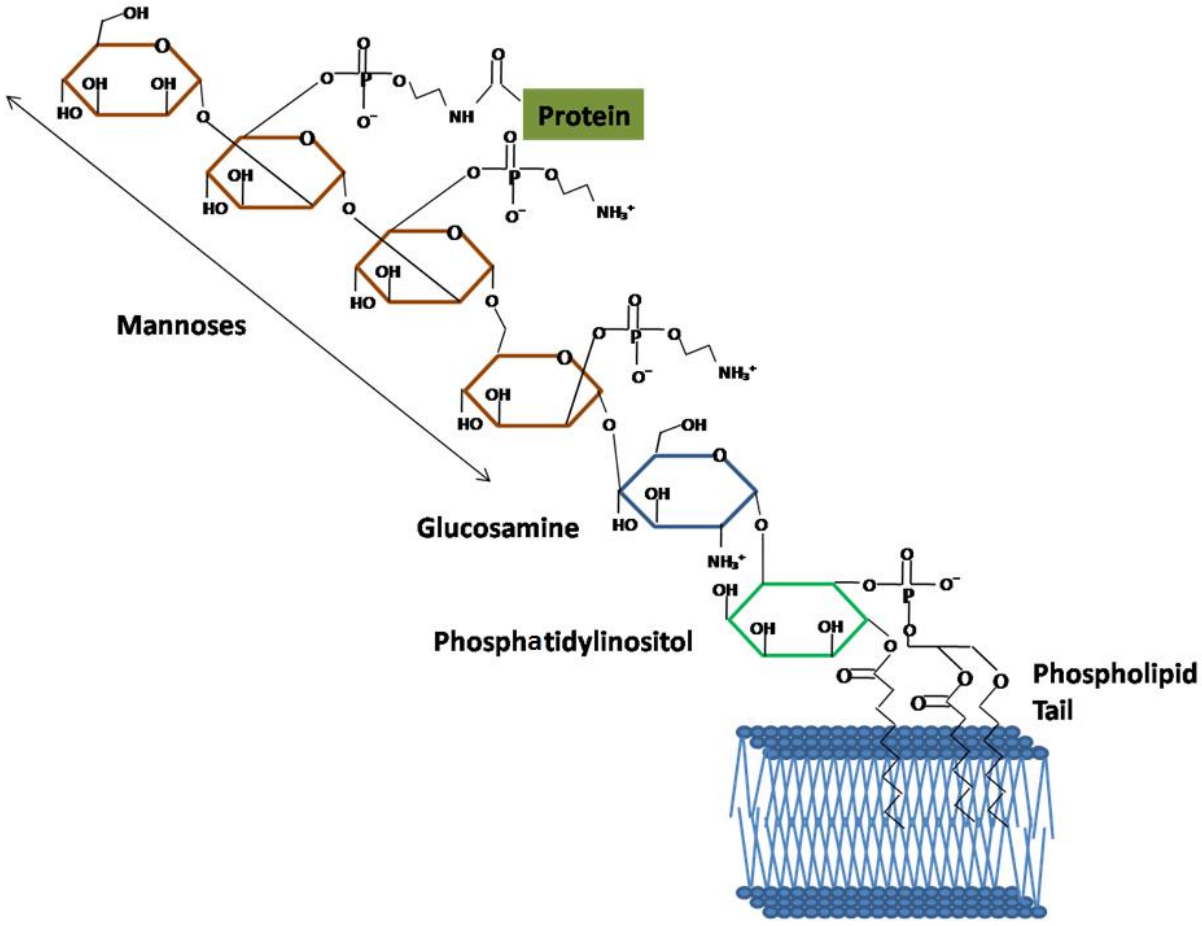

Figure I.1: The schematic diagram of GPI anchor in yeast. It represents that the GPI anchor consists of phosphatidylinositol linked to the glucosamine, four mannose residues, ethanolamine phosphates attached to all mannose residues. The ethanolamine phosphate attached to the third mannose is covalently linked to the protein.

I.2.1.1 GlcNAc-PI synthesis

GlcNAc-PI synthesis is the first step of GPI anchor biosynthesis involving the transfer of GlcNAc (*N*-acetyl glucosamine) from UDP-GlcNAc to PI (Phosphatidylinositol) which occurs in the cytosolic side of ER (Watanabe et al., 1998). This step is catalyzed by a six subunit GPI-GnT enzyme complex comprising of PIG-A/Gpi3, PIG-C/Gpi2, PIG-H/Gpi15, PIG-P/Gpi19, PIG-Q (hGpi1)/Gpi1 and PIG-Y/Eri1 mammalian proteins and their yeast homologs respectively (Inoue et al., 1996; Orlean and Menon, 2007; Watanabe et al., 1998). In mammalian cells, other than these six subunits there is one more subunit, DPM2, which is a non catalytic subunit, but which enhances the synthesis of GlcNAc-PI (Watanabe, 2000). PIG-A/Gpi3 has been identified as catalytic subunit in this complex and role of the other subunits has not been clearly delineated so far (Watanabe et al., 1998). It has been proposed that the other subunits of GPI-GnT complex might have a role in stabilization of the complex (Miyata et al., 1993). PIG-A is a 484 amino acid long transmembrane protein consists of a long N-terminal cytosolic region and a small C-terminal towards ER lumen. The N-terminal of mammalian PIG-A shows similarity with the bacterial GlcNAc transferase signifying the catalytic role of PIG-A in GPI-GnT complex (Kawagoe et al., 1994; Watanabe et al., 1998). Later studies shown that Gpi3 cross-links to a photoactivatable substrate analogue [P(3)-(4-azidoanilido)-uridine 5'-triphosphate (AAUTP)] of UDP-GlcNAc suggested that Gpi3 probably binding to UDP-GlcNAc and acts as a catalytic subunit for GlcNAc-PI synthesis (Kostova et al., 2000).

In *S. cerevisiae*, four subunits out of six are essential. Gpi1 and Eri1 are nonessential, although strains deficient in Gpi1 and Eri1 show temperature sensitive growth defects and low GlcNAc-PI synthetic activity *in vitro* (Leidich and Orlean, 1996; Sobering et al., 2004). Of these one or more accessory subunits (Gpi2, Eri1) appear to also be involved in regulating

Ras signaling (Sobering et al., 2004). In *C. albicans*, as mentioned earlier, it has been shown that CaGpi2 subunit of GPI-GnT complex regulates hyphal morphogenesis through Ras1 signaling and CaGpi19 subunit of GPI-GnT complex regulates ergosterol biosynthesis through Erg11 (Victoria et al., 2012; Yadav et al., 2014). Interestingly, in *S. cerevisiae*, the GPI-GnT complex and Ras are mutually negative regulators, each inhibiting the activity of the other (Sobering et al., 2004) whereas work from our lab indicate that in *C. albicans* they act as mutual activators of one another (Jain P, 2017, Unpublished Ph.D. thesis).

I.2.1.2 GlcNAc-PI de-*N*-acetylation

The second step of GPI anchor biosynthesis is de-*N*-acetylation, which involves the removal of acetyl group from GlcNAc-PI thus forming GlcN-PI. This reaction is catalyzed by PIG-L/Gpi12 in mammals/yeast, respectively (Watanabe et al., 1999). PIG-L/Gpi12 has a large cytosolic domain which catalyzes this reaction in the cytosolic side of ER and the 3'OH group of GlcNAc is very critical for the substrate recognition by *de-N-acetylase* enzyme (Pottekat and Menon, 2004; Smith, 2001). The de-*N*-acetylase activity of PIG-L/Gpi12 was confirmed by expressing rat PIG-L in *E. coli* and performing de-*N*-acetylase activity assay. Mutant Chinese hamster ovary (CHO) cells (defective in de-*N*-acetylation step) were complemented by cDNA of rat PIG-L suggesting that this protein by itself had de-*N*-acetylase activity. PIG-L was also found to be essential for viability in yeast and mammals (Nakamura et al., 1997; Watanabe et al., 1999). In *Trypanosoma brucei* and *Leishmania major*, GlcNAc-PI de-*N*-acetylase is catalyzed by TbGpi12 and LmGpi12, respectively. It has been shown that the cell surface expression of GPI anchored proteins in mammalian PIG-L deficient cells were complemented by TbGpi12 and LmGpi12 (Chang et al., 2002). Studies from our lab on *Entamoeba histolytica* showed that the cytoplasmic catalytic domain PIG-L had basal de-*N*-acetylase activity at optimal pH 5.5 in the absence of metal ions, which was enhanced by divalent cations (Ashraf et al., 2011). These divalent cations stimulated the

catalytic rate without affecting the enzyme-substrate affinity of *Entamoeba histolytica* PIG-L (Ashraf et al., 2011). Later, by mutational studies the residues (Asp-46 and His-140) which are critical for PIG-L catalysis in *Entamoeba histolytica* were identified (Ashraf et al., 2013). In *C. albicans*, the null mutant of CaGpi12 shows lowered de-*N*-acetylase activity, growth defect, cell wall defect and filamentation defect. This indicates that apart from catalyzing the de-*N*-acetylation reaction, CaGpi12 may have some additional role in growth, cell wall integrity and filamentation as well (Yadav U, 2017, Unpublished Ph.D. thesis).

This step shows species specific differences in terms of substrate specificity (Sharma et al., 1999; Smith, 2001). Due to the differences in the substrate for de-*N*-acetylation step between *T. brucei* and mammals, a parasite specific substrate based suicide inhibitor has been designed (Smith, 2001). Recently, a new compound salicylic hydroxamic acid has also been identified as a de-*N*-acetylase inhibitor for *T. brucei* (Urbaniak et al., 2014).

I.2.1.3 Flipping of GlcN-PI

Flipping of GlcN-PI is a very crucial step for PI acylation and further mannosylation. After completion of first two steps in the cytosolic side of ER, GlcN-PI is flipped into the luminal side of ER with the help of flippase enzyme, which has not been identified till date (Orlean and Menon, 2007; Watanabe et al., 1998). By using the fluorescent GPI probe, it was shown that flipping of GlcN-PI is a bidirectional and ATP independent process (Pomorski and Menon, 2006; Vishwakarma and Menon, 2005).

I.2.1.4 Inositol acylation

Acylation of GlcN-PI occurs at the second hydroxyl group of inositol in the lumen of ER, which is catalyzed by PIG-W/Gwt1 (inositol acyltransferase) in mammals and yeast, respectively (Costello and Orlean, 1992; Roberts et al., 1988). Acylation of GlcN-PI makes it resistant to PI specific phospholipase C cleavage (Costello and Orlean, 1992; Roberts et al., 1988; Umemura et al., 2003). Species-specific divergence has been observed in inositol

acylation reaction. In case of yeast and mammals, acylation of PI moiety occurs before the first mannosylation reaction whereas in case of trypanosomes, it occurs after the first mannosylation reaction (Murakami et al., 2003). It has been reported that the acylation of PI moiety in trypanosomes requires the fourth hydroxyl group of first mannose residue (Urbaniak et al., 2008). In yeast, acylation of GlcN-PI was found to be very critical for cell growth and viability whereas in mammals, it is essential for cell surface expression of proteins. In mammals, defect in *PIG-W* doesn't affect mannosylation, but affects the ethanolamine-phosphate (Etn-P) addition at the third mannose (Man-3) which is essential for the linkage of GPI anchor to proteins (Murakami et al., 2003). Recently, E1210 has been identified as an inhibitor for *GWT1* of *C. albicans* and *GWT1* of *A. fumigates* (Watanabe et al., 2012).

I.2.1.5 Mannosylation

Dolichol phosphate mannose (Dol-P-Man) acts as a donor for mannose residues, which is synthesized by Dol-P and GDP-Man in the cytosolic side of the ER and then flipped into the luminal side of ER (Abeijon and Hirschberg, 1992). In mammals, three mannose residues are attached to the GPI precursor, whereas in yeast a fourth mannose residue is critical for the attachment of GPI anchor to the proteins (Grimme et al., 2001). The presence of a fourth mannose in mammals has also been observed in a tissue-specific manner (Taron et al., 2004). Mannosylation reactions are catalyzed by membrane bound mannosyltransferase enzymes in the luminal side of ER. The PIG-M/Gpi14 encodes for the GPI-MT-I (GPI-Mannosyltransferase-I), which catalyzes the addition of the first mannose residue to GlcN-acyl-PI *via* α-1,4 linkage in mammals/yeast (Kim et al., 2007; Maeda, 2001). In mammals, another subunit PIG-X associates and stabilizes PIG-M, thus involved in mannosylation (Ashida, 2005; Kim et al., 2007). In *S. cerevisiae* Pbn1 is the functional homologue PIG-X and is involved in stabilization of ScGpi14 (Ashida, 2005). In *T. brucei*, the first mannose

addition occurs on the GlcN-PI before acylation (Güther and Ferguson, 1995). In *T. brucei* some GlcN-PI synthetic analogs have been reported which inhibit the first mannosylation reaction but do not do so in mammals suggesting that in *T. brucei* the first mannosylation step could be a potent drug target (Smith et al., 1999). In mammals, it has been reported that PIG-M gene consists of multiple transmembrane domains. It also has a functionally essential DXD motif towards the luminal side of ER which gives the indication that the mannosylation reaction occurs in the lumen of ER (Maeda, 2001). As mentioned earlier, studies from our lab have shown that CaGpi14 catalyzes the first mannosylation reaction in *C. albicans*. CaGpi14 was able to restore the defects of conditional lethal *Scgpi14* mutant of *S. cerevisiae* (Singh et al., 2016). The *CaGPI1*4 mutant exhibits growth defects, cell wall defects as well as the hyphal morphogenesis defects in *C. albicans* and has its HOG1 pathway activated (Singh et al., 2016).

The second mannose residue addition is catalyzed by GPI-MT-II *via* α-1,6 linkage at the first mannose in all organisms. PIG-V/Gpi18 encodes for GPI-MT-II to catalyze the second mannosylation in mammals and yeast, respectively (Fabre et al., 2005; Kang et al., 2005). PIG-V also has two functionally essential domains localized towards ER lumen which indicates that the second mannosylation also occurs in ER lumen (Kang et al., 2005). PIG-V/Gpi18 mutants lead to the accumulation of Man_1-GPI intermediate along with the first ethanolamine-phosphate (Etn-P) (Fabre et al., 2005). In mammals PIG-V could recognize both Man_1-GPI and Etn-P-Man_1-GPI intermediates for the addition of the second mannose, whereas yeast Gpi18 strictly requires Etn-P-Man_1-GPI for the addition of the second mannose (Fabre et al., 2005; Kang et al., 2005).

PIG-B/Gpi10 catalyzes addition of the third mannose residue to Man-2 *via* α-1,2 linkage (Sütterlin et al., 1998; Takahashi et al., 1996). PIG-B rescues the lethal defects of *GPI10* deletion suggesting that Gpi10 is a functional homologue of human PIG-B in yeast

(Sütterlin et al., 1998). In human, PIG-B has a short cytosolic domain, a single transmembrane domain and a long ER luminal domain. The mutant of PIG-B lacking its cytosolic domain was found to be active, indicating that the cytosolic domain of PIG-B is not essential and the luminal domain is functionally important for the activity of PIG- B (Takahashi et al., 1996). The mutants of PIG-B/Gpi10 have been shown to accumulate Man_1-Man_2-GlcN along with Etn-P at the first mannose residue (Sütterlin et al., 1998).

Unlike mammals, GPI precursor of yeast requires addition of the fourth mannose residue. The addition of the fourth mannose was found to be critical for modification of the third mannose with Etn-P. In *S. cerevisiae* Smp3 catalyzes addition of the fourth mannose residue to Man-3 *via* α-1,2 linkage (Grimme et al., 2001). The *smp3* mutant leads to the accumulation of GPI precursor consisting three mannoses modified with EthN-P either on its first mannose or the second mannose. Thus Smp3 prevents the formation of GPI precursor with four mannoses which is accumulated in *gpi13* mutant (Grimme et al., 2001). Gpi13 is required for the addition of EthN-P on third mannose which occurs after addition of fourth mannose suggesting that fourth mannose is important for the addition of EthN-P to third mannose of GPI precursor in yeast (Grimme et al., 2001). In *C. albicans*, a functional homologue of *S. cerevisiae* Smp3 has been identified, which is required for GPI precursor assembly. The *CaSMP3* rescued the growth defects and GPI precursor assembly defects of *S. cerevisiae smp3* mutant suggesting that CaSmp3 is a functional homologue of *S. cerevisiae* Smp3 (Grimme, 2004). The suppression of *CaSMP3* expression results in accumulation of GPI precursor consisting three mannoses and ethanolamine phosphate on its first mannose. Other than GPI precursor assembly defects, it also leads to growth defects, cell wall defects and cell inviability (Grimme, 2004). As mentioned earlier in mammals the presence of a fourth mannose residue appears in a tissue specific manner i.e. in brain and colon tissue (Taron et al., 2004). Human *SMP3* complements the defects of yeast *SMP3* mutant strain and

is able to promote addition of the fourth mannose residue indicating that the hSmp3 is a homologue of *S. cerevisiae* Smp3 (Taron et al., 2004).

I.2.1.6 Etn-P (Ethanolamine-phosphate) addition

Ptd-Etn (phosphatidylethanolamine) acts as a donor for Etn-P. The addition of Etn-P to the mannose residues is catalyzed by Ptd-Etn transferases (Menon and Stevens, 1992). The Ptd-Etn transferases have been predicted to be having multiple transmembrane domains and a large luminal domain (Benachour et al., 1999). The first Etn-P addition occurs on the 2-OH group of Man-1, which is catalyzed by PIG-N/Mcd4 in mammals and yeast, respectively, whereas this modification has not been identified in protozoan's (Hong et al., 1999; Wiedman et al., 2007). Mutant strain of PIG-N showed the absence of Etn-P addition at the first mannose, however the further steps of GPI assembly are not affected, indicating that in mammals, addition of Etn-P to the first mannose is not crucial for the GPI assembly (Hong et al., 1999). In *S.* cerevisiae, *MCD4* is required for the addition of Etn-P to Man-1 of GPI precursor and it is an essential gene for GPI assembly (Gaynor et al., 1999; Wiedman et al., 2007)). Mcd4 mutants were found to accumulate Man_2-GPIs lacking Etn-P on Man-1 suggesting that Mcd4 mediated modification is essential for addition of the third mannose to Man_2-GPIs (Wiedman et al., 2007). Mcd4 mutants mediated accumulation of Man_2-GPIs lacking Etn-P can be rescued by the overexpression of GPI10 (MT-III), indicating that Etn-P modification of the first mannose is not mandatory for further mannosylation reactions (Hong et al., 1999; Wiedman et al., 2007).

The second Etn-P addition occurs on the 6-OH group of Man-2, which is catalyzed by PIG-G (hGpi7)/Gpi7 in humans and yeast, respectively, whereas this modification has not been identified in protozoan's (Imhof et al., 2004; Shishioh et al., 2005). In mammals, the activity of hGpi7 is stabilized by the association of PIG-F (Shishioh et al., 2005). Similarly in yeast, the PIG-F homologue Gpi11 deficiency affects the EtN-P addition on Man-2 of GPI

precursor through Gpi7 (Taron et al., 2000). Mutant strain of human *gpi7* leads to the accumulation of Man_3-GPI intermediate having Etn-P at Man-1 and Man-3 but not on Man-2, whereas the yeast *gpi7* mutant strain leads to the accumulation of Man_4-GPI intermediate having Etn-P at Man-1 and Man-3 but not on Man-2, suggesting that addition of the second Etn-P on Man-2 occurs after addition of Etn-P on Man1 and Man3 of GPI precursor (Imhof et al., 2004; Shishioh et al., 2005). The mutant strain of *gpi7* showed sensitivity to CFW, reduced remodeling of lipid moiety with ceramides and delayed transport of GPI-protein complex to Golgi complex, indicating that Gpi7 is essential for cell wall integrity, GPI remodeling and transport of GPI-protein complex from ER to Golgi complex (Benachour et al., 1999; Shishioh et al., 2005).

In fungus, pathogen associated molecular patters (PAMPs) are recognized by the host immune system against defense mechanism (Brown and Gordon, 2001; Shen et al., 2015). These PAMPs are mainly β-Glucans which is the component of fungal cell wall. In fungus, these PAMPs are masked by the GPI anchored cell wall proteins to evade the host immune system. A recent study has shown that deletion of CaGpi7 blocked the GPI anchoring of most of the cell wall proteins thus the cell wall β-Glucans of *C. albicans* was exposed and recognized by the Dectin-1 receptor, therefore the *C. albicans* cells were killed by host innate immune response mediated by neutrophils, macrophages, secretion of cytokines and interleukins (Shen et al., 2015).

Addition of the third Etn-P on Man-3 is catalyzed by PIG-O and PIG-F in mammals and GPI13 in yeast (Hong et al., 2000; Inoue et al., 1993; Taron et al., 2000). PIG-F was identified from class F Thy-1-deficient mutant murine cell line (Inoue et al., 1993). PIG-O was predicted to be having the catalytic site and the stability of the expression of PIG-O was found to be governed by the association with PIG-F, but PIG-F is stable in the absence of PIG-O (Hong et al., 2000). The *pig-o* and *pig-f* mutants were also found to be defective in

transfer of Etn-P to Man_3-GPIs (Hong et al., 2000). The *pig-o* mutant cells leads to decreased cell surface expression of GPI anchored proteins, while *pig-f* mutant cells completely lacked cell surface expression of GPI anchored proteins, suggesting that PIG-F also has some functional role apart from stabilizing PIG-O (Hong et al., 2000). In yeast, deletion of Gpi13 protein blocked the formation of GPI precursors consisting of Etn-P on Man-3, leading to the accumulation of Man_4-GPIs with Etn-P on the first mannose (Taron et al., 2000).

I.2.1.7 Attachment of GPI anchor to precursor proteins

GPI anchor modification of proteins occurs into the ER lumen, where the C-terminal GPI anchor signal sequence of protein is replaced with precursor GPI anchor (Sharma et al., 1999). After the assembly of GPI anchor, Etn-P present on the third mannose is linked to the protein *via* an amide bond. This reaction is catalyzed by GPI transamidase enzyme in the ER lumen. The proteins receiving the GPI anchor have certain characteristics features in its C-terminal signal sequence (SS) for the anchor attachment. The ω-site at which the C-terminal signal sequence is cleaved must have small amino acid residues (Ser, Gly, Ala, Asp, Asn or Cys) for the transamidase recognition (Micanovic et al., 1990; Moran et al., 1991). A flexible linker region is present at the upstream of ω site between positions ω-10 to ω-1, moderately polar spacer region present between positions ω+3 to ω+9 followed by a variable hydrophobic region (Orlean and Menon, 2007). Details of the signal sequence and ω-site will be discussed later in the text. The C-terminal signal sequence of the proteins is cleaved between ω (GPI anchor attachment site) and ω+1 site by transamidase enzyme and the GPI anchor precursor is attached with the protein. The GPI transamidase enzyme activates the carboxyl group of ω amino acid of the precursor protein (Maxwell et al., 1995; Ohishi et al., 2000). This activated carboxyl group of ω amino acid undergoes nucleophillic attack by amino groups of Etn-P present on Man-3 of GPI anchor, thereby forming amide bond between GPI anchor and precursor protein (Kinoshita, 2014; Maxwell et al., 1995; Sharma et

al., 1999). The amide bond is formed between the amino group of Etn-P attached to 6-OH of the third mannose of GPI anchor and the α-carboxyl group of ω amino acid of precursor protein (Maxwell et al., 1995; Sharma et al., 1999).

GPI transamidase complex consists of five subunits, PIG-K (Gpi8)/Gpi8, GAA1/Gaa1, PIG-S/Gpi17, PIG-T/Gpi16 and PIG-U/Gab1 in mammals and yeast, respectively (Fraering et al., 2001; Grimme, 2004). But the GPI transamidase enzyme complex in *T. brucei* differs from that of yeast/mammals. *T. brucei* GPI transamidase complex also consists of five subunits of which TbGPI8, TbGAA1 and TbGPI16 are homologous to the human PIG-K, GAA1 and PIG-T subunits and the two other subunits TTA1 and TTA2 are identified unique to *T. brucei* (Nagamune et al., 2003). The PIG-K/Gpi8 acts as the catalytic subunit and is essential for GPI transamidase enzyme complex (Meyer et al., 2000; Ohishi et al., 2000). All the other subunits act as accessory subunits stabilizing the GPI transamidase complex (Benghezal et al., 1996; Meyer et al., 2000; Ohishi et al., 2000). The PIG-K/Gpi8 shows sequence homology with cysteine proteases (Meyer et al., 2000; Ohishi et al., 2000). The cysteine-histidine dyad of PIG-K/Gpi8 is conserved in the cysteine protease family and mutations in cysteine-histidine dyad of PIG-K/Gpi8 led to inactivation of GPI transamidase enzyme complex suggesting that PIG-K/Gpi8 is the catalytic subunit for transamidation reaction (Meyer et al., 2000; Ohishi et al., 2000). In mammals, PIG-K is stabilized by a disulfide bond with PIG-T subunit, which is required for the GPI transamidase enzymatic activity (Ohishi et al., 2003). The same interactions were observed in *T. brucei* homologs also, where TbGpi16 forms a disulfide-linkage with TbGpi8 (Hong et al., 2006). Recently, it has been reported that the *S. cerevisiae* Gpi8 and Gpi16 subunits do not forms disulfide bond suggesting that interactions between the GPI transamidase is species specific (Yi et al., 2017). In yeast, it has been identified that the *gaa1* mutant is not affected in the synthesis of GPI precursor but is unable to transfer GPI precursor to the proteins requiring a

GPI anchor indicating that Gaa1 might also be involved in GPI anchor recognition (Hamburger et al., 1995).

I.2.1.8 Remodeling of GPI anchor after attachment to the protein

After the attachment of GPI anchor to proteins, the lipid as well as glycan part of GPI goes through remodeling process in the ER and Golgi. These modifications are important for the transport of the GPI anchored proteins from ER to Golgi and subsequently its attachment to the cell surface.

(i) Inositol deacylation in yeast and mammals

Removal of the acyl chain from the inositol of GPI anchor precursor is the first GPI remodeling step and is very critical for the packing of GPI linked proteins into COPII vesicles and their transport from the ER exit site. The inositol deacylation occurs after the attachment of GPI to protein, which is catalyzed by PGAP1 (Post GPI anchor Attachment to Protein 1) /Bst1 proteins in mammals/yeast respectively (Güther and Ferguson, 1995; Tanaka et al., 2004). CHO cells defective in GPI-deacylase activity showed resistant to the phospholipase-C and the cells with over expression of PGAP1 were sensitive to phospholipase-C, indicating that PGAP1 has deacylase activity (Tanaka et al., 2004). The strain having mutation in PGAP1 showed accumulation of GPI-protein complex in the ER lumen and delayed transport to the Golgi (Fujita et al., 2011; Tanaka et al., 2004). In yeast, Bst1 mutant may lead to the transport of the misfolded proteins from ER to Golgi complex which can be retrieved back to the ER and degraded by the proteasomal degradation pathway (Tanaka et al., 2004; Vashist et al., 2001). It has been reported that the mutation in Bst1 leads to delayed degradation of misfolded Gas1 protein indicating that Bst1 mediated inositol deacylation has a role in the proteasomal degradation (quality control) of misfolded proteins in ER lumen (Fujita, 2005).

(ii) Lipid remodeling

(a) Removal of Etn-P side chain from second mannose

Removal of Etn-P side chain from the second mannose of GPI anchor is very critical for the transport of GPI anchored proteins from ER to plasma membrane. This step is catalyzed by PGAP5 protein in mammals and Ted1 and cdc1 in yeast (Fujita et al., 2009, 2011). Defect in PGAP5 leads to the delayed transport of proteins from ER to plasma membrane (Fujita et al., 2009).

(b) Fatty acid remodeling

The fatty acid remodeling of GPI anchor is essential for the efficient transport of GPI anchored proteins from ER to Golgi and for their association into lipid rafts**.** In trypanosomes, deacylation and reacylation of the lipid chains occur at *sn*-1 and *sn*-2 positions in the ER lumen before attachment of GPI precursor to the proteins (Hong and Kinoshita, 2009). The long chain fatty acid at *sn*-2 position is replaced by the myristate (14 carbon saturated fatty acid). After that, *sn*-1 fatty acid is remodeled with a second myristate group (Hong and Kinoshita, 2009). In yeast, lipid remodeling occurs in both ER and Golgi complex (Muñiz and Zurzolo, 2014). Lipid remodeling of *sn-1* saturated fatty acid chain of GPI anchor does not occur in case of yeast (Orlean and Menon, 2007). In yeast, two ER localized enzymes (Per1 and Gup1) regulate the fatty acid remodeling of GPI anchor at *sn-2* position (Bosson et al., 2006). The fatty acid chain at *sn-2* position of diacylglycerol moiety of GPI anchor is first removed by Per1 enzyme. It is subsequently followed by the addition of long chain fatty acid (mostly ceramide and minor fraction of C26:0 fatty acid) mediated by Gup1 enzyme (Bosson et al., 2006; Fujita et al., 2006; Sipos et al., 1997). One more yeast protein Cwh43 has been identified, which is a homolog of mammalian PGAP2 protein (Ghugtyal et al., 2007). The Cwh43 is involved in introducing ceramide at the place of diacylglycerol of GPI anchor (Ghugtyal et al., 2007).

Similar to yeast, remodeling of *sn-1* saturated fatty acid chain does not occur in mammals as well (Orlean and Menon, 2007). In mammals PGAP3 is a Per1 orthologue which catalyzes the removal of the fatty acid chain from *sn-2* position of diacylglycerol of GPI anchor (Fujita et al., 2006). Subsequently, the PGAP2 catalyzes the addition of $C_{18:0}$ acyl chain at *sn*-2 position of diacylglycerol of GPI anchor (Tashima et al., 2006). In mammals, both PGAP3 and PGAP2 are Golgi localized proteins therefore the fatty acid remodeling occurs in Golgi apparatus.

I.2.1.9 Transport of GPI anchored protein from ER to the cell surface

As already mentioned GPI anchored proteins are synthesized and modified in the ER. After the remodeling the correctly folded proteins are packed into the COPII coated vesicles and transported from the ER (Castillon et al., 2011). Since GPI anchored proteins are the luminal proteins therefore they can't directly interact directly with the cytosolic COPII vesicles (Castillon et al., 2011; Fujita et al., 2011). Hence, some membrane receptors are required for the GPI anchored proteins to be recognized by the COPII vesicles. The members of P24 family (Emp24, Erv25, Erp1 and Erp2) acts as receptors for the GPI anchored proteins and are involved in their packing into COPII vesicles (Castillon et al., 2011). The inner layer of COPII vesicles is composed of Sar1 (a small GTPase), Sec23 and Sec24 proteins (Bi et al., 2002; Hughson, 2008). Sar1 and heterodimer of Sec23/Sec24 recruit the outer components (Sec13 and Sec31) of COPII vesicles at the ER exit site (ERES). Sec13 and Sec31 form a hetrotetramer and along with the other proteins form a COPII vesicle (Bi et al., 2002; Hughson, 2008). In case of yeast, the P24 family receptors recognize only those GPI anchored proteins which undergo the lipid remodeling. But in mammals, since the lipid remodeling occurs in the Golgi thus the P24 family receptors recognizes the GPI anchored proteins consisting of unsaturated fatty acid at *sn-2* position (Castillon et al., 2011; Fujita et al., 2011). The association and dissociation of GPI anchored proteins with the COPII vesicle

receptors is dependent on the pH of the compartment (Fujita et al., 2011). The basic pH of ER facilitates the association of GPI anchored proteins whereas the acidic pH of the Golgi facilitates the dissociation of the GPI anchored proteins from the COPII vesicles (Fujita et al., 2011). After the dissociation, P24 family proteins are recycled to ER *via* COPI vesicles along with unremodeled GPI anchored proteins (Aguilera-Romero et al., 2008; Castillon et al., 2011).

I.2.1.10 Cross linking of GPI anchored proteins with the cell wall in yeast

In yeast, GPI anchored proteins are targeted either to the plasma membrane or the cell wall. The final destination of GPI anchored proteins depends on the presence of the particular amino acids upstream to ω-site (GPI anchor attachment site) which has been described later in detail. For the cell wall anchoring of GPI anchored proteins, the GPI moiety gets cleaved between the GlcN and the first mannose residue and a glycosidic bond is formed between the reducing end of the mannose and the non-reducing end of β-1,6 glucan (polymer of glucose) chain in the cell wall of yeast. Further, reducing end of β-1,6-glucan was found to be linked to the non reducing end of β-1,3-glucan (Kapteyn et al., 1999, 2000; Kollar et al., 1997; Lu et al., 1995), and the non reducing end of β-1,3-glucan linked to the chitin *via* β-1,4 linkage which was confirmed by β-1,3-glucanase and chitinase treatment (Kollár et al., 1995). Most of the GPI anchored CWPs were found to be solubilized by β-1,3-glucanase and β-1,6-glucanase digestions suggesting that these proteins are covalently linked with β-glucans (Kapteyn et al., 1999). In *C. albicans*, about 88 % GPI anchored cell wall proteins are found to be covalently linked *via* β-1,6-glucan and β-1,3-glucan and among them, Als1 and Als3 adhesins contribute about 90 % of total covalently linked GPI anchored cell wall proteins (Kapteyn et al., 2000). In yeast several GPI anchored cell wall proteins are studied, which are required for adhesion and cell wall stability; a-agglutinin, α-agglutinin, Cwp1p, Cwp2p, Tip1, Tir1p, Tir2p, Icwp, Flo1p and Sed1p (Caro et al., 1997; Kondo and Inouye, 1991; Kowalski

et al., 1995; Lipke et al., 1989; Moukadiri et al., 1997; Roy et al., 1991; Shimoi et al., 1998; van der Vaart et al., 1995). The a-agglutinin and α-agglutinin are involved in the adhesion of opposite mating cells types of *S. cerevisiae* (Lipke et al., 1989; Roy et al., 1991). The Cwp1p and Cwp2p required for the stability of the cell wall in *S. cerevisiae* (Shimoi et al., 1995; van der Vaart et al., 1995). The Icwp is present in the inner leaflet of the cell wall and also play a structural role in the cell wall of *S. cerevisiae* (Moukadiri et al., 1997). The Sed1p found to be involved in the stability of the cell wall in the stationary phase of *S. cerevisiae* cells and protect the cell wall from the lytic enzymes such as zymolyase (Shimoi et al., 1998). *S. cerevisiae TIP1* is a stress responsive gene which highly expressed both cold shock as well as heat shock responses (Kondo and Inouye, 1991). The *TIR1* and *TIR2* are the homologue of *TIP1* and transcription of these genes is induced in response to cold shock (Kowalski et al., 1995). The *FLO* gene family encodes for five cell wall proteins known as flocculin (Flo1p, Flo5p, Flo9p, Flo10 and Flo11p), these proteins are mainly involved in adhesion (Bidard *et al*., 1995; Caro *et al*., 1997). Similarly, *C. albicans* also has several GPI anchored cell wall proteins; Als protein family, Hwp family, Hyr family, Ywp1, Eap1 and Ecm33 (Bailey et al., 1996; Bates et al., 2007; Gil-Bona et al., 2016; Hayek et al., 2010; Hoyer, 2001; Li et al., 2007; Nobile et al., 2006). The detailed studies of these proteins have been described later in the text.

I.3 Significance of GPI anchor and GPI anchored proteins

I.3.1 GPI anchor acting as an ER exit signal

GPI anchors may act as putative ER exit signals. It has been discussed above that how the GPI anchored proteins are transported out from the ER and what receptors are involved in their transport. Defects in the GPI anchor biosynthesis results in blocking the attachment of GPI to the proteins thus blocking the exit of proteins from ER (Doering and Schekman, 1996). If mutations block the cleavage of C-terminal hydrophobic signal sequence from the

protein, the protein would be retained in the ER lumen along with the GPI attachment signal sequence and these proteins would be recognized by the quality control system of ER as unfolded proteins. This would result in retention of protein in the ER until their proper folding or else they would go into the degradation pathway (Ellgaard and Helenius, 2003). In early studies, it was shown that yeast cells growing in inositol-deprived conditions (which will block the GPI anchor biosynthesis) halted the segregation of Gas1p into the COP-II coated vesicles, suggesting that GPI anchor acts as a marker for the ER exit signal for the proteins (Doering and Schekman, 1996).

I.3.2 GPI anchor may alter the conformation of protein

GPI anchors may change the structure and conformation of the protein to which they are attached (Paulick and Bertozzi, 2008). It has been shown that OX7 antibody specifically binds to GPI anchored Thy-1 protein but not to the Thy-1 protein after removal of lipid by the action of phospholipase-C. CD analysis of protein in both conditions showed the conformational differences and suggested that due to the structural differences antibody couldn't bind to Thy-1 protein after phospholipase-C treatment. These results imply that the GPI anchor alters the conformation of proteins (Barboni et al., 1995).

I.3.3 GPI anchors determine the fate of cells and are associated with diseases

It has been reported that the GPI anchor biosynthesis is crucial for cell growth and viability, development and cell viability in eukaryotes (Ferguson, 1999; Leidich et al., 1994). In mammals GPI anchor biosynthesis is required for embryonic development. It has been reported that the defect in GPI anchor biosynthesis leads to embryonic lethality (Kawagoe et al., 1996; Nozaki et al., 1999).

Defects in the mammalian GPI anchor biosynthesis pathway leads to several diseases. PIG-A has been identified as the catalytic subunit of the GPI-GnT complex. Defect in PIG-A leads to the paroxysmal nocturnal hemoglobinuria (PNH). PNH is a clonal hematopoietic stem cell disorder which leads to intravascular hemolysis, thrombosis and bone marrow failure (Brodsky, 2006; Johnston et al., 2012). Over expression of PIG-P has been found to cause fetal Down syndrome (Ferrando-Miguel et al., 2004). Down regulation of PIG-M gene causes thrombosis and seizures, whereas hyperphosphatasia and mental retardation has been reported due to mutation in PIG-V and PIG-O (Almeida et al., 2009, 2006; Krawitz et al., 2012). Mutation in PIG-N gene causes multiple congenital anomalies-hypotonia-seizures syndrome, epilepsy and neurological impairment (Maydan et al., 2011; Ohba et al., 2014). Apart from this, defects in expression of PIG-L and PIG-Y were observed in Burkitt's lymphoma (Hu et al., 2009). Heterozygous mutation in PIG-L causes CHIME syndrome which leads to mental retardation, ear abnormalities and heart defects (Ng et al., 2012). The homozygous mutation in PIG-T gene leads to intellectual disability syndrome which is characterized by hypotonia, seizures and hypophosphatasia (Kvarnung et al., 2013). Over expression of GAA1 has been identified to be involved in oncogenesis (Jiang et al., 2007).

In *S. cerevisiae*, GPI anchor biosynthetic pathway is critical for cell viability and cell wall biogenesis as well (Leidich et al., 1994, 1995). The *gpi1*, *gpi2* and *gpi3* mutants were unable to form GlcNAc-PI *in vitro*. The single mutants of *gpi1*, *gpi2* and *gpi3* showed temperature sensitive growth while the double mutants were inviable suggesting that GPI anchor biosynthesis is essential for growth of *S. cerevisiae* (Leidich et al., 1994, 1995). In *C. albicans*, defects in GPI anchor biosynthesis leads to growth defect, cell wall defects, defects in filamentation and inviability (Grimme, 2004; Shen et al., 2015; Singh et al., 2016; Yadav et al., 2014). Depletion of *CaSMP3* affected growth, cell wall biogenesis and viability of *C. albicans* (Grimme, 2004). The *Cagpi2* mutant is affected growth, viability, filamentation and

altered cell wall integrity in *C. albicans* (Yadav et al., 2014). Recently, it has been reported that the *Cagpi14* mutant leads to growth defect, cell wall defects and affected hyphal morphogenesis (Singh et al., 2016). As mentioned earlier, CaGpi7 depleted *C. albicans* cells blocked the localization of GPI anchored proteins to the cell wall and were killed by the host immune response (Shen et al., 2015). In fungi GPI anchored proteins play very important role in virulence. There are several GPI anchored proteins which act as virulence factors. These proteins are involved in the adhesion and invasion of fungal species into the host cells which will be discussed later in detail.

In pathogenic protozoan, GPI anchored proteins are required for their protection from host immune defense system and their survival in the host. For example, in *T. brucei* the variable surface glycoprotein (VSG) is a GPI anchored protein, which forms the coat protein (Ferguson, 1999; Masterson et al., 1989). *T. brucei* frequently changes these coat proteins to avoid the host recognition which helps it in establishing infections in the host (Carrington et al., 1991; Doering et al., 1990; Ferguson, 1999). Likewise, *Leishmania* also expresses GPI-anchored proteins on its surface (Medina-Acosta et al., 1989; Schneider et al., 1990). These proteins provide protection from the host immune system, and help in the virulence of the organism. A cell surface protein gp63 helps in the survival of *Leishmania* promastigotes in the host (Medina-Acosta et al., 1989). These parasite shows differences in the GPI biosynthetic pathway which has been discussed earlier in the text. In these parasites, structural differences of GPI anchors, differences in enzyme specificity with reference to the respective human orthologs and the difference in proteins associated with these anchors make this pathway a probable drug target against parasite infections (Eisenhaber et al., 2003).

I.3.4 Hydrolytic enzymes

Some GPI anchored proteins are hydrolytic enzyme, such as alkaline phosphatase, acetylcholinesterase and 5'-nucleotidase in mammals (Low, 1989). Alkaline phosphate catalyzes the hydrolysis of phosphomonoesters, acetylcholinesterase hydrolyses acetylcholine and 5'-nucleotidase catalyzes the hydrolysis of ribose and deoxyribose at 5' carbon of nucleotides (Cousin et al., 1998; Massoulie, 2002; Millan, 2006; Zimmermann, 1992).

I.3.5 Signal transduction

Membranes are rich in lipid rafts, which are made up of microdomains of sphingolipids and cholesterol (Pike, 2003). These lipid rafts are composed of sterols, lipids and proteins and involved in signal transduction (Pike, 2003). GPI anchored proteins are normally associated with lipid rafts and are involved in signaling (Sangiorgio et al., 2004). Some GPI anchored proteins (CD14, CD48, CD55 and CD59 molecules from human and Thy-1 and Ly-6 molecules from mouse) was found to be having protein tyrosine kinase activity which activates signaling pathways (Shenoy-Scaria et al., 1992; Stefanová et al., 1991).

I.3.6 Adhesion molecules and cellular communication

GPI anchor proteins are present on the cell surfaces, and play a role in adhesion and cellular communication. Several GPI anchored proteins DAF, Thy-1 and NCAM are move in membrane rapidly and facilitates cellular interactions (Low and Saltiel, 1988; Paulick and Bertozzi, 2008; Saha et al., 2015).

I.3.7 Formation of coat proteins

Protozoans are the best example of presenting antigenic variant coat proteins. In *Trypanosoma brucei*, the variable surface glycoprotein (VSG) forms the coat, which protects

the microorganism from the host defense system. This microorganism frequently changes their coat proteins, which help to avoid the host recognition (Carrington et al., 1991; Ferguson et al., 1985).

I.3.8 Role in prion disease

It has been reported that GPI anchor attachment leads to conformational changes in some proteins; for example the normal form prion protein (PrPC) converts into pathogenic form (PrPSc) (Taylor and Hooper, 2006). These conformational changes are the main cause of prion disease. In prions disease, the prion proteins make insoluble aggregates of disordered structures and deposit as plaque leading to neurodegeneration (Taylor and Hooper, 2006).

I.4 Role of GPI anchor attachment signal sequences

Proteins destined to be GPI anchored possess two signal sequences: N-terminal signal sequence and C-terminal signal sequence. N-terminal signal sequence is the ER targeting signal for GPI proteins. Initially, it was proposed that after entry of the proteins into the ER lumen, N-terminal signal sequence is cleaved by N-terminal signal peptidase (Gerber et al., 1992). In later studies, it was identified that the cleavage of N-terminal signal sequence is not a prerequisite for GPI anchoring of proteins. A study attempted to make fusion proteins of neural endopeptidase (a transmembrane protein, attached to the membrane with intact N-terminal signal sequence) and decay accelerating factor (GPI anchored protein) (Howell et al., 1994). They constructed three expression vectors encoding a wild type neural endopeptidase (NEP), a GPI anchored neural endopeptidase and a double anchored (anchored *via* both GPI and uncleaved N-terminal signal sequence) endopeptidase (DA-NEP). When these fusion proteins were expressed in CHO-1 cells they observed similar levels of expression of proteins anchored by three different ways to the membrane; *via* uncleaved N-terminal signal sequence, *via* GPI anchor and *via* double anchored (Howell et al., 1994). The

cell surface expression of these proteins was further confirmed by the treatment with PI-PLC (used for GPI anchored proteins cleavage) and toluene/trypsin (used for transmembrane proteins cleavage) (Howell et al., 1994). These results suggest that the cleavage of N-terminal signal sequence is not essential for GPI anchoring of proteins.

C-terminal signal sequence is the second and very critical signal sequence for the GPI anchoring of proteins. The sequence of C-terminal signal is not highly conserved but the structural features of the sequence is conserved (Mayor and Riezman, 2004; Orlean and Menon, 2007). After translocation of proteins into the ER lumen, the C-terminal signal sequence of precursor protein is recognized and replaced with the preformed GPI anchor by the GPI-transamidase enzyme (Weihofen et al., 2000). In this reaction, the transamidase recognizes the ω site (GPI anchor attachment site at C-terminal of protein) where the peptide bond breaks and an amide bond forms between amino group of ethanolamine-phosphate (Etn-P attached at the third mannose of GPI anchor) and α-carboxyl group of C-terminal amino acid of precursor protein (Mayor and Riezman, 2004; Mayor et al., 1991; Vainauskas and Menon, 2006).

For efficient GPI anchoring of proteins, the C-terminal signal sequence has some specific characteristic features as described below (Figure I.2).

- A flexible linker region must be present upstream of the ω site between positions ω-10 to ω-1, consisting of polar amino acid residues (Orlean and Menon, 2007).
- The ω-site must have amino acid residues with small side chains such as Ser, Gly, Ala, Asp, Asn or Cys (Micanovic et al., 1990; Moran et al., 1991).
- The ω+1 position is not restricted to amino acid residues with small side chains. When the ω+1 of truncated form of placental alkaline phosphatase (miniPLAP)

was mutated with Asp, Ser, Cys, Met, Thr, Glu, Arg, Trp and Pro, it was observed that all the amino acids allowed the GPI anchoring of protein except proline (Gerber et al., 1992; Orlean and Menon, 2007).

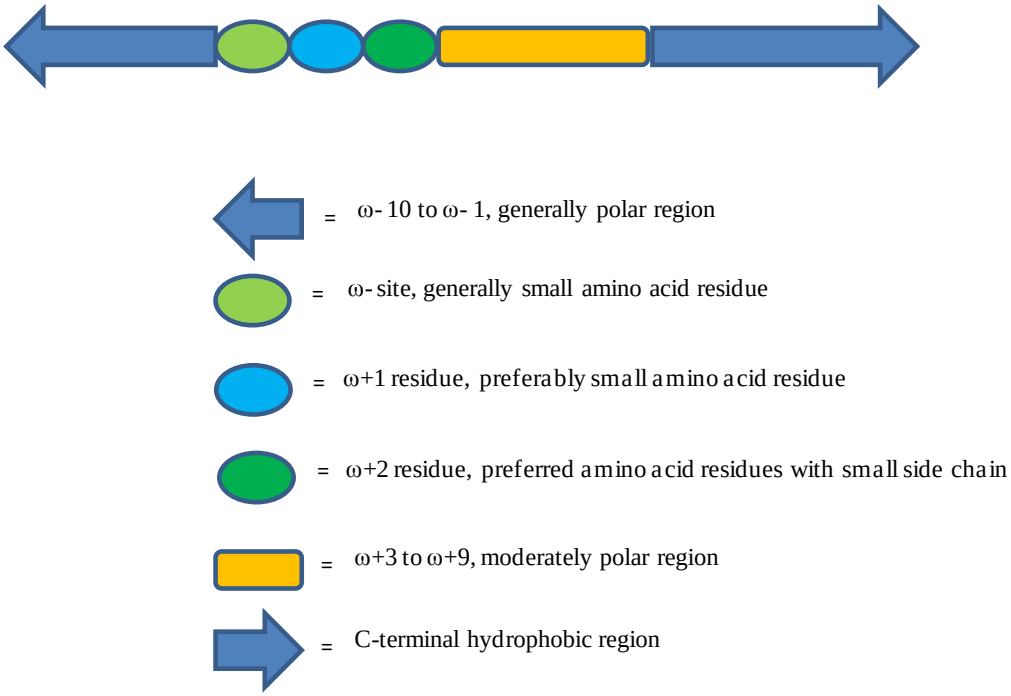

Figure I.2: Schematic representation of the C-terminal signal sequence. The figure shows the polar region at the N-terminus of ω-site, moderately polar region towards the C-terminus of ω site which is followed by hydrophobic stretch towards the extreme C-terminus.

- The ω+2 position (downstream of ω site) is the most restrictive, and requires the presence of amino acids with small side chains such as Gly or Ala. The ω+2 amino acid of placental alkaline phosphatase (PLAP) mutated with Ser, Val and Thr was found to be less effective for GPI anchoring as compared to wild type PLAP which consists Gly at ω+2 position (Kodukula et al., 1993; Orlean and Menon, 2007).
- The spacer region consists of moderately polar amino acid residues from ω+3 to ω+9 position, which is followed by a stretch of variable hydrophobic amino acid residues (Orlean and Menon, 2007).

Several studies have been shown that the ω site is very critical for the transamidase recognition. It has been defined by site directed mutagenesis in several studies. In a fusion of human growth hormone (secretary protein) with decay accelerating factor (GPI anchored protein)] when the ω site amino acid of the fusion protein was mutated with amino acids with small side chains like Ser, Ala, Asp, Asn and Gly, effective GPI anchoring was observed while Val, Cys and Glu residues were partially effective for GPI anchoring (Moran et al., 1991).

The same studies have been done in Gas1 protein (GPI anchor protein) of *S. cerevisiae* as well. By site directed mutational analysis in Gas1 protein, it has been shown that the ω site prefers amino acids with the short side chains such as Asn, Ser, Gly, Ala, Asn and Cys. Asn residue (present in wild type Gas1 protein) was identified as the most effective ω site amino acid for GPI anchoring. Ser and Gly residues were observed to be less effective than Asn but more effective ω site amino acids than Ala and Cys for effective GPI anchoring (Nuoffer et al., 1993).

The specific location of the C-terminal signal sequence has also been characterized. GPI anchor signal sequence is normally present at the extreme C-terminus of proteins, but it has been shown that the position of the GPI anchor signal sequence at the C-terminus of proteins is not strictly required. An internally localized GPI anchor signal sequence in a fusion protein [human growth hormone (hGH) fused with the C-terminus of DAF] also showed efficient GPI anchoring of DAF protein, indicating that GPI anchor signal sequence is not position-specific in mammals (Caras, 1991).

It has been shown that the GPI anchor attachment site is present 10-12 residues away from a hydrophobic sequence and the hydrophobic sequence in GPI anchor signal sequence is critical for effective GPI anchoring of proteins (Caras, 1989). The hydrophobic patch consists of approximately 10-20 hydrophobic amino acid residues. The minimum requirement of the

hydrophobic sequence varies for different proteins. For example, CD46 protein required minimum 11 amino acid long hydrophobic stretch for GPI anchoring (Coyne et al., 1993). In case of bovine liver 59-nucleotidase, a minimum sequence that is 13 amino acids long (Furukawa et al., 1994), and in placental alkaline phosphatase (PLAP) protein a sequence that is at least 17 amino acids long is required for proper GPI anchoring (Berger et al., 1988). Deletion of the hydrophobic patch from the GPI signal sequence led to the secretion of DAF, whereas replacement of the hydrophobic stretch with a general hydrophobic sequence resulted in correct processing of DAF protein (Caras and Weddell, 1989). Same studies were performed in hGH-DAF fusion protein, where the hydrophobic stretch of DAF was replaced with the signal peptide of hGH, resulting in the efficient GPI anchoring of DAF protein. All these studies suggested that the nature of the hydrophobic stretch is essential for GPI anchoring but not its specific sequence.

I.5 Differential location of GPI anchored proteins in mammals and yeast

In mammals, GPI anchored proteins are differentially sorted to the apical and basolateral membranes of polarized epithelial cells (Stoops and Caplan, 2014). The segregation of the apical and basolateral proteins occurs at the level of trans-Golgi network (Cao et al., 2012; Wandinger-Ness et al., 1990). Proteins sorted to the apical or basolateral membrane have different sorting signals (Paladino et al., 2006; Stoops and Caplan, 2014). Basolateral sorting signals reside within the cytoplasmic domain of the proteins, which are short amino acid motifs particularly tyrosine based and dileucine based motifs (Le Bivic et al., 1991; Miranda et al., 2001). While, apical sorting signal may reside in any domain of the protein (Chuang and Sung, 1998; Lin et al., 1998). Several studies have been shown that GPI anchored proteins are mostly sorted to the apical membrane in polarized epithelial cells (Lisanti et al., 1988, 1989). Distribution of the GPI anchored proteins on the apical surface of

the polarized Madin-Darby canine kidney (MDCK) cells was detected by using the PI-PLC mediated release of GPI anchored proteins (Lisanti et al., 1988). The apical targeting of GPI anchored proteins was further confirmed by making chimeric proteins (Lisanti et al., 1989). The endogenous decay accelerating factor DAF (GPI anchored protein) was found to be apically sorted in epithelial cells. In chimeric proteins (GPI anchor attachment signal sequence of DAF attached to a basolateral proteins herpes simplex glycoprotein D and to a secretory protein human growth hormone) the GPI anchor attachment signal of DAF sorted the basolateral and secretory proteins to apical surface of the polarized MDCK cells (Lisanti et al., 1989). Association of GPI anchored proteins with the detergent resistant microdomains suggested that the transport of GPI anchored proteins to the apical membrane is mediated by the association with lipid rafts (Lipardi et al., 2000). Lipid raft are composed of sphingolipids and cholesterol microdomains which are known to play a role in molecular trafficking and cell signaling (Simons and Ikonen, 1997). Association of the GPI anchored proteins with the lipid rafts is mediated by the GPI anchor in the trans-Golgi network and then delivered to the apical membrane of polarized epithelial cells (Harder and Simons, 1997; Simons and Ikonen, 1997). Cholesterol depletion was found to affect the oligomerization of apically sorted proteins suggested that raft association of the proteins at the Trans Golgi network is essential for apical sorting of GPI anchored proteins (Paladino et al., 2004). The same observations has been found in a recent studies performed by the same group (Paladino et al., 2014). Along with lipid rafts association, oligomerization of the GPI anchored proteins is also essential for the apical sorting of GPI anchored proteins (Paladino et al., 2004, 2007). Oligomerization of the GPI anchored proteins leads to the stabilization of the protein in lipid rafts (Paladino et al., 2004). It was observed that the apical as well as basolateral GPI anchored proteins associated with detergent resistant microdomains in Golgi apparatus, but only apically sorted proteins oligomerized and not the basolateral proteins suggesting that oligomerization is

required only for the apically sorted GPI anchored proteins (Paladino et al., 2004). The same group also reported that each GPI anchored protein makes homo-clusters (oligomerized by using same GPI anchored protein species) in the Golgi apparatus. Then they reach the plasma membrane of polarized MDCK cells where they formed cholesterol dependent hetero-clusters (formed by the homoclusters consisting different GPI anchored proteins). Whereas in non-polarized MDCK cells GPI anchored proteins are transported in monomeric form and they were unable to form hetero-clusters (Paladino et al., 2014).

GPI-CWPs are found to be either localized on plasma membrane or on cell wall of fungal cells. It has been reported that some amino acid residues within the C-terminal signal sequence critically determine the final destination of the GPI anchored proteins. Mutational studies in *S. cerevisiae* showed that Leucine (L), Valine (V) or Isoleucine (I) residues at positions ω-4/5 and Asparagine (N) or Tyrosine (Y) at position ω-2 site is critical for targeting GPI-protein to the cell wall. Yap3 (plasma membrane localized GPI anchored protein) contains Val or Ile residues at position ω-4 or ω-5 site and Val or Tyr at position ω-2 site resulting in the cell wall localization of this protein. Dibasic amino acid residues (Lys and Arg), KK or KR, at ω-1 and ω-2 site acts as a signal for plasma membrane localization (Hamada et al., 1998, 1999). In *C. albicans*, KK/SS and FE dipeptides at ω-1 and ω-2 were found to be acting as a signal for plasma membrane and cell wall respectively (Mao et al., 2008). It suggested that dibasic residues at ω-1 and ω-2 sites target the proteins to plasma membrane whereas hydrophobic residues target to cell wall in *C. albicans* (Mao et al., 2008).

I.6 GPI anchored proteins in *Candida albicans*

In eukaryotes, 1 % of all proteins or 10-20 % of membrane proteins are postranslationally modified by GPI anchors. In eukaryotes, from protozoa to mammals, several GPI anchor proteins have been reported (Ferguson and Williams, 1988; Nosjean et

al., 1997; Taylor and Hooper, 2011). In *C. albicans* GPI anchored proteins perform different functions and plays very important role in virulence. In a recent review, it has been reported that *C. albicans* consists of approximately 115 GPI anchored proteins. Among them the function of about 66 % proteins are unknown, about 13% genes having function in cell wall biogenesis, 11 % genes are involved in cell-cell interactions and adhesion and 9.5 % genes having enzymatic functions (Richard and Plaine, 2007). Some of the GPI anchored proteins include the following: The Als protein family consists eight members, which are involved in adhesion and cellular aggregation (Hoyer, 2001; Rauceo et al., 2004). Hwp family proteins (Hwp1, Hwp2and Rbt1), Hwp1 is involved in biofilms formation, Hwp2 is involved in filamentation and invasion and Rbt1 is also involved in adhesion and biofilms formation (Hayek et al., 2010; Monniot et al., 2013; Nobile et al., 2006). EAP1 is involved in adhesion and biofilms formation ((Li et al., 2007). Ecm33 is involved in morphogenesis and cell wall integrity (Gil-Bona et al., 2016). The detailed study of all the GPI anchored proteins is given below in the text.

I.7 *ALS* gene family in *Candida albicans*

ALS (Agglutinin like Sequence) family consists of GPI-anchored surface glycoproteins in *C. albicans*. Als family consists of eight (Als1-Als7 and Als9) different adhesins encoded by eight different *ALS* genes (Hoyer et al., 2008). These adhesins have several functions like adhesion, aggregation and biofilm formation on the host cell surfaces (Gaur et al., 1999; Sundstrom, 1999; Klotz and Lipke, 2010). Initially, nine genes (*ALS1* to *ALS9*) were considered in *ALS* gene family (Hoyer, 2001), but in later studies, *ALS3* and *ALS8* were found to be present on the same loci and so *ALS8* gene was removed from the family (Zhao, 2004).

I.7.1 Structural features of *ALS* genes

Structural features are conserved in all the *ALS* family genes (Hoyer et al., 2008). The *ALS* genes consist of three main domains: a highly conserved N-terminal domain, variable central domain and C-terminal domain (Figure I.4). The N-terminal domain is most conserved among all the *ALS* genes where the central domain is highly variable due to the presence of inconsistent number of tandem repeat sequences. The C-terminal domain is also moderately variable among all *ALS* genes.

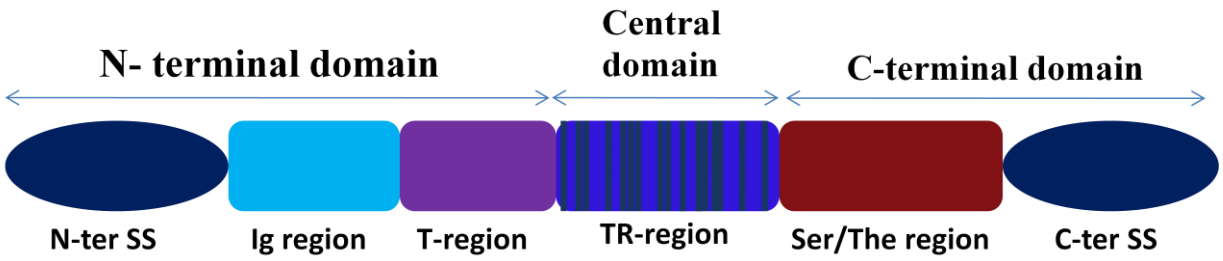

Figure I.3: The schematic diagram of structure of ALS domains.

I.7.1.1 N-terminal signal sequence

N-terminal signal sequence is a short stretch of amino acids, which acts as an ER targeting signal for the pre-pro-proteins. After entry of the proteins into the ER lumen, the N-terminal signal sequence is cleaved by N-terminal signal peptidase. Thus, it doesn't exist in the pre-protein and mature protein (Gerber et al., 1992).

I.7.1.2 N-terminal ligand binding domain

It is approximately 330 residues long, globular Ig-like structure, which is conserved (approximately 55 % - 90 % identical) across the *ALS* gene family (Rauceo et al., 2006; Klotz and Lipke, 2010). This region consists of ligand binding site which is responsible for the involvement of Als proteins in adhesion of *C. albicans* on the host cell surface (Loza et al., 2004). The N-terminal region of Als proteins consists of three Ig like domains which shows

similarity with α-agglutinin protein (adhesin glycoprotein which is responsible for mating of two haploid cells) of *S. cerevisiae*, suggesting the adhesion function of N-terminal domain of Als proteins (Hoyer et al., 1995; Lipke et al., 1989). Studies have been shown that the N-terminal of Als proteins is enough for adhesion to the substrates. Circular dichroism (CD) analysis of Als5 protein showed that the N-terminal of Als5 protein is β-sheet rich domain, which is similar to the α-agglutinin protein of *S. cerevisiae*, suggesting the role of Als5 in adhesion (Hoyer and Hecht, 2001). In later studies Loza *et al*., showed that deletion and insertion mutations in N-terminal of Als1 protein abrogated the adhesion of Als1 protein to endothelial cells (Loza et al., 2004). A recent study also showed that the N-terminal of Als9-2 [Als9-2 consists extra nucleotide region at the 3' end known as Variable Block 1 (VB1) and Variable Block 2 (VB2) which is absent in Als9-1 variant (Zhao, 2003)] protein is β-sheet rich domain and the Lys59 residue (present within N-terminal domain) is suggested to be important for binding to peptide ligands (Salgado et al., 2011) due to a salt bridge that Lys makes with the C-terminal carboxylate group of the peptide ligand (Salgado et al., 2011). To validate the importance of Lys59 in terms of ligand recognition, Als5 K59A mutant was made in our lab and it was observed that this residue is not involved in ligand recognition in Als5 although it is involved in aggregation (Faiz and Komath unpublished data).

Als proteins have been shown to bind to a broad range of substrates. Sheppard *et al*., has shown the ligand binding activity resides within the N-terminal domain of Als proteins (Sheppard et al., 2004). Despite having high similarity between the sequences of Als5 and Als6 proteins *S. cerevisiae* expressing Als5 binds to both epithelial cells and gelatin, while cells expressing Als6 binds only to gelatin (Sheppard et al., 2004). To identify these differences, chimeras were made by swapping N-terminal regions of Als5 and Als6. It was observed that the chimeric proteins showed the ligand specificity based on the N-terminal

domain of the Als protein (Sheppard et al., 2004). These results suggested that Als proteins show different substrate specificity which lies within the N-terminal domain of Als proteins.

I.7.1.3 T (Threonine rich) domain

T-domain is a threonine rich 104 residues long and highly conserved (90 %-98 % identical) region across the *ALS* gene family. Due to the abundance of threonine residues, this region is highly O-glycosylated (Rauceo et al., 2006). In 2008, Otoo *et al* identified the heptapeptide (IVIVATT) within the T region which is conserved in *ALS* gene family (Otoo et al., 2008). TANGO (discussed later) analysis identified that this heptapeptide has high tendency for β-aggregation and suggested the role of T-domain in protein aggregation. Staining of synthetic peptides (containing amyloid forming region) or cells expressing Als5 fragment ($Als5^{20-431}$ and Als520-664) with amyloid specific dyes such as thiofalvin T and Congo red suggested the role of the T-domain in β-aggregation (Otoo et al., 2008).

I.7.1.4 Tandem repeat domain

The tandem repeat domain was first identified in the sequence of *ALS1* gene, which consists of head-to-tail repeats of 108 base pair long sequence (Hoyer et al., 1995). This region is heavily glycosylated due to high content of Ser/The residues, which help in proper functioning and maintaining the secondary structure of Als proteins (Frank et al., 2010; Rauceo et al., 2006). Due to the presence of variable number of tandem repeats, this region is least conserved among the Als proteins. Tandem repeats are found to be responsible for allele or strain specific variability in Als proteins (Hoyer et al., 1995). The number of tandem repeats corresponds to the extent of adhesion and aggregation properties. Protein with more tandem repeats was found to be more adhesive and aggregating suggesting that TR domain governs the aggregation properties (Rauceo et al., 2006). Tandem repeat was also found to be involved in adhesion and cellular aggregation of Als proteins. In *in-vitro* assays, Als5 (Ig-T-TR) protein was found to be strongly bound to fibronectin as compared to Als5 (Ig-T). It

suggested that TR region enhances the adhesion of Als proteins (Frank et al., 2010; Rauceo et al., 2006). Same results were found in *in-vivo* assays. *S. cerevisiae* cells expressing Als5 (Ig-T-TR) was found to be more adherent to fibronectin and mediated more cellular aggregation as compared to cells expressing Als5 (Ig-T) (Rauceo et al., 2006).

Based on the cross hybridization of tandem repeats, Als adhesins have been sub-divided into three subfamilies. Tandem repeats of *ALS1, ALS2, ALS3* and *ALS4* genes cross hybridize with *ALS1*, so they belong to one subfamily (Hoyer, 2001; Hoyer et al., 1998a). Tandem repeats of *ALS6* and *ALS7* cross hybridize with *ALS5* gene so *ALS5, ALS6* and *ALS7* genes belong to another subfamily. The tandem repeat sequence of *ALS9* were found to be unique and *ALS9* belongs to a separate subfamily (Hoyer, 2001; Hoyer and Hecht, 2000).

I.7.1.5 C-terminal stalk (Ser/Thr) region

This is the highly O-glycosylated Ser/Thr rich region which constitutes about 50 % of total size of protein. This is very long and very less conserved region among all Als proteins (Klotz and Lipke, 2010; Dranginis et al., 2007; Hoyer et al., 1998). Glycosylation makes this region flexible so that the N-terminal of proteins can be extended away from the cell surface. Thus the N-terminus can be easily accessible and interact with ligands on host cell surfaces (Klotz and Lipke, 2010).

I.7.1.6 C-terminal signal sequence

As explained earlier, the C-terminal signal sequence is prerequisite for proteins to be GPI anchored. The amino acid sequence of C-terminal signal sequence is not conserved but the nature of amino acids is conserved among the GPI anchored proteins (Orlean and Menon, 2007). In Als protein family of *C. albicans*, GPI-anchor attachment signal sequence (SS) is a 20-22 amino acid long sequence (Hoyer et al., 1998a). Previous report from our lab has shown that the Als5 protein consist a 20 amino acid long GPI anchor attachment signal

sequence (Ahmad et al., 2012). The characteristic features of the C-terminal signal sequence have been discussed in detail earlier in the text.

I.7.2 Genetic variability in *ALS* genes

The sequence of all the members of *ALS* gene family is similar. Despite this, these genes have high genetic variability due to the presence of variable number of tandem repeats (36 amino acids long) in the central domain. This variability could be strain specific or allele specific. Thus, one gene could have different sizes in two strains or in two alleles of the same gene in the same strain (Hoyer et al., 1995). Based on cross hybridization of tandem repeats, these adhesins are subdivided into three subfamilies. Tandem repeats of *ALS1-ALS4* genes cross hybridize with *ALS1*, so *ALS1-ALS4* belongs to one subfamily (Hoyer, 2001; Hoyer et al., 1998a). The tandem repeats of *ALS6* and *ALS7* were found to be cross hybridizing with *ALS5* gene, so *ALS5-ALS7* genes has been placed into another subfamily. *ALS7* is also an exceptional adhesion in some senses. Despite having tandem repeats within the central domain, *ALS7* has tandem repeats in the C-terminal region also (Hoyer, 2001; Hoyer and Hecht, 2000). Als7 consist of 15 nucleotide long repeats, which encode for the VASES protein sequence. Due to the presence of variable number of tandem repeats, this sequence could be differing in length between 411- 441 nucleotide base pairs (Hoyer and Hecht, 2000).

The tandem repeat sequences of *ALS9* were found to be unique and *ALS9* was classified in a separate subfamily (Gaur and Klotz, 1997; Hoyer, 2001; Hoyer and Hecht, 2000). *ALS9* gene shows greater allelic variability than any other member of *ALS* family. Based on the variability in the 3' domain, *ALS9* further has been divided into two subfamilies i.e. *ALS9-1* and *ALS9-2*. It has been reported that *ALS9-2* have extra sequences in two regions [Variable block 1 (VB1) and Variable block 2 (VB2) at its 3' end. *ALS9-2* consist extra 144 and 177 nucleotide base pair in VB1 and VB2 respectively. Despite this *ALS9-1* and *ALS9-2* shows differences in tandem repeat region as well. It has been found that *ALS9-1* has long

tandem repeats whereas *ALS9-2* consists of short tandem repeats. Als9 also shows the sequence variability within 5' domain as well. The sequence within 5' domain of Als9-1 and Als9-2 was found to be 11 % variable in SC5314 strain of *C. albicans* (Zhao, 2003).

I.7.3 Functional role of individual member of Als family of *Candida albicans*

I.7.3.1 Als1

At the very beginning *ALS1* gene was identified and it was found that the N-terminus and C-terminus of Als1 protein resembled with the sequence of AGα1 protein of *S. cerevisiae* (Hoyer et al., 1995). In *S. cerevisiae* AGα1 protein (adhesion glycoprotein) involved in mating of two haploid cells, contributes to adherence (Lipke et al., 1989). The sequence similarity of Als1 protein with AGα1 protein suggested that it too may participate in adhesion of *C. albicans* (Hoyer et al., 1995). Heterologous expression of *ALS1* in non adherent *S. cerevisiae* showed increased adherence to the epithelial and endothelial cells (Fu et al., 1998). Further, the adherent properties of Als1 were confirmed by deletion as well as over expression of *ALS1* gene. The a*ls1* null mutant showed reduced adhesion to endothelial cells as compared to wild type strain, whereas adhesion was enhanced in *ALS1* overexpressing strain as compared to wild type strain (Fu et al., 2002).

Sheppard's group has also shown the adhesion properties of heterologously expressed Als1 protein. They performed adhesion assay with extracellular matrix proteins (gelatin, laminin and fibronectin) as well as with vascular epithelial and endothelial cells where they observed that *S. cerevisiae* expressing Als1 protein showed adherence to all the substrates (Sheppard et al., 2004). Another report showed that the purified N-terminal (18-432 residues) of Als1 protein binds to the extracellular matrix proteins laminin and fibronectin in micromolar concentrations (Donohue et al., 2011). They also showed that the purified N-

terminal domain of Als1 protein binds to fucose containing glycans indicating that Als1 protein acts as a fucose-specific lectin (Donohue et al., 2011).

The cell surface expression of Als1 protein has been confirmed by antibody binding. The monoclonal antibody against the N-terminal domain (17–432) of Als1 binds to the Als1 protein expressed on blastospores or germ tubes of *C. albicans* (Fu et al., 2002). The *als1/als1* mutant strain of *C. albicans* didn't show binding with the monoclonal antibody. It suggested the specificity of monoclonal antibody and the cell surface expression of Als1 protein (Fu et al., 2002). Coleman *et al* also did similar experiment (Coleman et al., 2010). They observed that yeast as well as hyphae forms expressing Als1 shows binding to anti-Als1 antibody. They also showed that the anti-Als1 monoclonal antibody inhibited the adhesion of *C. albicans* to buccal epithelial cells, suggesting that Als1 protein is mainly involved in adhesion (Coleman et al., 2010).

It was also found that Als1 expression is associated with growth stage and growth medium. As mentioned above, Als1 was found to be expressed in both yeast as well as hyphae forms but highly expressed during biofilm growth (Coleman et al., 2010; Nobile et al., 2008). Expression of Als1 was found to be slower during progressive growth of culture while it increased with the transfer of cells (either in yeast or hyphae form) from the overnight saturated culture into the fresh medium (Coleman et al., 2010; Green et al., 2005).

Als1 protein was also tested as a vaccine against *Candida* infections in mice (Ibrahim et al., 2005; Spellberg et al., 2005). It has been shown that the 20 µg of recombinant N-terminal Als1 protein (administered intraperitoneal as well as subcutaneous) was sufficient to improve the survival of mice suffering from disseminated candidiasis. Vaccine based improvement in survival of mice was observed in B-cell deficient mice but not in T-cell deficient mice which suggested that the mechanism of protection is a cell mediated

phenomena; rather than developing immunity against *C. albicans* (Ibrahim et al., 2005; Spellberg et al., 2005). Apart from this, in a very recent study, it has been shown that Als1 and Als3 expressing *C. albicans* isolates from vaginitis were sensitive to fluconazole. It suggested that Als1 and Als3 could be efficient drug targets as well (Roudbarmohammadi et al., 2016).

Als1 protein is also involved in producing inter species communities on the host surfaces (Hoyer et al., 2014). Als1 protein was found to interact with the SspB protein of *S. gordonii* (Hoyer et al., 2014). In the double mutant of *als1/als3* very less interactions were observed between *C. albicans* and *S. gordonii*, whereas the reintegration of Als1 protein significantly increased the interactions suggesting that Als1 plays a role in adhesion of *C. albicans* to *S. gordonii*.

I.7.3.2 Als2

Als2 protein was found to be expressed in biofilms and has a role in adhesion of *C. albicans*. The expression of *ALS2* has not been detected in *in vitro* conditions implying that *in vivo* signals might be essential for its expression (Hoyer et al., 1998a; Zhao, 2005). The null mutant of *als2* has not been studied so far indicating that *ALS2* might be an essential gene for *C. albicans*. However, conditional null (*als2Δ/PMAL2-ALS2*) mutant has been studied (Zhao, 2005). Conditional null mutant showed decreased adhesion to endothelial cells and reconstituted human epithelium (RHE) cells, reduced RHE destruction, impaired and slower germ tube formation in glucose containing medium suggesting that the Als2 protein also functions as an adhesion in *C. albicans* (Zhao, 2005). The null mutant strain of *als4* showed increased levels of *ALS2* expression and vice-versa, suggesting that Als2 complements the function of Als4 (Zhao, 2005).

I.7.3.3 Als3

ALS3 expression was found to be associated with hyphae (Hoyer et al., 1998b). Expression of ALS3 was detected by GFP fluorescence in hyphae form all over the biofilms catheter model (Zhao, 2006). The *als3* null mutant was defective in biofilm formation and overexpression of Als3 restored biofilm formation in biofilm defective strain *in vitro* as well as *in vivo* suggesting that Als3 is involved in biofilms formation (Nobile et al., 2006, 2008; Zhao, 2006). Overexpression of *ALS3* in a biofilms defective *efg1* null mutant restored biofilm biomass equal to wild type but didn't form hyphae, suggesting the role of Als3 protein in biofilm formation is not dependent on hyphal formation (Zhao, 2006).

S. cerevisiae expressing Als3 protein showed adherence to extracellular matrix proteins (gelatin, laminin and fibronectin), epithelial and endothelial cells (Sheppard et al., 2004). Murciano *et al* (2012) described a role for Als3 in adhesion, cell damage and cytokine production in oral epithelial cells (Murciano et al., 2012). A recent research showed that a defect in the N-terminal domain of Als3 (peptide binding cavity) leads to defects in adhesion similar to that seen in a*ls3* null mutant (Lin et al., 2014). Peptide binding cavity (PBC) mutated Als3 strain was non-adherent on human pharyngeal epithelial, umbilical vein endothelial cells and human buccal epithelial cells suggesting that an intact PBC in Als3 was required for adhesion of *C. albicans* to host cells. The same work also studied the contribution of amyloid forming region (AFR) of Als3 in adherence. Mutations in AFR (destroyed amyloid potential) didn't alter adhesion, suggesting that AFR is not involved in adhesion of Als3 (Lin et al., 2014).

Cell surface expression and adhesion of Als3 protein has been confirmed by antibody staining. Monoclonal antibody against the N-terminal of Als3 protein binds to germ tube of wild type *C. albicans* cells expressing Als3 and blocked the adhesion to buccal epithelial as

well as vascular endothelial cells, whereas the antibody was unable to binds with *als3* null mutant of *C. albicans*, suggesting the cell surface expression of Als3 protein and its role in adhesion (Coleman et al., 2009).

Als3 is also known as an invasin. Invasion is a process where fungal cells enter to the epithelial as well as endothelial cells of host tissues and cause deep infections. *S. cerevisiae* strain expressing Als3 protein is involved in endothelial cell invasion (Sheppard et al., 2004). Als3 binds to E-Cadherin on epithelial cells and N-Cadherin on endothelial cells, which induces endocytosis of *C. albicans via* clathrin dependent endocytosis (Phan et al., 2007). Molecular modelling studies have suggested that the binding parameters of N-terminal of Als3 with E-Cadherin and N-Cadherin are comparable to that observed for Cadherin-Cadherin interactions in the host (Phan et al., 2007).

Als3 protein is also involved in the formation of inter-species communities of *C. albicans* with other species. It has been shown that Als3 protein of *C. albicans* interacts with SspB proteins of *S. gordonii* (human flora resides in oral cavity) which causes more persistent and more adherent stable communities on the host cells (Bamford et al., 2015). The *als3* null mutant strain was unable to interact with the SspB proteins of *S. gordonii* (Silverman et al., 2010). To identifying the interacting regions in *C. albicans*, deletion constructs of Als3 were made. The Δ166–225, Δ218–285, Δ270–305 and Δ277–286 deletion strain prevented the binding of *S. cerevisiae* expressing these Als3 variant to *S. gordonii*, indicating that these regions were involved in formation of mixed species communities (Bamford et al., 2015). On the other hand the deletion mutant in amyloid region (Δ325–331) didn't show any difference in interaction of Als3 protein with SspB proteins of *S. gordonii*, suggesting that the inter-species interactions are not dependent on the amyloid region (Bamford et al., 2015).

Besides acting as an adhesin and invasin, Als3 is also involved in iron acquisition from host ferritin (Almeida et al., 2008). The epithelial cells incubated with *C. albicans* in ferritin depleted condition were protected whereas the cells in ferritin enriched condition were susceptible to damage, which suggested that *C. albicans* could use epitheilial ferritin as a iron source. This was confirmed by using an iron chelator bathophenanthroline disulphonic acid which sequesters the extracellular iron. In an *in vitro* assay, *C. albicans* cells incubated with ferritin in the presence of bathophenanthroline disulphonic acid grew well suggesting that ferritin is a source of iron for *C. albicans* (Almeida et al., 2008). *C. albicans als3* null mutant showed poor growth on agar plats enriched with ferritin, which was improved by the reintegration of the one allele of *ALS3* in the strain, again suggesting that Als3 protein acts as a receptor for ferritin (Almeida et al., 2008).

Als3 protein could be a potential vaccine against *Candida* infections. The recombinant N-terminal of Als3 protein improves the survival of mice suffering from disseminated, oropharyngeal and vaginal candidiasis by generating cell mediated as well as humoral immunity (Spellberg et al., 2006).

I.7.3.4 Als4

ALS4 expression was found to be associated with growth phase of the cells. Its expression was observed in mid-log phase of cells and the highest expression was seen towards stationary phase (Hoyer et al., 1998a; Zhao, 2005). The transcription of *ALS4* was found to be temperature dependent. It was greater in cells grown at the temperature of 30 °C as compare to those grown at 37 °C (Zhao, 2005).

Als4 protein is also involved in adhesion of *C. albicans* (Zhao, 2005). The *als4* null mutant showed reduced adhesion to vascular endothelial cells as compare to wild type, but not to buccal epithelial cells and RHE cells. These differences in adhesion

properties are due to complementating the function of *als4* mutant by increased expression levels of Als2 (Zhao, 2005). As mentioned previously, *als4* null mutant showed 2.8 fold increased levels of *ALS2* expression and the *als2* null mutant showed 3.2 fold increased levels of *ALS2* expression, suggesting that the Als4 complements the function of Als2 protein in *C. albicans* and vice-versa (Zhao, 2005). The localization studies of Als4 protein has also been done using anti-Als4 monoclonal antibody. Expression of Als4 has been observed in yeast as well as hyphae cells (Coleman et al., 2012). Anti-Als4 monoclonal antibody was found to associate with Als4 protein expressed on the yeast form of cells as well as on germ tubes (Coleman et al., 2012). The yeast culture grown at the temperature of 30 °C showed higher abundance of Als4 protein as compare to that at 37 °C, suggesting the temperature dependent expression of Als4 protein. It was also observed that the expression of Als4 was detected at the very early time point of inoculation of yeast cells into germ tubes inducing media. At later time points Als4 was detected proximal to mother yeast cells, which remained till hyphae maturation (Coleman et al., 2012).

I.7.3.5 Als5

ALS5 was initially identified as *ALA1*, but later it was named as ALS5 (Gaur et al., 1999). It is involved in adhesion and aggregation of *C. albicans* on the host cell surface (Gaur and Klotz, 1997). The adherence properties of Als5 have been studied by heterologous expression of Als5 in *S. cerevisiae* (non-adherent) as a model organism. *S. cerevisiae* cells were transformed with *ALS5* from the low copy number vector. *S. cerevisiae* expressing Als5 showed adhesion to human buccal epithelial cells and magnetic beads coated with extracellular matrix proteins (fibronectin, collagen type IV and laminin), but didn't show aggregation (Gaur and Klotz, 1997). It suggested that the low levels of Als5 expression was

enough for adhesion but not sufficient for cellular aggregation. Later when the same group transformed *S. cerevisiae* cells with *ALS5* from a high copy number vector, they observed adherence to extracellular matrix protein as well as cellular aggregation, suggesting that the adhesion and aggregation depends on the concentration of the protein (Gaur et al., 1999). This idea was further supported by expressing Als5 under GAL promoters [GALS (weak), GALL (moderate), and GAL1 (strong) promoter]. They observed adhesion and aggregation in cells grown in galactose media, which could be correlated with promoter strength (Gaur et al., 1999). Sheppard *et al* reported that *S. cerevisiae* cells expressing Als5 shows adherence to extracellular matrix proteins (gelatin, laminin and fibronectin) as well as with vascular epithelial and endothelial cells (Sheppard et al., 2004). Similarly, an another report also found that *S. cerevisiae* strain over expressing Als5 showed adherence on the fibronectin coated magnetic beads and formed large aggregates. These effects were inhibited by polyclonal antibody against Als1 protein, indicating that Als5 expression is essential for adhesion and cellular aggregation (Rauceo et al., 2004). It was also shown that Als5 mediated adhesion of *S. cerevisiae* cells on extracellular matrix proteins coated beads was resistant to vigorous vortexing, was stable between pH-4 to pH-8, but greatly reduced at pH-9 or higher and reversibly inhibited in the presence of 6 M urea or 50% formamide (hydrogen bond disrupting agents) (Gaur et al., 1999).

Zhao *et al*., performed adhesion studies of Als5 protein in *C. albicans* and found contradictory results. *C. albicans* strain with deletion of Als5 resulted in increased adhesion on epithelial as well as endothelial cells as compare to control strain, indicating the anti-adhesion function of Als5 (Zhao et al., 2007a). The authors suggested that, there could be the possibility of up regulation of some other adhesins in deletion strain of Als5. They also observed that *C. albicans* strain with deletion of Als5 showed slower growth. Which was

restored by reintegration of wild type allele of *ALS5*, indicating that Als5 might be involved in cell growth of *C. albicans* (Zhao et al., 2007a).

So far, several studies have characterized different deletion mutants of Als5 protein. It has also been shown that the C-terminal signal sequence is critical for GPI anchor attachment to the proteins (Moran and Caras, 1991; Moran et al., 1991). But, not a single report has successfully characterized the full length Als5 protein *in vitro*. Previous studies from our lab have reported the characterization of full length Als5 protein (Ahmad et al., 2012). One of my seniors (Dr. Faiz Ahmad) has shown that apart from acting as a signal for GPI anchor attachment, C-terminal signal sequence also dictates the conformation and function of Als5 protein *in vitro* (Ahmad et al., 2012). This has been described later in the text.

I.7.3.6 Als6

Als6 protein was identified by Hoyer and Hecht (Hoyer and Hecht, 2000). Als6 protein also has a role in adhesion. Its transcription levels were extremely low in *C. albicans* cells isolated from infected buccal RHE cells, suggesting that low levels of Als6 are sufficient for function (Green, 2004). *S. cerevisiae* strain over expressing Als6 showed adherence to gelatin (extracellular matrix protein) (Sheppard et al., 2004). *C. albicans* strain with deletion of Als6 resulted in increased adhesion on epithelial as well as endothelial cells as compare to control strain of Als6 (Zhao et al., 2007a). These results were contradictory to the known function of adhesins. The authors suggested that, this could be either due to the compensation by another Als protein or it could be that Als6 has an anti-adhesion function in *C. albicans* (Zhao et al., 2007a). Further, Als6 deleted strain of *C. albicans* showed slower growth, suggesting that Als6 might be associated with cell growth as well (Hoyer et al., 2008).

I.7.3.7 Als7

ALS7 was also discovered along with *ALS6* in 2000 by Hoyer and Hecht (Hoyer and Hecht, 2000). Its expression is found to be at very low levels and the detection of its transcript levels were very difficult from the buccal RHE cells infected with *C. albicans* (Green, 2004). Overexpression of *ALS7* in *S. cerevisiae* strain didn't improve adherence properties and deletion of *ALS7* resulted in increased adhesion on epithelial and endothelial cells, suggesting that Als7 has an anti-adhesion role in *C. albicans* (Sheppard et al., 2004; Zhao et al., 2007a).

I.7.3.8 Als9

The null mutant of Als9 was defective in adhesion to human vascular endothelial cells (Zhao et al., 2007b). The reintegration of wild type Als9-2 allele restored the adhesive properties, but reintegration of Als9-1 allele was unable to restore adhesion. Adhesion was also restored by the fusion gene consisting of 5' domain of Als9-2 and central domain and 3' domain of Als9-1. These results suggested that Als9-1 and Als9-2 govern different functions due to the allelic variability within the 5' domain of Als9 (Zhao et al., 2007b).

Heterozygously expressed Als9 protein showed adherence on laminin, suggesting that Als9 has a role in adhesion (Sheppard et al., 2004). Molecular modelling and molecular dynamics simulations studies suggested that Lys59 of Als9-2 makes the salt-bridge with the C-terminal carboxylate group of the peptide ligand (Salgado et al., 2011). To validate the importance of Lys59 in terms of ligand recognition, Als5 Lys59Ala mutant was heterologously expressed in bacterial cells and purified in our lab for ligand-binding studies. The results suggested that Lys59 is involved in aggregation, but not in ligand binding (Faiz and Komath unpublished data).

I.7.4 Functional diversity of Als proteins

The functional aspects of the individual Als protein has been described above in the detail. Here, we have given a brief summery for all the Als proteins. All the members of Als proteins are diverse in terms of functions. Als proteins has been shown different substrate specificities. Sheppard's group studied substrate binding of all heterologously expressed Als proteins except Als2 and Als4 due to problems in their amplification. They observed that all the Als proteins have different substrate specificities (Sheppard et al., 2004). Chimeras were made by swapping of N-terminals of Als5 and Als6 and it was observed that the ligand binding specificity lies within the N-terminus of Als proteins (Sheppard et al., 2004). Later on, the role of Als2 and Als4 was reported by Zhao *et al.*, which has been discussed in the text above (Zhao, 2005). Some other studies have also shown the functional aspect of domain or truncated proteins, which has already been discussed in the text above (Loza et al., 2004; Rauceo et al., 2006).

I.7.5 Amyloid like aggregation by Als proteins

Amyloids are the fibers of insoluble form of proteins, which is found in several neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease (Koo et al., 1999). The proteins involved in the amyloid formation consist of β-sheets rich amyloid forming sequences. Amyloid specific dyes (Congo Red, Thiaflavin T and ANS) bind to the amyloid aggregates and cause inhibition of aggregation. Als protein mediated aggregates have similar characteristics to the amyloid aggregates, which are governed by amyloid forming sequences (Otoo et al., 2008; Rauceo et al., 2004). *S. cerevisiae* strain expressing Als5 showed ANS and Congo Red mediated reduction in aggregation and increased fluorescence of ANS in aggregating cells, suggesting that Als5 forms amyloid aggregates (Rauceo et al., 2004).

Otoo *et al* identified an amyloid forming sequence (IVIVATT) within the T domain of Als5 protein (Otoo et al., 2008). TANGO analysis predicted that, this region contributes to 93 % of β-aggregation in Als5 protein. This amyloid forming region (IVIVATT) was found to be highly conserved within the Als family of proteins. They also proposed that the amyloid forming region leads to the conformational changes in proteins and thus promotes aggregation (Otoo et al., 2008). A synthetic 13-mer peptide of Als5 protein consisting of the amyloid forming sequence formed aggregates that had enhanced absorbance of Congo Red and enhanced the fluorescence of Thioflavin T confirming that this sequence mediated amyloid like aggregtion (Otoo et al., 2008).

Similar to the synthetic peptide of Als5p, aggregates mediated by purified fragments of Als5 protein (Als5p20-431 and Als5p20-664) also formed fibrils (Otoo et al., 2008). They also bound amyloid specific dyes (Congo Red and Thioflavin T). *C. albicans* Als1 and Als5 proteins expressed in *S. cerevisiae* cells also exhibited similar results (Ramsook et al., 2010).

The aggregation mediated by the amyloid forming region (IVIVATT) was further confirmed by mutational approaches (Garcia et al., 2011). Cells expressing $Als5^{V326N}$ (mutation in amyloid patch) mutant protein showed very small aggregates as compare to cells expressing wild type Als5 (Garcia et al., 2011). Amyloid perturbing dyes Congo red and Thioflavin T inhibited the aggregation of cells expressing wild type Als5, but didn't show any effect on the aggregation of cells expressing $Als5^{V326N}$ mutant protein (Garcia et al., 2011). *S. cerevisiae* cells expressing $Als5^{V326N}$ incubated with the synthetic amyloid forming sequence of wild type Als5 protein ($SNGIV^{326}IVATTRTV$) formed large aggregates similar to wild type Als5 protein, suggesting that Als proteins mediated aggregation is dependent on amyloid formation (Garcia et al., 2011; Lipke et al., 2014). Later, Garcia *et al* develop a fluorescent peptide from *C. albicans* Als5 which consisting the amyloid sequence (Garcia-Sherman et al., 2014). This peptide bound to the *S. cerevisiae* cells expressing Als5. This

peptide also bound to the yeast and hyphae form of *C. albicans* which expresses Als1 and Als3 proteins, respectively, whereas the null mutants of a*ls1* and *als3* shown reduced fluorescence indicating that the fluorescent peptide binds to Als1 and Als3 proteins. The authors suggested that the amyloid sequence of Als1, Als3 and Als5 are similar therefore the Als5 fluorescent peptide would bind to Als1 expressing yeast and Als3 expressing hyphae form of *C. albicans* (Garcia-Sherman et al., 2014).

I.7.6 Biofilm formation in *Candida albicans*

Biofilms are a very important aspect of fungal infections. Biofilms are formed by association of multiple layers of fungal species. Biofilm formation occurs in a sequential manner and starts with adhesion of the cells on to abiotic or biotic surfaces. Adherent cells lead to cell proliferation across the surface, which is followed by the yeast to hyphae transition and accumulation of extracellular matrix in mature biofilms (Finkel and Mitchell, 2011). Genes related to hyphal formation, adherence, amino acid metabolism and drug resistance are found to be up-regulated during biofilm formation (Desai and Mitchell, 2015; García-Sánchez et al., 2004; Nett et al., 2009). Biofilms shows resistance against antifungal drugs. Some factors such as complex structure of biofilms, accumulation of extracellular matrix and up-regulation of drug efflux pumps make the biofilms resistant (Finkel and Mitchell, 2011). *C. albicans* was found to be resistant to fluconazole, amphotericin B, nystatin and voriconazole (Fanning and Mitchell, 2012). Nobile *et al*, reported that Als3 is involved in biofilms formation *in vitro* as well as *in vivo* and it is expressed under Bcr1 transcription factor. The *in vitro* studies showed that *als3* null mutant was unable to form biofilms and overexpression of *ALS3* rescued the defect in biofilms formation. The *in vivo* studies showed that overexpression of *ALS3* in bcr1 null mutant (biofilms defective) rescued the biofilm defects (Nobile et al., 2006). They also observed that *als3* null mutant formed biofilms *in vivo*, the authors suggested that other Bcr1 targeting genes (Hwp1 and Als1)

compensating the Als3 biofilm defects. In later studies, the same group reported that Als1 also has a role in biofilms formation. They observed that *als1 als3* null mutant strain was defective in biofilm formation. The mutant strains with one functional copy of either *ALS1* or *ALS3* (*als1 als3+ALS1* or *als1 als3+ALS3*) showed very thin layer of biofilms, suggested that two functional alleles of *ALS1* or *ALS3* are required for biofilms formation (Nobile et al., 2008). Other members of Als family also support biofilms formation. Biofilm defects in *als1 als3* null mutant strain was rescued by overexpression of other Als genes *in vitro*. *In vivo*, Als6, Als7 and Als9 were able to rescue the biofilm defect in *als1a als3* null mutant strain, Als5 rescued partially whereas Als2 and Als4 didn't rescue the defect (Nobile et al., 2008).

Hwp1 (hyphae specific protein) also plays a critical role in biofilm formation *in vitro* as well as *in vivo* (Nobile et al., 2006). Hwp1 expression is regulated by Brc1 transcription factor (Nobile and Mitchell, 2005). The *hwp1* null mutant showed defect in biofilms formation. Whereas, the overexpression of *HWP1* in *bcr1* null mutant strain improve adherence to the catheter *in vivo* (Nobile et al., 2006). Nobile *et al* showed the complementary function of Hwp1 and Als1/Als3 in biofilms formation. They show that the wild type strains of each produced perfect biofilms but the mutants (*hwp1* null mutant and *als1 als3* null mutant) were defective in biofilms formation. But, the mixture of both the mutants was able to form the biofilms similar to wild type strains indicating that the Hwp1 and Als1/Als3 complement each other in biofilm formation (Nobile et al., 2006).

C. albicans forms mixed species biofilms. These mixed species biofilms include other *Candida* species or bacterial species. These biofilms can affect *C. albicans* mediated biofilm positively or negatively. *C. albicans* was found to be making inter species communities with *S. gordonii* in oral cavity of humans (Silverman et al., 2010). *C. albicans* Als1 and Als3 protein interacts with the SspB protein of *S.* gordonii, which causes more persistent and more adherent stable communities on the host cells (Bamford et al., 2015; Hoyer et al., 2014).

Staphylococcus epidermidis forms mixed biofilm with *C. albicans* and was found to improve antifungal resistance (Adam et al., 2002). Some other bacterial species negatively affects *C. albicans* mediated biofilms. *Pseudomonas aeruginosa* was found to form biofilms on the filaments of *C. albicans* and kill the fungal cells, which support its own growth in the host (Hogan and Kolter, 2002). *Streptococcus thermophilus* was found to be releasing biosurfactants thus inhibiting adherence of *C. albicans* on silicon rubber (Busscher et al., 1997).

I.8 Non-Als adhesin proteins in *Candida albicans*

Apart from Als family of adhesins, some other adhesin (non-Als adhesins) proteins are also reported. These non-Als adhesins are also involved in adhesion and virulence of *C. albicans*. These are mentioned below:

I.8.1 *SAP* (secreted aspartyl proteinases) family

Secreted aspartyl proteinases (SAP) are a family of 10 genes (*SAP1* to *SAP10*) encoding for proteins, which act as hydrolytic enzymes in *C. albicans* (Stehr et al., 2000). Among all the Sap proteins, Sap9 and Sap10 are GPI anchored, whereas Sap1 to Sap8 are non-GPI anchored proteins (Richard and Plaine, 2007). Sap proteins are considered to be virulence factors in *C. albicans* (Naglik et al., 2003). Sap proteins are involved in adherence and morphology switching (Hube et al., 1994; Ollert et al., 1993). *C. albicans* showed reduced adherence to the human epidermal cells in the presence of a Sap protein inhibitor known as pepstatin A (el-Maghrabi et al., 1990; Ollert et al., 1993).

Sap proteins shows differential expression depending on morphological forms and environmental conditions. Sap1 to Sap3 proteins are highly expressed in the yeast form of cells, whereas expression of Sap4 to Sap6 proteins has been observed in hyphae formation at

neutral pH (Felk et al., 2002; Hube et al., 1994). Sap proteins also show pH dependent heterologous expression. Sap1 to Sap3 shows expression at low pH, whereas Sap4 to Sap6 proteins show expression at high pH. This property of Sap proteins helps *C. albicans* to infect the host within a wide range of pH (Hube et al., 1994). Sap proteins also show differential expression in white and opaque cells. Sap1 and Sap3 proteins are expressed during the switching of white and opaque forms, whereas Sap8 protein expressed only in opaque form of cells (Chaffin, 2008; Hube et al., 1997).

I.8.2 Hwp (Hyphal wall protein) family

HWP family consists of three members *HWP1*, *HWP2* and RBT1, which encodes for Hwp1, Hwp2 and Rbt1 proteins. All these proteins are hyphae specific proteins (de Groot et al., 2013). Hwp1 is involved in stabilizing the strong attachment of *C. albicans* cells to buccal epithelial cells (Staab et al., 1999). The *hwp1* null mutant strain shows greatly reduced attachment to host buccal epithelial cells (Staab et al., 1999). The N-terminal of Hwp1 shows sequence similarity with proline rich proteins (PRP) of mammals (Staab et al., 2004). Proline rich proteins act as a substrate for TGase (transglutaminase) enzyme, which helps in cross linkage of proteins to epithelial cells (Bradway and Levine, 1993; Lee et al., 2000). Therefore, Hwp1 was expected to mimic the function of proline rich proteins as well. It cross linked to buccal epithelial cells with the help of TGase (transglutaminase) enzyme and enhanced the adherence of *C. albicans* to host tissues (Staab et al., 1999). Hwp1 also plays a critical role in biofilm formation. As mentioned earlier, the *hwp1* null mutant was found to produce thin biofilms with less hyphal biomass, whereas the reconstituted strain produced biofilms similar to the wild type strain (Nobile et al., 2006). As previously mentioned, the null mutants of *hwp1* and *als1 als3* produced thin biofilms with less hyphal biomass whereas the mixture of both the mutant strains produced biofilms similar to the wild type strain,

suggesting that Hwp1 and Als1/Als3 have a complementary function in terms of biofilms formation (Nobile et al., 2006, 2008).

HWP2 (Hyphal wall protein2) encodes for a 908 amino acid long GPI anchored protein. The N-terminal as well as C-terminal signal sequences of Hwp2 shows homology with Hwp1 protein (De Groot et al., 2003). The role of Hwp2 proteins has been identified in filamentation, adhesion to epithelial and endothelial cells, invasion, biofilms formation and drug resistance of *C. albicans* (Hayek et al., 2010; Younes et al., 2011). The *hwp2* null mutant showed defect in filamentation, reduced adhesion, reduced invasion on solid medium and decreased biofilms formation; it also showed reduction in virulence in a mice model of candidiasis (Hayek et al., 2010; Younes et al., 2011). Peter Hayek's group identified a 37 amino acid long stretch in Hwp2 protein, which is highly homologous to Hwp1 and Rbt1 protein. This region contains a five amino acid long sequence (AIVVT), which is most similar to IIVVTT sequence of Als9 protein. Using TANGO analysis, as mentioned earlier, it was identified that IIVVTT sequence has approximately 96 % beta-aggregation potential (Otoo et al., 2008). Since AIVVT sequence is highly similar to IIVVTT sequence of Als9 protein, the authors proposed that Hwp2 protein is possibly involved in protein aggregation as well (Hayek et al., 2010).

I.8.3 *EAP1* (Enhanced Adherence to Polystyrene1)

EAP1 encodes for a GPI anchored cell wall protein in *C. albicans*. Eap1 is expressed on the cell surface of yeast as well as hyphal forms (Li and Palecek, 2003). Eap1 protein is involved in adhesion to polystyrene and epithelial cells (Li et al., 2007). The *eap1* null mutant strain showed decreased adherence to polystyrene plate and defect in biofilm formation as compare to the wild type *in vitro* as well as *in vivo* (Chaffin, 2008; Li et al., 2007). TANGO prediction identified that Eap1 also consists several amyloid forming stretches with high beta-

aggregation potential. Congo red absorption and thioflavin T fluorescence observations showed that these high beta-aggregation potential peptides were able to form insoluble amyloids (Ramsook et al., 2010).

I.8.4 Iff/*HYR* family (Hyphally regulated proteins family)

The *IFF/HYR* family encodes for 12 proteins. Among all, 10 genes are predicted to be GPI anchored and 2 genes (*IFF10* and *IFF11*) are non-GPI anchored (Richard and Plaine, 2007). All the members of HYR family show high sequence similarity in their N-terminal (de Groot et al., 2013). Hyr1 and Iff11 are hyphae specific proteins, but Iff11 is a secretory protein (de Groot et al., 2013). The functional role of Hyr1 and Iff11 has been identified in cell wall organization and enzymatic activities (Bailey et al., 1996; Bates et al., 2007).

I.8.5 *YWP1* (Yeast form Wall Protein)

YWP1 encodes for Ywp1 protein, which is a GPI anchored protein present on the cell surface of yeast form of cells (Chaffin, 2008; Granger, 2005). The *Ywp1* null mutant of *C. albicans* didn't affect the growth and morphology but cells were more adherent to polystyrene and formed intense biofilms consisting yeast form of cells, suggesting the anti-adhesive role of Ywp1 protein (Granger, 2005). The authors suggested that Ywp1 protein must be involving in the dispersion of yeast form of the *C. albicans* cells so that it can cover the maximum possible cell surface of the host. Recently, the same group shown that the actopic expression of *C. albicans YWP1* under the promoter of *HWP1* (hyphal wall protein 1 gene) inhibited adhesion on mammalian cells confirmed the anti-adhesion function of Ywp1 protein (Granger, 2012).

I.8.6 *ECM33* (Extra cellular matrix protein)

ECM33 encodes for a GPI anchored cell wall protein known as Ecm33 (Chaffin, 2008; Martinez-Lopez, 2004). It is involved in cell wall integrity, morphogenesis and stress tolerance in *C. albicans*. The deletion of *ECM33* resulted in defects in morphology of yeast as well as hyphal form, cell wall integrity and sensitivity to stress responses (Gil-Bona et al., 2016; Martinez-Lopez, 2004).

I.9 Objectives of the study

Als protein family members act as very important virulence factors which mainly contribute to the adhesion and aggregation properties of *C. albicans* (Calderone and Fonzi, 2001; Hoyer, 2001; Rauceo et al., 2004, 2006). Als proteins have also found to be involved in biofilm formation and invasion of *C. albicans*, which has been described earlier in the text (García-Sánchez et al., 2004; Nobile et al., 2008; Sheppard et al., 2004). Some studies have been shown that N-terminal domain of Als proteins could act as an effective vaccine against candidiasis. *C. albicans* expressing Als proteins were found sensitive to antifungal drugs, suggesting that Als adhesins could be a potential drug target against *Candida* infections (Ibrahim et al., 2005; Roudbarmohammadi et al., 2016; Spellberg et al., 2006). Thus, there is an urgent need to explore the characteristics of Als adhesins in more detail, in order to better understand its function as well as to develop strategies to overcome the burden of fungal infections.

Given that the Als family has a set of 8 closely related proteins that exhibit compensatory regulations and at times also show redundancy in function, studying them *in vivo* limits our understanding of the characteristics of a specific Als protein. An *in vitro* approach, on the other hand, would allow us to study individual proteins. However, Als proteins are extremely large proteins and are a challenge to express heterologously in

bacterial systems. Thus, although several reports described characterization of individual domains of Als proteins, until recently none of the studies described purification and characterization of a full length Als protein.

Our lab reported the first successful cloning, expression and characterization of an Als protein (Als5) (Ahmad et al., 2012). Full length Als5 (with and without the C-terminal signal sequence (SS) was successfully cloned with an N-terminal GST-tag in the pGEX-6P-2 vector. These two proteins were expressed and purified from *E. coli* using GST-affinity chromatography.

Using purified GST-Als5 and GST-Als5-SS proteins, functional and structural comparisons were done to understand whether the C-terminal signal sequence in any way affected the conformation or function of the protein. Collagen adhesion and aggregation assays (the functional properties of Als5) showed that GST-Als5 was more functional than GST-Als5-SS. Circular dichroism (CD) analysis showed that there were differences in the secondary structure of both proteins. Als5 had a pre-molten globular structure, whereas Als5-SS had a well folded α- helical conformation. These studies suggested that the differences in the functionalities of the Als5 variants were due to the differences in conformation of Als5 and Als5-SS. It was proposed that the C-terminal signal sequence may be folding back and interacting with the amyloidogenic region in the N-terminal domain (IVIVATT) of Als5-SS thus creating a torsion strain in the Als5-SS protein leading to a different conformation as compared to the conformation of Als5 as shown in Figure I.4. It was suggested that the α-helical conformation of the protein masks the amyloidogenic patch on Als5-SS making it unable to exhibit aggregation and adhesion properties.

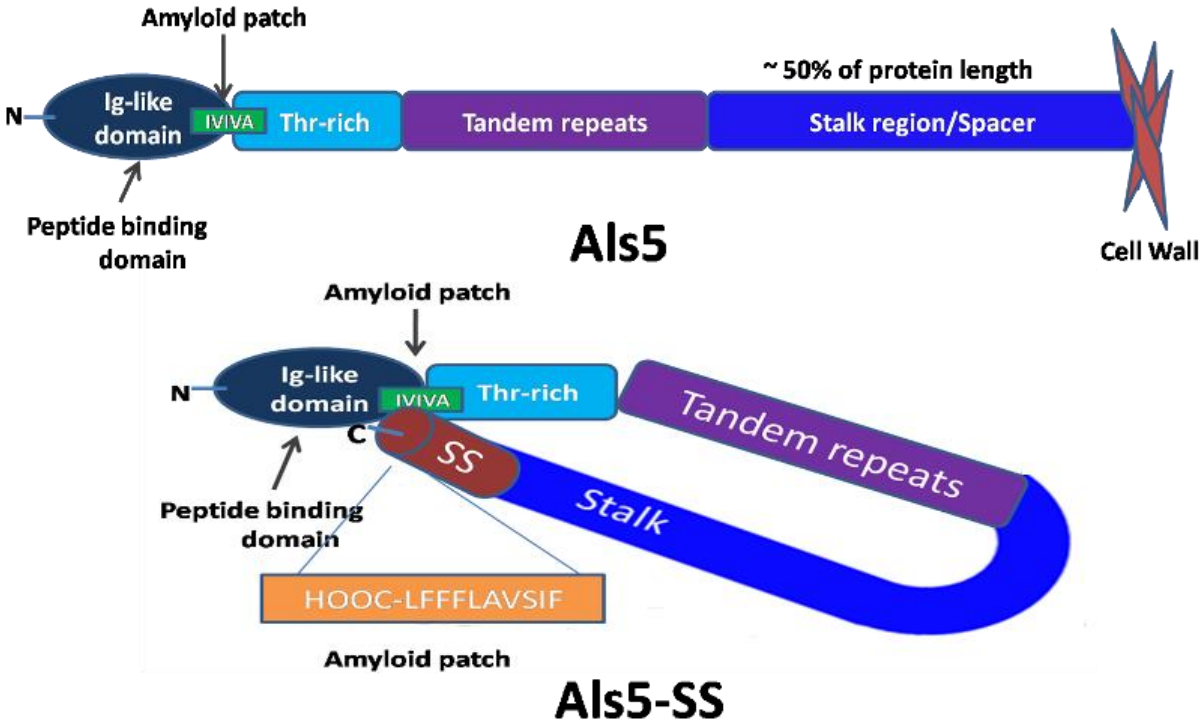

Figure I.4: Model showing the interaction of signal sequence with the amyloid patch thus both the patches get buried inside the protein resulting in a well folded conformation of Als5-SS. In Als5, however, due to the lack of signal sequence, the amyloid patch gets exposed on the surface and thus gets different conformation.

The major focus of the present study was to test this model. Two site directed mutations in the signal sequence of Als5-SS (L1326R and F1327R) and one site directed mutation in the amyloid patch Als5-SS (V309N) were generated based on TANGO predictions that these mutations would abrogate beta-aggregation potential of the SS/amyloid patch. The proteins were expressed and purified from *E. coli* and studied. We observed that these mutations did induce functionality in the protein as expected. *In-vivo* studies of Als5-SS and its mutants expressed in *Saccharomyces cerevisiae YPH501* strain were also carried out. We observed that the mutations do not affect the GPI anchor modification and the cell surface expression of Als5 protein. In addition, we attempt to introduce site directed mutations in the peptide binding pocket to identify the amino acid residues critical for peptide binding. The results of these studies are described in subsequent chapters.

Chapter II

Material and Methods

II.1 Materials

To create mutations in the Als5 proteins, primers were synthesized from Sigma-Aldrich or from Xcelris genomics. All the chemicals were purchased either from MERK, SRL, Sigma-Aldrich or from Fisher Scientific. All media components were purchased from HIMEDIA. DH5α and BL21 (DE3) as well as Glutathion-CL-Agarose beads were purchased from Bangalore Genei. Protease inhibitor cocktail was from Sigma-Aldrich. DNA ladder were from Thermo-Scientific whereas protein markers were from Genetix. Restriction enzymes, Ribonuclease-A, T4 DNA ligase and DpnI enzymes were either from Thermo-Scientific or NEB. XT-20 polymerase was purchased from Bangalore Genei. Anti-GST antibody was from Santa Cruz; production of anti-Als5 antibody (polyclonal) was outsourced to Merck India Ltd. and TRITC labeled secondary antibody was from Bangalore Genei.

II.2 Vectors

II.2.1 pGEX-6P-2 vector

pGEX-6P-2 vector was purchased from GE-Healthcare and used for the construction of GST fusion proteins by inserting the gene of interest between BamHI and XhoI restriction sites. Expression of fusion proteins were done under the control of the tac promoter, which was induced by the isopropyl- β-D-thiogalactoside (IPTG), a lactose analogue. This vector also contains an internal lacI gene, whose gene product act as a repressor protein and binds to the operator region of the tac promoter. Thus prevents the expression of fusion protein until induced by IPTG. The vector map is mentioned below (Figure I.1).

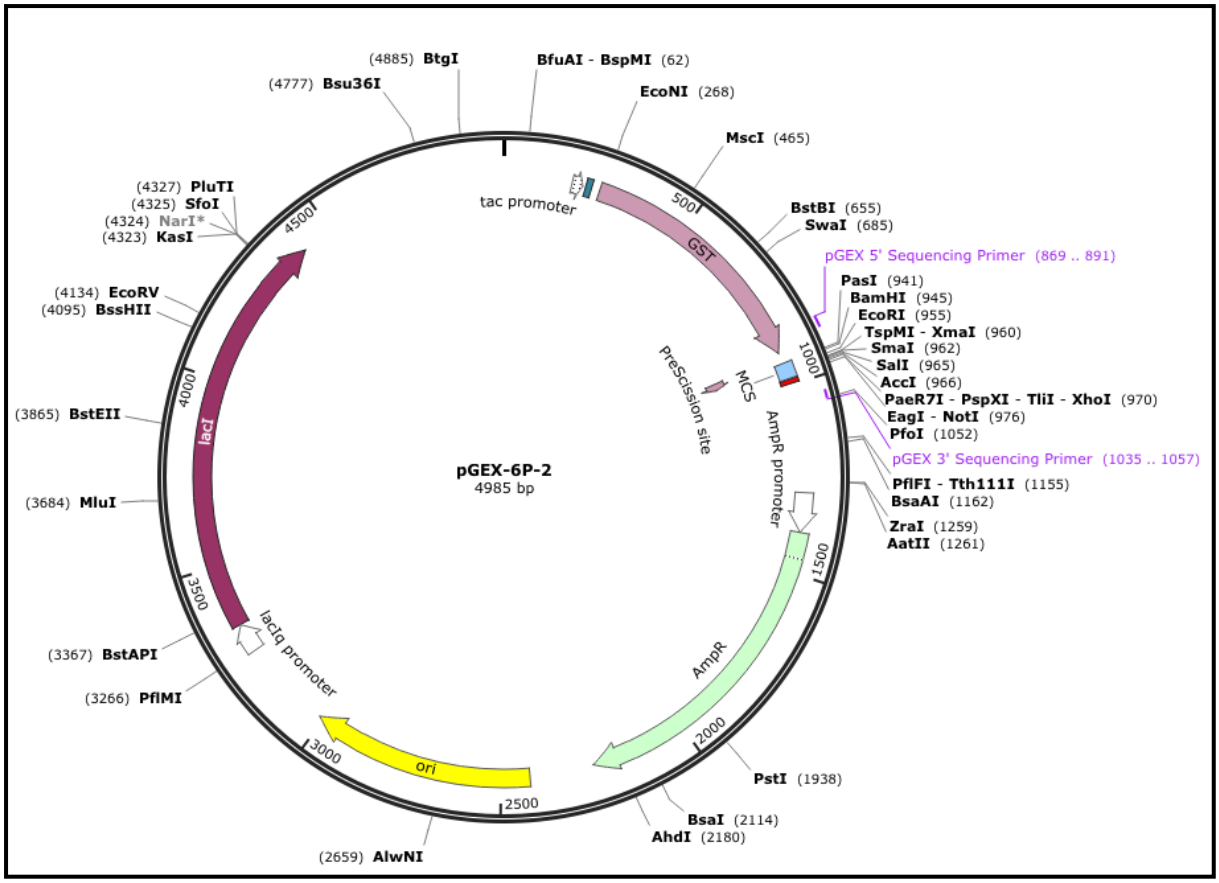

Figure II.1: Vector Map of pGEX-6P-2 used in this study.

II.2.2 pYES2 vector

pYES2 vector (Invitrogen™) was used for the expression of recombinant proteins (Als5-SS, Als5-SS V309N, Als5-SS L1326R and Als5-SS F1327R) in *Saccharomyces cerevisiae*. It contains a Gal promoter, so genes cloned downstream of this promoter showed expressed in the presence of galactose only. It contains the *URA3* gene as a selection marker, which has been used for the selection in yeast. It also contains 2µ origin to maintain high plasmid copy number. Apart from that it consists of the multiple cloning site and ampicillin selection marker for the cloning purpose in bacterial systems (Figure II.2).

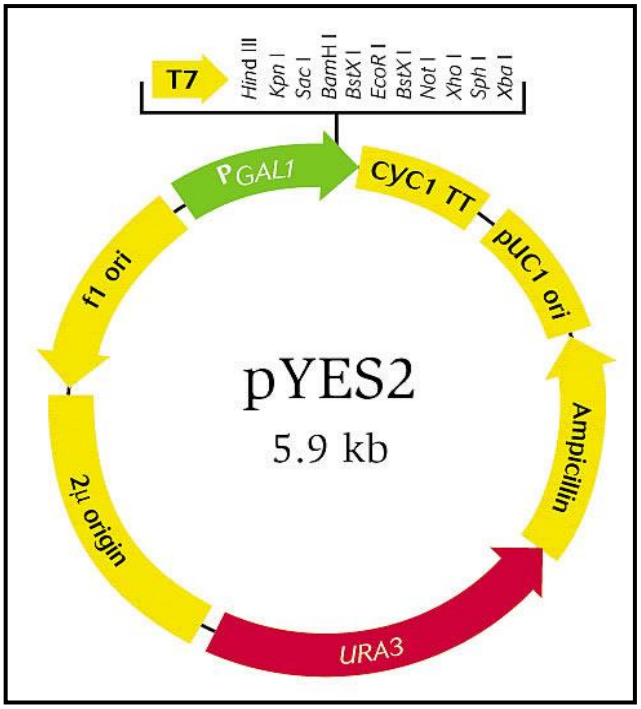

Figure II.2: Vector Map of pYES2 used in this study.

II.3 Primers

Based on the sequence of the gene from *Candida* genome database (CGD), the following forward and reverse primers were designed and used in this study for cloning purpose. The primers were synthesized from (Sigma and Xcelris genomics) in lyophilized form and dissolved in sterile TE buffer at 100 µM stock concentrations. The primers were then diluted in sterile water to make 2 µM (working concentration) used for PCR amplification. The sequence of primers used in this study has been mentioned below (Table II.1).

Table 1: List of primers used in this study.

Primer Name	Primer sequence
Als5-SS FP	5’GCGGGATCCATGATTCAACAATTTACATTGTTATTC3’
Als5-SS RP	5’GCGCTCGAGTCATAGAAAGAAGAATAATGCAACG3’
Als5-SS L1326R FP	5'CTTAAATTTATTAGCGTTGCACGGTTCTTC3’
Als5-SS L1326R RP	5’CTCGAGTCATAGAAAGAAGAACCGTGCAAC3’
Als5-SS F1327R FP	5’AAATTTATTAGCGTTGCATTACGGTTCTTT3’
Als5-SS F1327R RP	5’CCGCTCGAGTCATAGAAAGAACCGTAATGC3’
Als5-SS S170Y FP	5’CAAAGTGGGTATTTGACTACTTACAGATTT3’
Als5-SS S170Y RP	5’ATTGAGACTCGGCATAAATCTGTAAGTAGT3’
Als5 3729 FP	5’TGAAGAATCTATTATGAATCCTGAT3’
Ura3 FP	5’GGGTAATAACTGATATAATTAAAT3’
Ura3 RP	5’GATTCGGTAATCTCCGAACAG3’

II.4 Methods

II.4.1 Tools

II.4.1.1 TANGO analysis

TANGO is an aggregation prediction software tool, which calculates the aggregation potential of the regions of protein (tango.crg.es/protected/correctlogin.jsp). It looks for the secondary structures (α-helix, β-turns and β-sheets) of the protein and predicts the β-aggregation potential of that particular region. To create the site directed mutations, we replaced each and every residue with all 20 amino acids and looked for the aggregation potentials. For our experimental needs, we chose those mutations which showed the lowest aggregation potentials.

II.4.1.2 GPI-SOM and BIG-PI predictor

These are the softwares which predict the ω-site or GPI anchor modification site in the protein sequences (http://gpi.unibe.ch and http://mendel.imp.ac.at/gpi/fungi_server.html). GPI-SOM is based on Kohonen self-organizing maps or Kohonen neural networks, while

BIG-PI algorithm is based on sequence analysis from experimentally known proteins (Eisenhaber et al., 1999; Kohonen, 2001).

II.4.1.3 Molecular modelling and molecular dynamics simulations

(i) Model generation of Als5 and Als5 S170Y mutant

The structure of N-terminal domain of the *C. albicans* Als9-2-apo form (PDB ID 2Y7N) was used for the construction of three dimensional structure Als5Nt. The modelled protein shared 66% identity and 78% similarity with Als9-2 (Ahmad et al., 2012). We used flexible backbone protein structure modeling and mutations were incorporated using the Rosetta Backrub protocol (Lauck et al., 2010) which uses Monte-Carlo simulated annealing using the Rosetta all-atom force field. Rosetta all-atom force field was applied to model conformational changes, in the neighbourhood of mutated region using Rosetta Backrub protocol.

(ii) Classical molecular dynamics and enhanced sampling using accelerated molecular dynamics

To understand the effect of the mutation on structures of the protein compared to the wild type, models of the wild type Als5 protein along with the S170Y mutant were subjected to molecular dynamics simulations. The lowest energy model of the mutant was taken from the cluster of 100 structures used for MD simulations. We performed all-atom MD simulations for 50 ns using the following initial structure: i) NtAls5 model ii) S170Y mutant. After obtaining the lowest weighted scored structure, the hydrogen atoms were added using the tleap tool of amber14. The system was solvated using atomistic TIP3P water in a box with edges at least 12 Å from the protein followed by electrostatic neutralization by addition of Cl^- ions. Energy minimization was first conducted by the steepest descent method and then

switched to conjugate gradient after 2500 steps for a total of 5000 steps with 0.1 kcal·mol^{-1}·Å^{-2} restraints on the protein molecule. Long-range coulombic interactions were handled using the particle mesh Ewald summation. For equilibration and subsequent production runs, the shake algorithm was employed on all atoms covalently bonded to a hydrogen atom, allowing for an integration time step of 2 fs. The system was gently annealed for a period of 50 ps using a Langevin thermostat with a coupling coefficient of 1.0 ps^{-1} and 500 ps of density equilibration with weak restraints on the protein molecule. The system was again equilibrated for 10 ns without any restraints. The production dynamics was run without any restraints for a total of 50 ns on each system. The coordinates and energy values were collected every 10 ps throughout the simulations. The pmemd.cuda module of the amber14 package was used to run all the production dynamics. The amber ff14SB force field was used to describe the protein molecule for all the MD simulations.

(iii) Accelerated molecular dynamics

After cMD simulations, we performed aMD simulations on all systems to discover the longtime conformational changes that are difficult to sample using small scale classical molecular dynamics. An average dihedral energy and total potential energy was computed from 50 ns of cMD and used as a reference for the aMD simulation. The aMD simulation was carried out using the exact same conditions as described above starting from the equilibrated simulation used for the 50 ns cMD simulation. Accelerated MD modifies the energy landscape by adding a boost potential, ΔV(r), to the original potential energy surface when V(r) is below a pre-defined energy level E, where α modulates the depth and the local roughness of the energy basins on the modified potential.

$$\Delta V(r) = \left\{ \begin{array}{ll} 0, & V(r) \geq E \\ \dfrac{(E - V(r))^2}{\alpha + (E - V(r))}, & V(r) < E \end{array} \right\}$$

We employ the dual-boost implementation of aMD to boost both dihedral and total potential energy force field terms to promote side chain dihedral angle rotations and diffusive transitions, respectively. We set the parameters E and a for our systems by defining these variables for the dihedral and total potential energy components with respect to the number of residues in the system, N res , and the number of atoms in the system, N atoms , respectively.

$$E_{DIHED} = \langle V_{DIHED}(r) \rangle + 2.5 \times N_{res}$$

$$\alpha_{DIHED} = 1.25 \times N_{res}$$

$$E_{TOT} = \langle V_{TOT}(r) \rangle + 0.17 \times N_{atoms}$$

$$\alpha_{TOT} = 0.17 \times N_{atoms}$$

Table 2: Accelerated molecular dynamics simulations analysis.

System	Total Atoms	Average EPtot	Average Dihedral	Alpha D	Alpha P	Ethreshold P	Ethreshold D
Als5-Nt	49169	-149419.60	3022.03	207.9	9833.8	−145358.07	4061.53
Als5-Nt-S170Y	49116	-119270.44	3031.88	207.9	9823.2	−115199.06	4071.381

The aMD simulations were carried for both the system at 200 ns. The total simulation length for all the system is 1 micro second. The analysis of the MD simulations including root mean square deviation (RMSD), root mean square fluctuation (RMSF), radius of gyration (ROG) and accessible surface area (SASA) calculations was carried out using the cpptraj program. The 3D molecular graphics were displayed using pymol software.

II.4.2 PCR amplification to generate site directed mutations in Als5-SS

Previously in our lab, the wild type *ALS5-SS* was cloned in pGEX-6P-2 by Dr. Faiz Ahmad. To create the site directed mutation *ALS5-SS* was amplified along with the vector

(pGEX-6P-2) with the mutagenic primers mentioned above (Table II.1). Primers were designed on the basis of coding sequence of the *ALS5-SS* from the *Candida* genome database (CGD). The parental copy of the coding sequence was digested using DpnI enzyme and then DH5α strain of *E. coli* was transformed with the Dpn1 digested products. The colonies obtained after transformation were screened by colony PCR using gene specific forward and reverse primers of *ALS5-SS* and the colonies confirmed by the colony PCR were further confirmed by DNA sequencing.

II.4.3 Competent cells preparation

Competent cell preparations were done by following calcium chloride method with slight modifications (Tang et al., 1994). DH5α strain of *E.coli* was inoculated in 10 ml of LB media and incubated in shaker at 37 °C overnight. For secondary culture 1 % of primary culture was used and the culture was grown at 37 °C till OD_{595nm} came to 0.5 (approximately 2-3 hrs). The culture was then incubated in ice for 15 minutes and centrifuged at 6000 rpm for 5 minutes at 4 °C. Now 6 ml of 0.1 M $CaCl_2$ (chilled) and 24 ml of 0.1 M $MgCl_2$ (chilled) was added to the cells, incubated in ice for 30 minutes and centrifuged at 6000 rpm for 5 minutes at 4 °C. The supernatant was removed and 1.75 ml of 0.1 M $CaCl_2$ and 0.75 ml of glycerol (ice cold) was added. The cells were aliquoted and stored at -80 ºC. All the steps were carried out in 4 °C and in sterile conditions carefully.

II.4.4 Transformation of DH5α strain of *E.coli* with Dpn1 digested products

The protocol for transformation of *E. coli* cells was followed that reported by Inoue *et al* with slight modifications (Inoue et al., 1990). The Dpn1 digested products were added to the competent cells of *E.coli* (DH5α strain) and incubated in ice for 30 minutes. Heat shock was given to the cells at 42 °C for 90 seconds and transferred to ice immediately. Then 1 ml

of LB medium was added to each tube and incubated at 37 °C for 1 h and centrifuged at 5000 rpm for 5 minutes at room temperature (RT). The supernatant (900 µl) was discarded and the pelleted cells were resuspended in remaining 100 µl medium. The cells were spread on LB-agar plate having ampicillin (100 µg/ml) as a selection marker and incubated at 37 °C overnight. The colonies obtained were confirmed by colony PCR. The plasmids were isolated from positive colonies and further confirmed by DNA sequencing.

II.4.5 Screening of colonies by Colony PCR

The colonies obtained after transformation were streaked on LB-ampicillin plate and a small inoculum of each of the streaked colonies was taken in 50 µl of autoclaved water and heated at 95 °C for 10 minutes. Samples were centrifuged at 5000 rpm for 5 minutes at RT and 10 µl of the supernatant was taken as a template. The PCR reaction cocktail was prepared (Table A.I.6.1) and PCR amplification was done by using gene specific primers in appropriate PCR conditions (Table A.I.6.2). The PCR products were run on 0.8 % agarose gel to confirm the positive colonies.

II.4.6 Agarose gel electrophoresis

The PCR amplicons were checked by agarose gel electrophoresis. The PCR products were mixed with DNA loading dye and loaded on 0.8 % agarose gel (prepared with 1 X TAE). The bands became visible under UV light by using EtBr (Ethedium Bromide) in the gel.

II.4.7 Confirmation of clones by DNA sequencing

Plasmids were isolated from positive colonies confirmed by colony PCR. The site directed mutations were confirmed by DNA sequencing by using primer (Als5 3729 FP) described in Table II.1.

II.4.8 Plasmid Isolation

The alkaline lysis method was used for plasmid purification (Sambrook and Russell, 2001). The colonies were inoculated in 10 ml of LB media having 100 µg/ml ampicillin and incubated at 37 ºC overnight. Bacterial cultures were centrifuged at 5000 rpm at 4 °C for 10 minutes. Supernatant was removed and pellet was resuspended in 200 µl of ice cold solution I (mentioned in Appendix). The samples were transferred into 1.5 ml micro centrifuge tubes and 400 µl of freshly prepared solution II (mentioned in Appendix) was added and mixed properly by inversion. Then 300 µl of solution III (mentioned in Appendix) was added in all the tubes and incubated for 5 minutes on ice and centrifuged at 14,000 rpm for 15 minutes at 4 °C. The supernatant was transferred to fresh micro centrifuge tube and RNase treatment was given at 37 °C for 45 minutes. Then Phenol: chloroform solution (1:1 ratio) was added and again centrifuged at 10,000 rpm for 5 minutes at 4 °C. The aqueous layer was transferred to a fresh micro centrifuge tube and equal volume of chloroform (to remove phenol) was added and centrifuged at 10,000 rpm for 10 minutes at RT. The upper aqueous layer was again transferred into fresh micro centrifuge tube, equal volume of iso-propanol was added to each tube, and incubated at -20 °C for 30 minutes (to precipitate plasmid DNA). It was again centrifuged at 14,000 rpm for 10 minutes at RT. The supernatant was removed and pellet was washed with 70 % ethanol (1 ml). The supernatant was removed and the pellet was air dried for 10-15 minutes and resuspended in 20-30 µl of dH_2O and stored at -20 °C for further use.

II.4.9 Expression and purification of Als5-SS mutants

The expression of Als5-SS mutants were done in BL21 strain of *E.coli.* First, BL21 strain of *E.coli* was transformed with all the plasmids having the required inserts (pGEX-6P-2 Als5-SS V309N, pGEX-6P-2 Als5-SS L1326R and pGEX-6P-2 Als5-SS F1326R).

Competent cell preparation and the transformation protocol was followed the same as described earlier in the Methods section. The colony obtained after transformation were inoculated in LB medium with the appropriate antibiotic. The primary cultures were incubated at 37 °C overnight at 220 rpm shaking. For secondary culture, 1 % of primary culture was used and incubated at 37 °C at 220 rpm for 3 hours till OD_{600nm} reached 0.6, then induced with 0.1 mM IPTG at 16 °C for 4 hours and cells were harvested at 8500 rpm at 4 °C for 10 minutes. Pellet was dissolved in lysis buffer [50 mM sodium phosphate buffer (pH 8.0), 150 mM NaCl, 10 µM PMSF, 0.1 mg/ml lysozyme and 5 % glycerol], incubated at 4 °C on rocker for 1 hour, and then the cells were lysed using a digital probe sonicator (30 sec ON and 30 sec OFF for 7 minutes). Cell lysate was then centrifuged at 8500 rpm for 45 minutes at 4 °C. The supernatant was transferred on to glutathione-agarose beads and incubated for 4 hours on rocker at 4 °C. Now the beads were washed using wash buffer [50mM sodium phosphate buffer (pH 8.0), 3 M NaCl], and then protein was eluted using elution buffer [50 mM sodium phosphate buffer (pH 8.0), 150 mM NaCl, 10 mM glutathione, 20 % glycerol]. The purified proteins were run on 6 % SDS polyacrylamide gel. The bands were observed by staining with Coomassie brilliant blue R250. The compositions of staining and destaining solutions have been mentioned in the Appendix.

II.4.10 Western blotting

It is a technique to identify the proteins based on antibody detection. First the proteins were separated based on the size by SDS-PAGE. PVDF membrane was activated in methanol for 15 minutes and rinsed 2-3 times with water. The protein bands were transferred from the gel to the PVDF membrane. To transfer the protein bands from the gel to membrane, we prepared the stack in a sequential manner as mentioned below-

Cathode (Negative) ➡ Black pad ➡ White pad ➡ Filter paper ➡ Gel ➡ PVDF Membrane ➡ Filter paper ➡ White pad ➡ Black pad ➡ Anode (Positive)

The stack was fitted to the transfer apparatus and run for 3 hrs at 100 V. The membrane was removed and blocked with blocking solution (5 % skimmed milk in PBS) for 1 hr at room temperature. The membrane was washed thrice with wash buffer (PBS with 0.05 % Tween 20) and incubated with anti-GST antibody (1:1000) for 1 hr at room temperature. The antibody was dissolved in antibody buffer mentioned in Appendix. Again the membrane was washed trice with wash buffer and incubated with HRP conjugated secondary antibody for 1 hr at room temperature. Again the membrane was washed 3-4 times with wash buffer and the protein band was developed on an X-ray film using ECL kit following the manufacturer's protocol.

II.4.11 Dialysis bags activation

The dialysis membranes were boiled in buffer (1 % $NaHCO_3$ + 1 mM EDTA) for 10 minutes. The buffer was decanted and the membranes were again boiled for 10 minutes in buffer containing 1 mM EDTA. The buffer was decanted and the membrane was stored at 4 °C in autoclaved water before use.

II.4.12 Secondary structure analysis using CD spectroscopy

The concentrations of proteins (GST-Al5-SS L1343R and GST-Als5-SS F1344R) were measured using Bradford reagent. The dialysis bags were activated and filled with the respective protein samples. The proteins were dialyzed using dialysis buffer [50 mM sodium phosphate buffer (pH 8.0), 150 mM NaCl, and 20 % glycerol] to remove glutathione. After dialysis concentration for each protein was adjusted to 0.09 mg/ml and spectra recorded at 25° C on Chirascan (Applied Photophysics) CD spectrophotometer. All the spectra were recorded between 200-260 nm in a 1mm path length cuvette with 1 nm step length, where

spectrum of buffer was taken for background correction. The final spectrum was an average of five repeat scans. GST spectrum was subtracted from GST-Als5-SS L1343R and GST-Als5-SS F1344R spectrum to get the spectrum of Als5-SS L1343R and Als5-SS F1344R using Pro-Data software which is inbuilt within the instrument and the secondary structure was predicted using K2D prediction software (http://dichroweb.cryst.bbk.ac.uk/html/home.shtml). The CD analysis of Als5-SS V309N was done by Dr. Faiz Ahmad.

II.4.13 TEM for Aggregation studies

JEOL 2100F was used for TEM studies. Proteins (0.09 mg/ml) were incubated at 37 °C for different time points (12 hrs, 24 hrs, 1week and 2 week) before analysis. Similarly these studies were done with synthetic peptides having both mutations, which were incubated with purified Als5 protein for 12 hrs and 24 hrs. Carbon coated copper grid was used for spotting of protein and then stained with 2 % uranyl acetate and dried, then the samples were recorded using TEM at 200 kV accelerating voltage.

II.4.14 Adhesion assay

Protocol for the adhesion assay was followed that reported by Gaur *et al*., with slight modifications (Gaur et al., 1999). A 96 well plate was coated with 0.5 mg/ml of collagen type-IV and incubated at 4 °C overnight. Next day all the wells were washed three times with PBS and two times with elution buffer and 200 µl of each protein (GST, GST-Als5-SS, GST-Als5-SS V309, GST-Als5-SS L1326R and GST-Als5-SS F1327R) with the concentration of 0.07mg/ml was added in the respective wells and incubated at 37 °C for 1 hour, where the GST was taken as a control for all the proteins. Control without primary antibody was also taken for all the proteins. 200 µl of 5 % skimmed milk in PBS was added to block the wells

and incubated at 37 °C for 1 hours and washed 5 times with antibody buffer (1 % skimmed milk in PBS + 0.025 % Twin-20). Anti-GST primary antibody (1:1000 diluted) was added in each well and incubated at 37 °C for 1 hour. Again all the wells were washed 5 times with antibody buffer and 200 µl of HRP-conjugated secondary antibody (1:20,000 diluted) was added and incubated at 37 °C for 1 hour. Again wells were washed 5 times with antibody buffer and 100 µl of Tetramethylbenzidine solution (1 mg/ml TMB in DMSO + 9 ml of 0.05 M phosphate citrate buffer + 2 µl H_2O_2) was added to each well and incubated at 37 °C for 1 hour. OD_{650nm} was recorded by plate reader (Multiskan™ GO).

II.5 Methods for *in-vivo* studies

II.5.1 Cloning of Als5-SS and its mutants in pYES2 vector

Als5-SS and its mutants (Als5-SS V309N, Als5-SS L1326R and Als5-SS F1327R) were cleaved from PGEX-6p-2 vector (all were inserted between BamH1 and Xho1) by BamH1 and Xho1 restriction enzymes and were ligated into pYES2 vector using T4 DNA ligase between the BamH1 and Xho1 restriction sites. The clones were confirmed by double digestion with BamH1 and Xho1 restriction enzymes. The ligation (A.I.6.4) as well as double digestion (A.I.6.3) reaction cocktail has been mentioned in the Appendix.

II.5.2 Double digestion of pYES2, pGEX-6p-2-Als5-SS, pGEX-6p-2-Als5-SS V309N, pGEX-6p-2-Als5-SS L1326R and pGEX-6p-2-Als5-SS F1327R

To excise the genes from pGEX-6p-2 vector, the constructs were double digested. To insert all the genes into pYES2 vector, along with the pYES2 vector also double digested. Two restriction enzymes BamHI and XhoI were used for double digestion of all the plasmids to create the sticky ends. The double digestion reaction cocktail was made and incubated the mixture for 5-6 hours at 37 °C (A.I.6.3).

II.5.3 Elution of double digested pYES2 vector, Als5-SS, Als5-SS V309N, Als5-SS L1326R and Als5-SS F1327R

Double digested products which are mentioned above were run on agarose gel and the bands of insert as well as vector were excised using a scalpel under the UV transilluminator. DNA was eluted using Qiagen gel extraction kit following the manufacturer's protocol.

II.5.4 Ligation of Als5-SS, Als5-SS V309N, Als5-SS L1326R and Als5-SS F1327R into pYES2 vector

All the genes were ligated into pYES2 vector. For the ligation reaction 1:5 ratio (vector Vs insert) was taken (A.I.6.4). The ligation reaction was incubated in cooling water bath at 22 °C overnight. The DH5α strain of *E.coli* was transformed with ligated products and the colonies obtained were screened by colony PCR in a manner similar to that described earlier in the Methods section. The clones were further confirmed by double digestion with BamH1 and Xho1 restriction enzymes (A.I.6.3).

II.5.5 Transformation of *S. cerevisiae* with pYES2, Als5-SS and its mutant

The transformation protocol was followed from Gietz and Schiestl (Gietz et al., 1995). The pYES2 alone (vector control), pYES2-Als5-SS and its mutants were transformed in YPH501 strain of *S. cerevisiae* using LiAc method (Lithium Acetate method). Primary cultures were inoculated in 10 ml of YPD medium at 30 °C at 220 rpm overnight. For secondary culture, 2 % of primary culture was used and incubated at 30 °C at 220 rpm for 3-5 hours till OD_{595nm} reached 0.5. Cells were pelleted down and washed with sterile water and resuspended in 1 ml of 100 mM LiAc (Lithium Acetate). Transformation mix (50 % PEG, 1M LiAc, SS DNA and plasmid) was added in the cell suspension and heat shock was given at 42 °C for 30 minutes. Now cells were pelleted down and washed with sterile water. Pellet was resuspended in sterile water and spread on SD Ura^- plate. The plates were incubated for

2-3 days at 30 °C to get transformants. Transformed colonies were confirmed by colony PCR amplification using URA3 specific primers for pYES2 and gene specific primers for all the inserted genes.

II.5.6 Confirmation of *S. cerevisiae* constructs from DNA isolation

We followed the Hoffman and Winston protocol for DNA isolation with slight modification (Hoffman and Winston, 1987). Cells from the streaked transformed colonies were grow overnight in 10 ml YPD medium at 30°C, 220 rpm. The culture was centrifuged at 5000 rpm for 5 minutes at 4°C and pallet was collected. Pallet was washed with distilled water and centrifuged at 5000 rpm for 5 minutes again. Then 500 µl of lysis buffer and 0.7 – 0.8 gm glass beads were added to the cells. Cells were lysed by three round of vortexing (1 minute vortex, 1 minute on ice). Then centrifuged again at 3000 rpm for 5 minutes at 4°C and collected the liquid phase in 2 ml microcentrifuge tubes. Now, 275 µl of ammonium acetate was added, incubated the tubes at 65°C for 5 minutes and kept on ice for 5 minutes. Then 500 µl of chloroform was added to each sample and centrifuged at 12000 rpm for 15 minutes at 4°C. The upper aqueous layer was collected in separate microcentrifuge tubes then 5 µl of RNase was added and kept at 37°C for 30 minutes. Equal volume of Chloroform was mixed and centrifuged at 10000 rpm for 10 minutes at 4°C. The upper aqueous layer was collected in separate 1.5 µl microcentrifuge tubes then equal volume of iso-propanol was added and kept at room temperature for 30 minutes for DNA precipitation. The mixture was centrifuged at 12000 rpm for 15 minutes at 4°C. Supernatant was discarded and the pallet was washed with 70 % ethanol. The pellet was air dried and stored in 50 µl of distilled water at 4°C.

II.5.7 Expression of Als5-SS and its mutants in *S. cerevisiae*

For protein expression, YPH 501 cells transformed with pYES2-Als5-SS, all mutants and pYES2 (vector control) were inoculated in 15 ml of SD Ura^- medium containing 2 %

glucose and were grown overnight at 30 °C. Cell with 0.5 OD_{595nm} were pelleted down and then resuspended in 50 ml of SD Ura^- medium containing 2 % of galactose (induction media) and allowed to grow overnight at 30 °C. Afterwards, cells were pelleted down and resuspended in phosphate- buffered saline (PBS) such that the OD_{595nm} reached 0.5. Then the cells were incubated at 30 °C for 1 hour with anti-Als5 antibody (1:50 diluted), followed by 3-4 times washing with phosphate- buffered saline (PBS). Now the cells were incubated at 30 °C for 1 hour with TRITC labelled secondary antibody (1:100 diluted), followed by 3-4 times washing with phosphate- buffered saline (PBS) and the cells were seen under fluorescence microscope (TiE, Nikon). After checking the expression microscopically, quantification of the expression levels was done by using NIS element AR analysis software.

II.5.8 Different assays performed to study the functionality of Als5-SS and its mutant proteins in *S. cerevisiae*

II.5.8.1 Adhesion Assay

Protocol for the adhesion assay was followed that reported by Gaur *et al*., with slight modifications (Gaur et al., 1999). All the *S. cerevisiae* strains expressing the different mutations of Als5-SS were inoculated in 15 ml of SD Ura^- medium containing 2 % glucose and were grown overnight at 30 °C. Cells with 0.5 OD_{595nm}, cells were pelleted down and then resuspended in 50 ml of SD Ura^- medium containing 2 % of galactose (induction media) and allowed to grow overnight at 30 °C, where the pYES2 was taken as a vector control. Cells were pelleted down followed by 3-4 times washing with phosphate buffered saline (PBS) and resuspended in PBS. Then a 96 well plate was coated with 0.5 mg/ml and 1 mg/ml of collagen type-IV and incubated at 4 °C overnight. Next day all the wells were washed three times with PBS. The cells (200 µl) of equal OD_{595nm} (0.5) were added in the respective wells and incubate at 37 °C for 1 hour, where the pYES2 (vector control) was taken as a

control. 200 µl of 5 % skimmed milk in PBS were added to block all the wells and incubated at 37 °C for 1 hour and washed three times with PBS. Anti-Als5 primary antibody (1:50 diluted) was added in each well and incubated at 37 °C for 1 hour. Again all the wells were washed three times with PBS and 200 µl of HRP-conjugated secondary antibody (1:20,000 diluted) was added and incubated at 37 °C for 1 hour. The wells were washed three times with PBS and 100 µl of Tetramethylbenzidine solution (1 mg/ml TMB in DMSO + 9 ml of 0.05 M phosphate citrate buffer + 2 µl H_2O_2) was added to each well and incubated at 37 °C for 1 hour. OD_{650nm} was recorded by plate reader (Multiskan™ GO).

II.5.8.2 Aggregation assay

Protocol for the adhesion assay was followed that reported by Rauceo *et al*., with slight modifications (Rauceo et al., 2004). For aggregation studies, *S. cerevisiae* strains expressing the different mutants of Als5-SS were inoculated in 15 ml of SD Ura^- medium containing 2 % glucose and were grown overnight at 30 °C. Cells with 0.5 OD_{595nm} were pelleted down and then resuspended in 50 ml of SD Ura^- medium containing 2 % of galactose (induction media) and allowed to grow overnight at 30 °C. Cells transformed with pYES2 were used as vector control. Afterwards, cells were pelleted down, washed with PBS and resuspended in phosphate buffered saline (PBS) such that the OD_{595nm} reached 0.5. All the cells were stained with 30 µM ANS (8-anilino-1-naphthalene-sulfonic acid) for 30 minutes and then washed 2-3 times with PBS. To see the aggregates, the cells were seen under a fluorescence microscope (TiE).

II.5.9 Statistical significance of data

Statistical significance (*p* value) of the data was calculated using student's t-test in Sigma Plot 8.0 software. The *p* value less than 0.05 is represented by *, *p* value less than 0.01 is represented by **, and *p* value less than 0.001 is represented by ***.

Chapter III

Characterization of Als5 mutants

in vitro

The *ALS* gene family encodes for eight adhesins in *C. albicans*. These adhesins perform several functions such as adhesion, invasion and aggregation and are thus involved in *C. albicans* pathogenesis. All Als members are highly homologous to one another and show compensatory regulations within the family (Hoyer and Hecht, 2001; Klotz and Lipke, 2010). *C. albicans* strains deletion with Als5, Als6 and Als7 result in increased adhesion to epithelial as well as endothelial cells as compared to control strain, suggesting that they may possess anti-adhesion function (Zhao et al., 2007a). However, as the authors suggest, it is also possible that there is complementary upregulation of other adhesins in these deletion strains. Such a compensatory mechanism has been previously shown between *ALS2* and *ALS4* at the transcript level (Zhao, 2005). So, using complementation studies to study a particular Als protein in *C. albicans* itself is a challenging task due to the presence of other Als proteins.

An alternative approach is to study the Als protein *in vitro* but this too has its problems. Several reports exist on the transcriptional studies of *ALS* genes of *C. albicans* (Cheng et al., 2005; Green, 2004; Green et al., 2005). RT-PCR studies showed the constitutive transcription of *ALS1-ALS5* and *ALS9* in *C. albicans* infected buccal epithelial cells, whereas the transcripts of *ALS6* and *ALS7* were relatively very low (Green, 2004). In another report, GFP (green fluorescent protein) reporter gene was cloned downstream of different *ALS* promoter genes. RT-PCR was done using GFP specific primers; transcription from the promoters of *ALS1*, *ALS2*, *ALS3* and *ALS9* was detected, whereas very less transcription from *ALS4* promoter was observed. In case of *ALS5, ALS6* and *ALS7* they didn't observe any transcription (Green et al., 2005). Similarly, RT-PCR from the samples collected from clinical isolates and murine vaginitis disease models showed transcription of *ALS1*, *ALS2*, *ALS3* and *ALS9* was detected very frequently, but detection of the transcript levels of *ALS4*, *ALS5*, *ALS6* and *ALS7* was very less (Cheng et al., 2005). These studies suggested that the low transcripts levels of *ALS4*, *ALS5*, *ALS6* and *ALS7* are sufficient to perform their

cellular functions. However, purification of Als proteins from its natural host is a challenging task since *C. albicans* shows very low levels of endogenous expression of *ALS* genes. Therefore, heterologous expression systems are an option for us to study a specific Als protein.

Characterization of full length Als proteins using heterologous expression systems are very difficult. Because, Als proteins are GPI anchored as well as very large in size, so heterologous expression and purification of Als proteins is a challenge. Since expression of these proteins is a major issue, most such studies so far have involved the *in vitro* characterization of individual domains of Als proteins rather than the full length Als protein (Donohue et al., 2011; Lin et al., 2014; Rauceo et al., 2006). Adhesion is a well known function for Als proteins and *in vitro* studies confirmed the adhesion function of Als proteins (Donohue et al., 2011; Rauceo et al., 2006). The N-terminal domain of Als5 was expressed and purified from *Pichia pastoris* expression system (Hoyer and Hecht, 2001). This domain is not known to be glycosylated (Hoyer and Hecht, 2001; Hoyer et al., 1998a). CD analysis of N-terminal domain of purified Als5 protein show that it is a beta sheet rich, immunoglobulin-like structure, similar to the other adhesins (Hoyer and Hecht, 2001). This suggested that Als5 protein too could work as an adhesin. Als proteins bind to several extracellular matrix proteins such as fibronectin, laminin and collagen. The TR domain (threonine rich repeat domain) purified from *S. cerevisiae* enhanced the fibronectin binding capability of *C. albicans* Als5 protein (Rauceo et al., 2006). Other Als proteins too have been similarly studied. Salgado *et al* used the bacterial expression system for the expression and purification of N-terminal Als9-2 protein and suggested that Lys59 (within the N-terminal domain) involved in the ligand binding (Salgado et al., 2011). On the other hand, studies from our lab suggested that Lys59 is not involved in ligand recognition in Als5 (Faiz and Komath unpublished data). Donhoue *et al* observed that, the N-terminal domain of Als1 protein

purified from *S. cerevisiae* showed binding to fucose containing glycans indicating that Als1 protein acts as a fucose-specific lectin (Donohue et al., 2011). It has also been shown that the N-terminal domain of Als1 and Als3 proteins improved the survival of mice suffering from candidiasis suggesting that these proteins could be potential vaccines as well (Ibrahim et al., 2005; Spellberg et al., 2006).

Like adhesion, β-aggregation is also a well-known functionality for Als proteins. Peter Lipke's group identified a heptapeptide (IVIVATT) with high beta aggregation potential within the T region of Als5 protein, which is conserved in *ALS* gene family. TANGO analysis predicted that, this heptapeptide region contributes to 93 % of β-aggregation in Als5 protein. They also observed that the Als5 protein ($Als5^{20-431}$ and $Als5^{20-664}$) purified from *S. cerevisiae* bound to amyloid specific dyes (Congo Red and Thioflavin T) suggesting their role in β-aggregation (Otoo et al., 2008). Later, the same group showed that Als5 (without C-terminal 68 residues) purified from *S. cerevisiae* was precipitated at submicromolar concentration from neutral buffer. These precipitates formed amyloids and showed binding with amyloid specific dyes (Ramsook et al., 2010). Als proteins have variable number of tandem repeats (Hoyer et al., 1995). The number of tandem repeats correlated with the extent of adhesion and aggregation. Proteins with more tandem repeats were found to be more adhesive and aggregating suggesting that TR domain governs the aggregation properties (Rauceo et al., 2006).

As we have described above none of the studies characterized full length Als protein *in vitro* till date. Our lab reported the first successful cloning, expression and characterization of Als5 protein (Ahmad et al., 2012). We chose *E. coli* strain for expression and purification of the Als5 protein for our studies. This was mainly because we wished to examine the role of the C-terminal signal sequence of Als5. Since *E. coli* doesn't have post translational machinery, the C-terminal signal sequence is not expected to be processed. Thus, Als5

protein with and without the C-terminal signal sequence could be expressed and purified from *E. coli* and comparative studies could be performed on the purified Als5 and Als5-SS proteins. A major drawback to this approach is the absence of post-translational glycosylation machinery in *E. coli* and therefore the absence of glycosylation on the proteins of interest. Since Als protein consists of a long Ser/Thr domain which is highly O-glycosylated and in *E. coli* glycosylation would not be occurred, thus it is quite possible that the endogenous conformation of the protein could be different from that observed *in vitro*.

Nevertheless, we hoped to obtain information regarding the conformation and function of the unglycosylated version of the protein as it would exist before it would be processed by the GPI transamidase machinery.

As also mentioned in the Introduction, full length Als5 (with and without C-terminal signal sequence) genes of *C. albicans* were cloned with N-terminal GST tag in pGEX-6P-2 vector in our lab. Both proteins (GST-Als5 and GST-Als5-SS) were expressed and purified from BL21 strain of *E. coli*. The functional and structural comparisons were performed for both GST-Als5 and GST-Als5-SS to see whether the C-terminal signal sequence affects the conformation or function of the protein. Besides biophysical and biochemical characterization, we performed adhesion assays using collagen type IV and aggregation assays. We observed that GST-Als5 adherence to human collagen type IV was much more than that of GST-Als5-SS. We obtained similar results in aggregation assays as well. Based on these studies we observed that GST-Als5 was more functional as an adhesin than GST-Als5-SS.

Our studies suggested that the presence of the C-terminal signal sequence was responsible for the reduced functionality. We showed that the C-terminal signal sequence was capable of inhibiting the aggregation of Als5 protein *in vitro* and proposed that this might be

the mechanism by which the pro-Als5 protein is kept away from aggregation in the ER just after translation and before it is processed by the GPI transamidase machinery (Ahmad et al., 2012).

Conformational studies using circular dichroism (CD) spectroscopy suggested significant differences in the secondary structures of GST-Als5 and GST-Als5-SS. Als5 had a pre-molten globular structure, whereas Als5-SS had a well folded α- helical conformation. Hence, we concluded that the differences in the functionalities of Als5 variants were due to the differences in conformation of Als5 and Als5-SS proteins.

Full length Als5-SS containing the C-terminal signal sequence has two stretches of hydrophobic patches capable of beta aggregation (Ahmad et al., 2012). The first of these is present in the SS itself as mentioned above, and the second is present toward the end of the N-terminal Ig-like domain of the protein. Thus, we hypothesised that C-terminal signal sequence could possibly fold back and interact with this amyloidogenic region (IVIVATT) in the N-terminal domain of Als5-SS. This interaction would force the protein to fold back upon itself, creating a torsional strain in Als5-SS leading to an apparently well-folded protein. Due to the masking of the amyloidogenic patch on the protein, Als5-SS is unable to exhibit aggregation and adhesion although it continues to bind to specific peptide ligands of Als5. On the other hand, in Als5 protein the N-terminal amyloidogenic patch of the Als5 exposed and is available for self-aggregation under appropriate concentration conditions (Figure I.6). The major focus of the present study was to test this model.

III.1 TANGO analysis to identify mutation that would reduce β-aggregation potential of Als5-SS

In our previous studies, we identified that Als5-SS protein has two domains with high β aggregation potential using TANGO analysis (Ahmad et al., 2012). One was found within the T (Threonine rich) domain, having a highly conserved IVIVA amyloidogenic patch and

another was found within the C-terminal signal sequence of the protein. As mentioned above, we hypothesized that these two domains interact with each other and govern the conformation and function of Als5-SS.

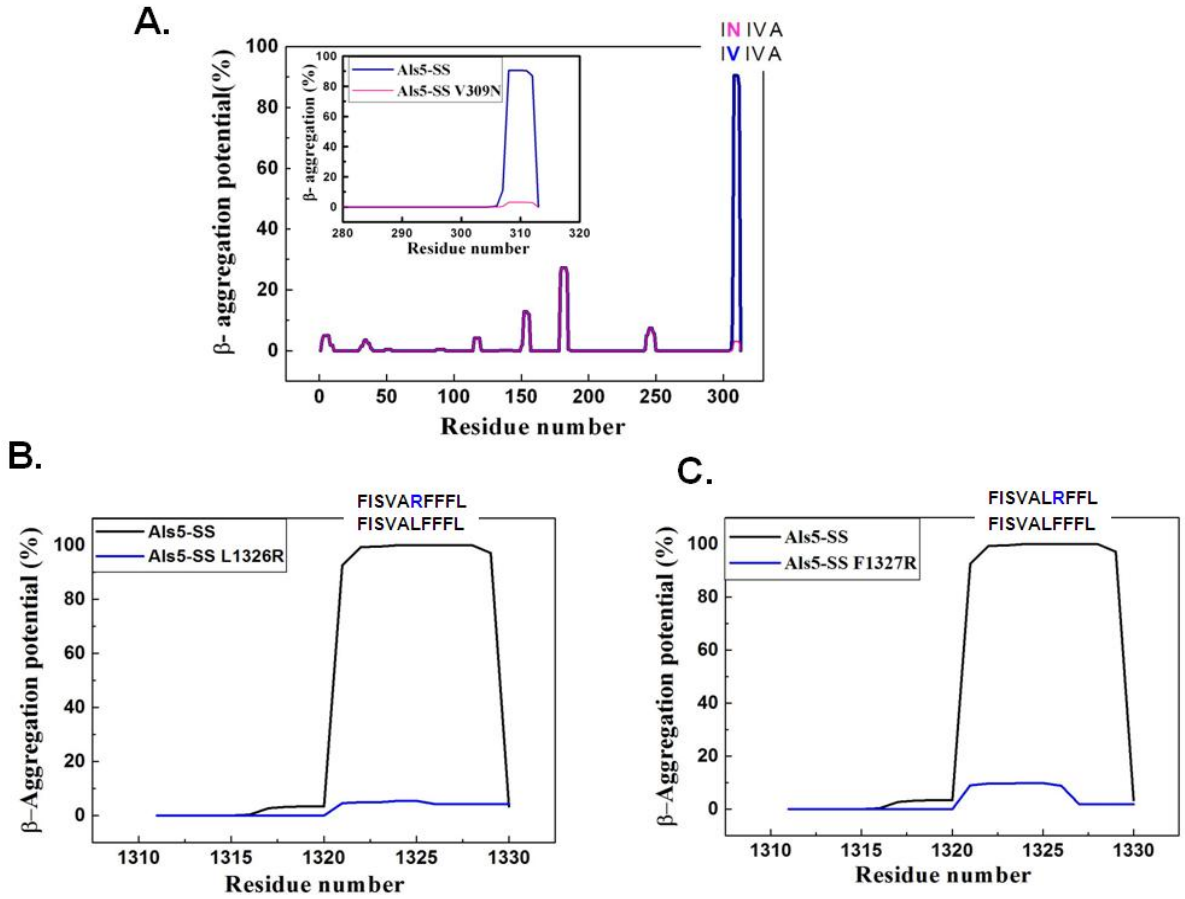

Figure III.1: TANGO prediction for the site directed mutations. (A) Als5-SS V309N (B) Als5-SS L1326R and (C) Als5-SS F1327R leading to the reduced β-aggregation potential for Als5-SS. In each mutant the β- aggregation potential was observed nearly zero as compared to the wild type Als5-SS.The sequences within which the mutations have been generated are also shown. In each figure the sequence below represents the wild type and the one above shows where the mutations have been generated.

To test our hypothesis, all the amino acid residues in both the amyloidogenic patches were replaced with charged residues individually and the aggregation potential was analyzed by TANGO predictor. We identified one site directed mutation in the amyloidogenic patch (Als5-SS V309N) and two site directed mutations in C-terminal signal sequence (Als5-SS

L1326R and Als5-SS F1327R) which could drastically reduce aggregation potential (Figure III.1).

III.2 Cloning, Expression and Purification of Als5-SS mutants

III.2.1 Cloning of Als5-SS mutants

The two site directed mutations [Als5-SS (L1326R and F1327R)] in the C-terminal signal sequence were generated and confirmed as described in Methods. Site directed mutation in the amyloid patch [Als5-SS (V309N)] was previously made and confirmed by Dr. Faiz Ahmed in our lab. In brief, the template (Als5-SS) was amplified from pGEX-6P-2-Als5-SS using primers with the specific mutations (Table II.1) and they gave us an amplified band of approximately 8.9 kb (Figure III.2.1a).

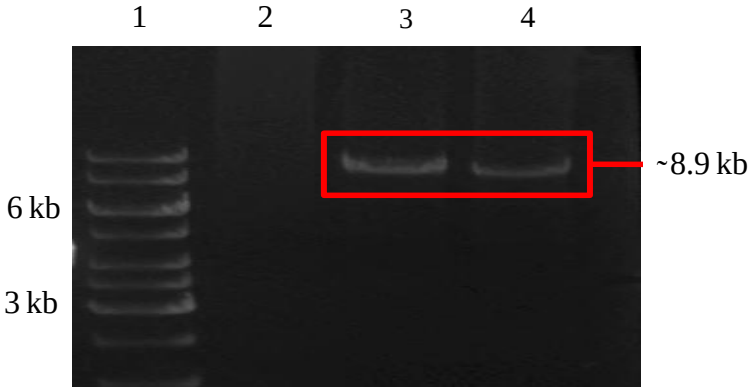

Figure III.2.1a: PCR amplification of Als5-SS L1326R and Als5-SS F1327R**.** Lane1: 1 kb DNA Ladder; Lane2: Negative control; lane3: Amplification of Als5-SS L1326R; Lane4: Amplification of Als5-SS F1327R.

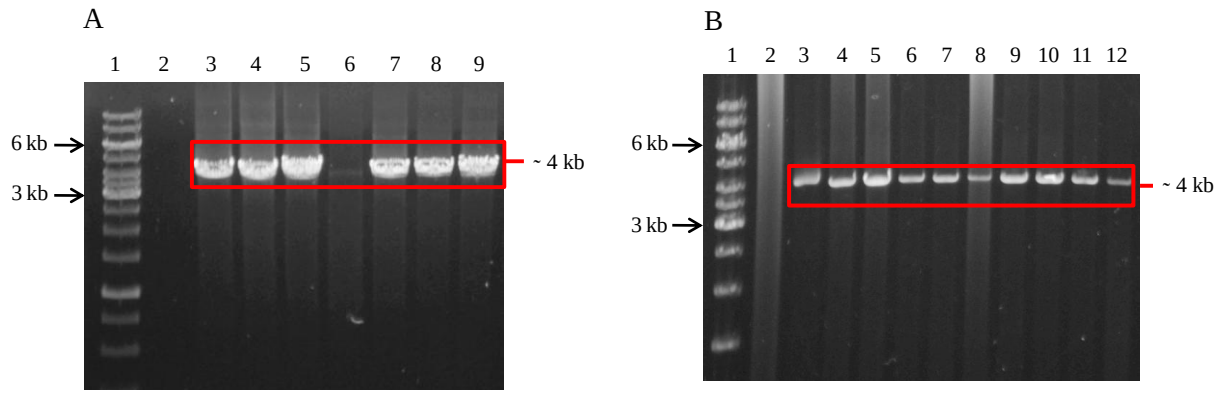

Figure III.2.1b: A. Colony PCR for the obtained colonies of Als5-SS L1326R after transformation. Lane 1: 1 kb DNA Ladder; Lane 2: Negative control; Lane 3-9: Screening of colonies obtained after transformation. B. Colony PCR for the obtained colonies of Als5-SS F1327R after transformation. Lane 1: 1 kb DNA Ladder; Lane 2: Negative control; Lane 3-12: Screening of colonies obtained after transformation.

The PCR amplified products were digested with Dpn1 enzyme to digest the template strands. DH5α strain of *E.coli* was transformed with the Dpn1 digested products. Colony PCR was done to screen the obtained colonies; bands of approximately 4 kb in case of each mutant confirmed the presence of Als5-SS proteins (Figure III.2.1b).

III.2.2 DNA sequencing for Als5-SS L1326R and Als5-SS F1327R mutants

DNA sequencing was done to further confirm the site directed mutations in Als5-SS (L1326R and F1327R). BLAST (Basic Local Alignment Search Tool) results are given below showing the sequence alignment between the query (sequence obtained after DNA sequencing) and the subject (sequence of wild type Als5-SS). As can be seen from these results, we were successful in generating two site directed mutants, one in which leucine was replaced by arginine and the second one in which phenylalanine was replaced by arginine (Figure III.2.2 a and III.2.2 b)

(i) DNA sequence alignment for Als5-SS L1326R

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Query  4     ATGCAACTAAC-ATGGATTTATCGCTACTTTATCACAAGCTCAAGTACCAAGTTCCTCGA  62
             ||| ||||||| ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3761  ATGAAACTAACAATGGATTTATCGCTACTTTATCACAAGCTCAAGTACCAAGTTCCTCGA  3820

Query  63    TTCATTCAGAGTTAATACTGACTACGACGGCTAAAACAACTGATGCTTCGATGAATGGAG  122
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3821  TTCATTCAGAGTTAATACTGACTACGACGGCTAAAACAACTGATGCTTCGATGAATGGAG  3880

Query  123   ACAGTGCTGCTTCAAACTCACAGCCAACCACATTAATTCAACAAGTAGCAACTTCCTCCT  182
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3881  ACAGTGCTGCTTCAAACTCACAGCCAACCACATTAATTCAACAAGTAGCAACTTCCTCCT  3940
```

```
Query  183   ACAATCAACCCCTTATTACCACTTATGCCGGATCTTCATCCGCCACTAAACATCCTTCCT  242
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3941  ACAATCAACCCCTTATTACCACTTATGCCGGATCTTCATCCGCCACTAAACATCCTTCCT  4000

Query  243   GGTTACTTAAATTTATTAGCGTTGCACGGTTCTTCTTTCTATGA  286
             |||| ||||||||||||||||||||    |||||||||||||||
Sbjct  4001  GGTTGCTTAAATTTATTAGCGTTGCATTATTCTTCTTTCTATGA  4044
```

Figure III.2.2a: Sequence alignment result for Als5-SS L1326R. Leucine was successfully mutated to arginine in Als5-SS background. As mentioned in the alignment TTA (codon encodes for leucine) is replaced by CGG (codon encodes for arginine).

(ii) DNA sequence alignment for Als5-SS F1327R

```
Query  7     AATGAAACTAACAAATGGATTTATCGCTACTTTATCACAAGCTCAAGTACCAAGTTCCTC  66
             |||||||||||| ||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3760  AATGAAACTAAC-AATGGATTTATCGCTACTTTATCACAAGCTCAAGTACCAAGTTCCTC  3818

Query  67    GATTCATTCAGAGTTAATACTGACTACGACGGCTAAAACAACTGATGCTTCGATGAATGG  126
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3819  GATTCATTCAGAGTTAATACTGACTACGACGGCTAAAACAACTGATGCTTCGATGAATGG  3878

Query  127   AGACAGTGCTGCTTCAAACTCACAGCCAACCACATTAATTCAACAAGTAGCAACTTCCTC  186
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3879  AGACAGTGCTGCTTCAAACTCACAGCCAACCACATTAATTCAACAAGTAGCAACTTCCTC  3938

Query  187   CTACAATCAACCCCTTATTACCACTTATGCCGGATCTTCATCCGCCACTAAACATCCTTC  246
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  3939  CTACAATCAACCCCTTATTACCACTTATGCCGGATCTTCATCCGCCACTAAACATCCTTC  3998

Query  247   CTGGTTGCTTAAATTTATTAGCGTTGCATTACGGTTCTTTCTATGA  292
             |||||||||||||||||||||||||||||||   ||||||||||||
Sbjct  3999  CTGGTTGCTTAAATTTATTAGCGTTGCATTATTCTTCTTTCTATGA  4044
```

Figure III.2.2b: Sequence alignment result for Als5-SS F1327R. Phenylalanine was successfully mutated to arginine in Als5-SS background. As mentioned in the alignment TTC (codon encodes for Phenylalanine) is replaced by CGG (codon encodes for arginine).

III.2.3 Expression and purification

Als5-SS V309N, Als5-SS L1326R and Als5-SS F1327R were expressed in BL21 strain of *E. coli* cells and purified as described in Methods. In brief, the BL21 strain of *E. coli* was transformed with pGEX-6P-2 plasmid incorporated with inserts of our interest. Primary cultures were grown for overnight at 37° C, the secondary culture was induced with 0.1 mM IPTG and grown at 16° C for 4 hrs. After expression, the cells were harvested and lysed. The expression of all the proteins was detected on SDS-PAGE. We observed the band of expected size (approximately 270 kDa) for all the proteins (GST-Als5-SS V309N, GST-Als5-SS L1326R and GST-Als5-SS F1327R in Figure III.2.3a (A), III.2.3b (A) and III.2.3c (A) respectively). Cell lysate from the uninduced secondary culture was taken as a negative control for protein expression. Als5-SS is a 1347 amino acids long protein and its molecular weight is 142 kDa. Since GST has a molecular weight of ~ 26 kDa, so the expected molecular weight for GST-Als5-SS V309N, GST-Als5-SS L1326R and GST-Als5-SS F1327 would be 168 kDa, but we obtained the bands of ~270 kDa in SDS-PAGE (Figure III.2.3a (A), III.2.3b (A) and III.2.3c (A) respectively). In previous studies, this anomalous mobility was attributed to the high content of polar amino acids, Ser/Thr, in the proteins (Rauceo *et al*., 2006). Als5 is a Ser/Thr rich protein and polar side chains of serine and threonine residues are known to interact with polyacrylamide gels resulting in higher than actual molecular weights for such proteins on SDS-PAGE.

After confirmation of protein expression by SDS-PAGE, the cell lysate were used and proteins were further confirmed by western blotting as described in Methods section (GST-Als5-SS V309N, GST-Als5-SS L1326R and GST-Als5-SS F1327R in Figure III.2.3a (C), III.2.3b (C) and III.2.3c (C) respectively). All the proteins were detected by anti-GST primary antibody and HRP conjugated secondary antibody.

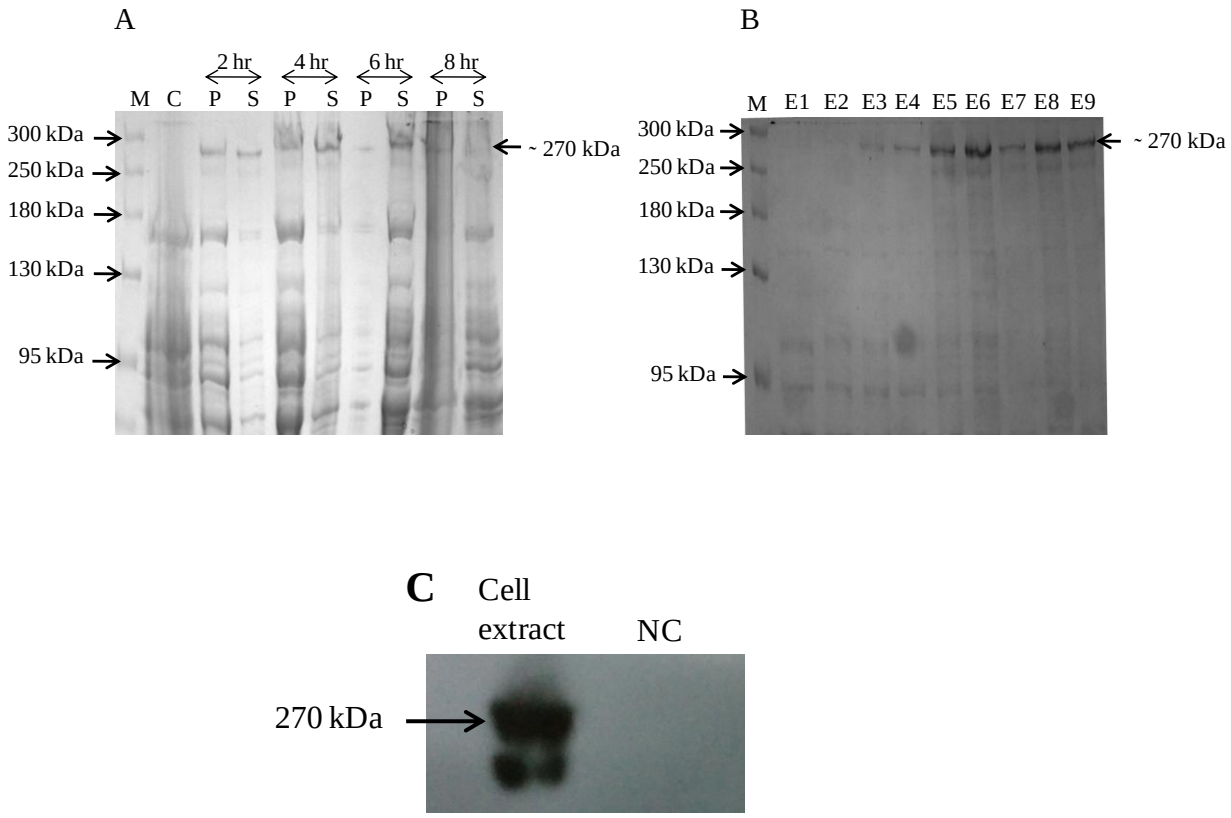


Figure III.2.3a: A. Expression of GST-Als5-SS V309N. M: Protein ladder; C: Control; S: Supernatant; P: Pellet. Expression was done at different time points- 4 hrs, 6 hrs and 8 hrs at 16° C, arrow indicates the band of approximately 270 kDa. B. Purification of GST-Als5-SS V309N. M: Protein ladder; Lane E1-E9: Eluted fractions of protein; we obtained the band of approximately 270 kDa. C. Detection of protein by western blotting from the cell lysate of GST-Als5-SS V309N.

Next, the cell lysate of all the constructs were used for protein purification. The cell lysate was poured on to Glutathion-CL-Agarose beads and all the proteins were purified by affinity chromatography as discussed in the Methods section. The purified proteins were detected by SDS-PAGE and we observed the expected band for all the proteins (GST-Als5-SS V309N, GST-Als5-SS L1326R and GST-Als5-SS F1327R in Figure III.2.3a (B), III.2.3b (B) and III.2.3c (B) respectively).

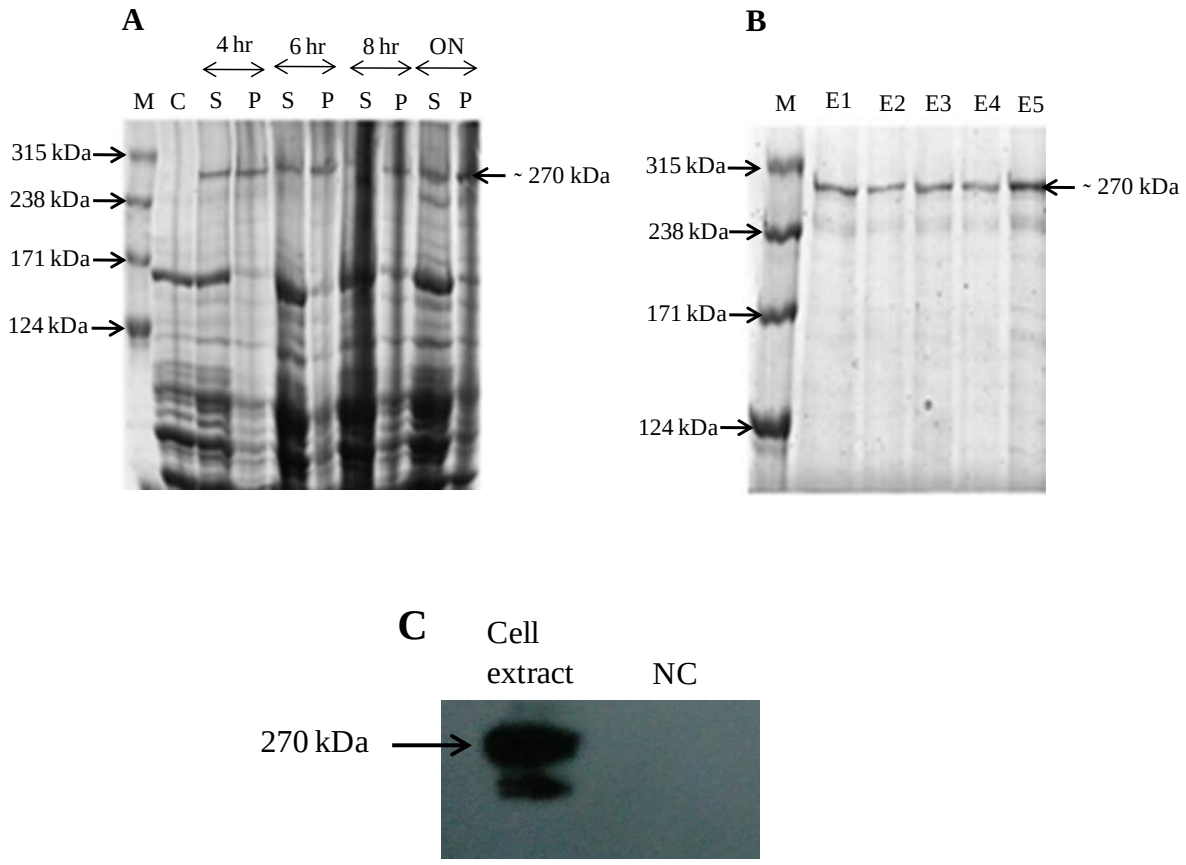
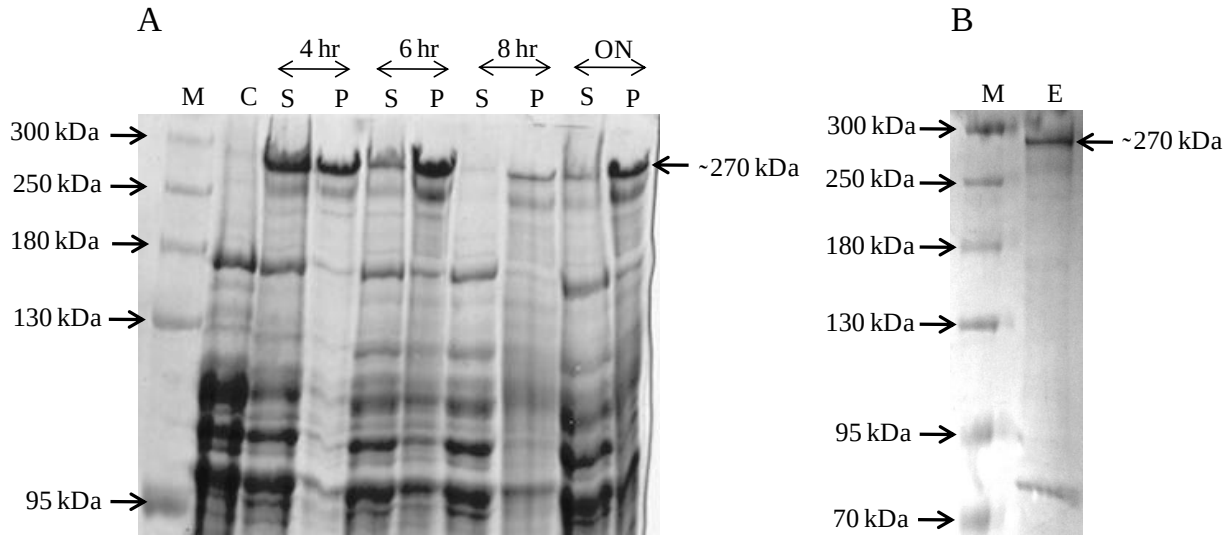


Figure III.2.3b: A. Expressions of GST-Als5-SS L1326R. M: Protein ladder; C: Control; S: Supernatant; P: Pellet. Expression was done at different time points- 4 hrs, 6 hrs, 8 hrs and O/N at 16°C, arrow shows band of our interest of approximately 270 kDa. B: Purification of GST-Als5-SS L1326R. Lane1: Protein ladder; Lane 2-6: different elutes of protein, and arrow shows band of approximately 270 kDa. C. Detection of protein by western blotting from the cell lysate of GST-Als5-SS L1326R.



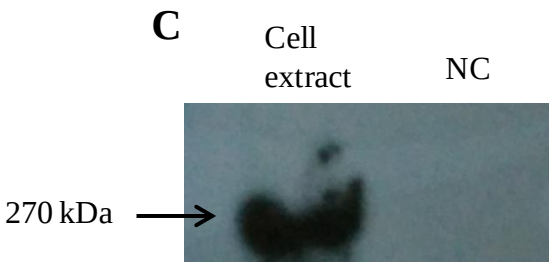


Figure III.2.3c: A. Expression of GST-Als5-SS F1327R. M: Protein ladder; C: Control; S: Supernatant; P: Pellet. Expression was done on different time points- 4 hrs, 6 hrs, 8 hrs and O/N at 16°C, arrow indicates band of approximately 270 kDa. B. Purification of GST-Als5-SS F1327R. M: Protein ladder; E: Eluted fraction of Als5-SS F1327R. C. Detection of protein by western blotting from the cell lysate of GST-Als5-SS F1327R.

III.3 Biophysical characterization for Als5-SS variants

III.3.1 Als5-SS mutant proteins had different conformations from that of wild type Als5-SS protein

The secondary structure analysis for Als5-SS and Als5-SS mutant variants were assessed by CD spectroscopy by using Chirascan CD spectrometer (Applied Photophysics). The protocol for CD analysis was followed as described in Methods section.

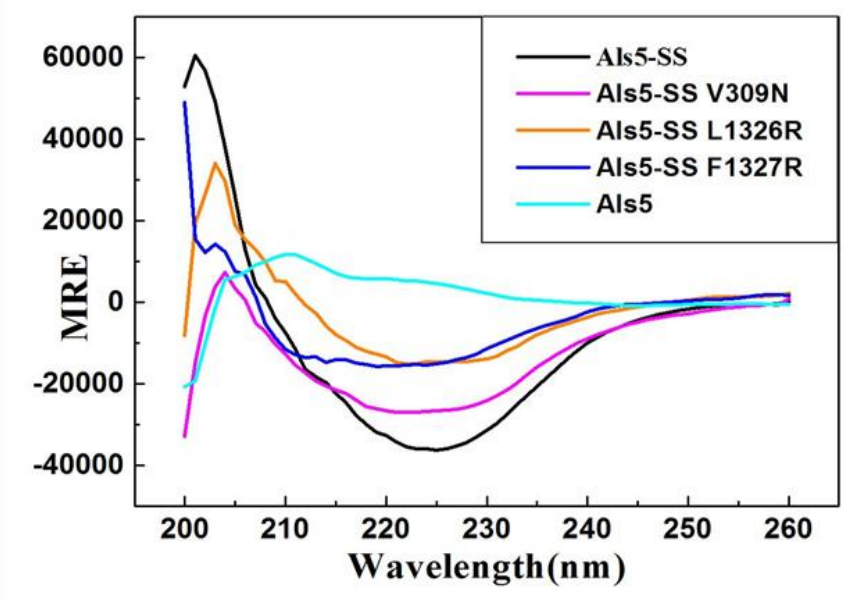


Figure III.3.1: Secondary structure prediction of proteins- GST-Als5, GST-Als5-SS, GST-Als5-SS V309N, GST-Als5-SS L1326R and GST-Als5-SS F1327R. The GST spectrum was subtracted from the spectra of all the proteins. The CD spectra of mutant proteins differed from that of GST-Als5-SS, suggesting that these mutations alter the conformation of the protein possibly by disrupting the interaction between the N-terminus amyloidogenic patch and C-terminal SS (Ahmad et al., 2015).

Briefly, we took approximately 0.09 mg/ml of dialyze proteins and CD spectra were obtained between 200-260 nm in a 1 mm path length cuvette with 1 nm step length. The final spectra were averages of five repeat scans and in all cases buffer background was subtracted. Since all the proteins were tagged with GST, hence the spectrum of GST was subtracted from the spectrum of each protein using Pro-Data software that came with the instrument.

As we discussed in the introduction as well that Als5-SS had a totally different secondary structure as compared to that of Als5 protein. The spectrum of the mutant proteins studied here were neither similar to Als5-SS nor to Als5 (Figure III.3.1).

It appeared that the site directed mutations in the amyloid patch and the C-terminal signal sequence altered the conformation of Als5-SS protein (Figure III.3.1), without completely unfolding it. The secondary structure content was measured by K2D (http://dichroweb.cryst.bbk.ac.uk/html/home.shtml) software which predict protein secondary structure from their CD spectra. It was observed that the secondary structure content of Als5-SS had 69 % α-helix, 4 % β- sheet and 27 % random coil. While the secondary structure content of Als5-SS V309N had 55 % α-helix, 9 % β- sheet and 36 % random coil, Als5-SS L1326R had 59 % α-helix, 8 % β- sheet and 33 % random coil and Als5-SS F1327R had 60 % α-helix, 7 % β- sheet and 33 % random coil.

Table 3: Secondary structure content of Als5-SS and its mutant varients.

Proteins	**α-helix**	**β- sheet**	**Random coil**
Als5-SS	69 %	4 %	27 %
Als5-SS V309N	55 %	9 %	36 %
Als5-SS L1326R	59 %	8 %	33 %
Als5-SS F1327R	60 %	7 %	33 %

The K2D prediction suggested that the alpha helical content of the mutant proteins is decreasing as compared to that of wild type Als5-SS while the random coil strctures are increasing. We have previously seen that Als5 lacking the SS has a CD spectrum that is mostly disordered (Ahmad *et al*., 2012). The CD spectrum of Als5 had 25.4 % β strand, 9.2 % α-helix, 43.8 % turn and 21.6 % disordered region. We have previously shown using PONDR-FIT (Xue et al., 2010) that the full length Als5 protein should in all likelihood be a natively unfolded protein (Ahmad et al., 2012).

III.4 Biochemical characterization for Als5-SS variants

III.4.1 Als5-SS mutants shows better adhesion to human collagen type IV than wild type

First of all, we observed the collagen type-IV binding ability of mutant proteins (Als5-SSV309, Als5-SS L1326R and Als5-SSF1327R) to see whether they mutants were altered in their adhesion properties as compared to that of wild type Als5-SS. Previous reports from our lab suggested that GST-Als5 showed greater adherence to collagen type IV than that of GST-Als5-SS (Ahmad et al., 2012).

We followed the protocol as described in the Methods section. In brief, a 96 well plate was coated with collagen type-IV and incubated at 4°C over night. Next day the wells were washed with PBS and proteins (GST, GST-Als5-SS, GST-Als5-SS V309, GST-Als5-SS

L1326R and GST-Als5-SS F1327R) were added in the respective wells and incubated at 37°C for 1 h. The wells were blocked with 5% skimmed milk and incubated at 37°C for 1 h and washed with antibody buffer. It was treated with anti-GST primary antibody and HRP-conjugated Secondary antibody and incubated at 37°C for 1 h. The HRP substrate tetramethylbenzidine solution was added to each well and incubated at 37° for 1 h and $OD_{650\ nm}$ was recorded by plate reader (MULTISCAN GO).

After performing adhesion assay for all the mutants (GST, GST-Als5-SS, GST-Als5-SS V309, GST-Als5-SS L1326R and GST-Als5-SS F1327R), we observed that the mutant proteins were adhering much better than Als5-SS (Figure III.4.1). These results support the hypothesis that the mutations could interrupt the interactions between amyloid patch and C-terminal signal sequence, thus exposing the aggregation-prone regions of the proteins.

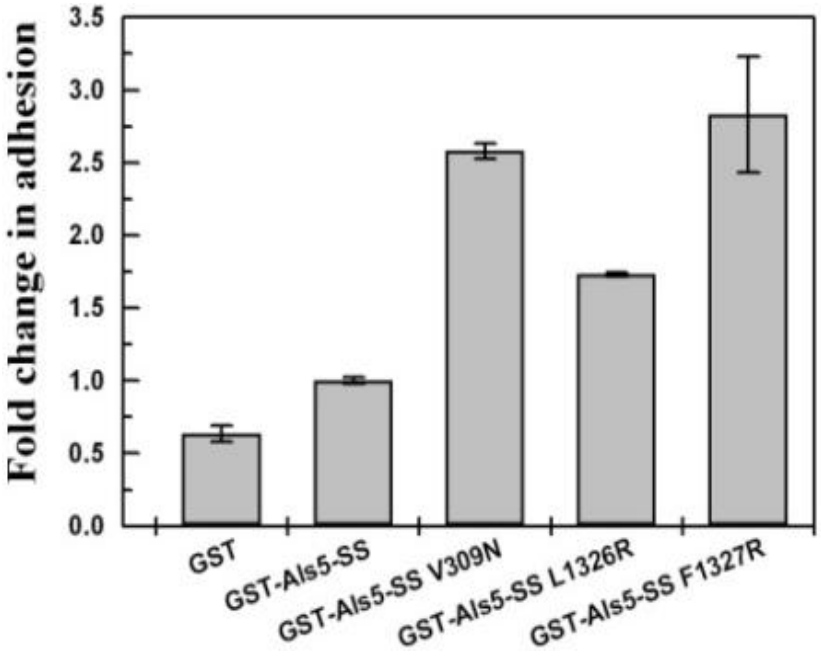


Figure III.4.1: Adhesion assay for Als5-SS and its mutant proteins. The mutant proteins showed better adherence to Collagen type IV as compare to GST-Als5-SS and GST alone (control) (Ahmad et al., 2015). This experiment was done twice and the average is plotted with standard deviation.

III.4.2 Als5-SS mutants variants showed greater aggregation than that of wild type Als5-SS

As we have already discussed in the Introduction chapter, the amyloidogenic regions and hydrophobicity of Als proteins are mainly involved in the aggregation of Als proteins and aggregation is a well known functional property for Als family of adhesin proteins. Hence, we next performed aggregation assays using transmission electron microscopy (TEM) as described in the Methods section. In brief, we purified GST-Als5 as well as GST-Als5-SS protein by affinity chromatography, incubated the proteins at 37°C for different time period (12 hrs, 24 hrs, 1 week and 2 weeks) and analyzed them by Transmission Electron Microscope (TEM). In previous studies from our lab, we observed that Als5 formed large aggregates, whereas GST (taken as control) and Als5-SS protein didn't show any aggregates under the same conditions, suggesting that Als5 protein was more functional as compared to that of Als5-SS (Ahmad et al., 2012). We hypothesised that the differences in the functionalities were due of the differences in the secondary structures of the two proteins.

To further confirm that only the short C-terminal signal sequence (SS) stretch of amino acids was preventing aggregation in case of Als5-SS, the same assay was performed with synthetic signal sequence peptide acting as a competitor in these assays. The synthetic signal sequence peptide was incubated with purified GST-Als5 at 1:1 molar concentration and was able to reduce the self aggregation of GST-Als5. We hypothesised that the synthetic signal sequence peptide is interacting with the amyloid patch of Als5, thereby reducing the self aggregation of GST-Als5 (Ahmad et al., 2012).

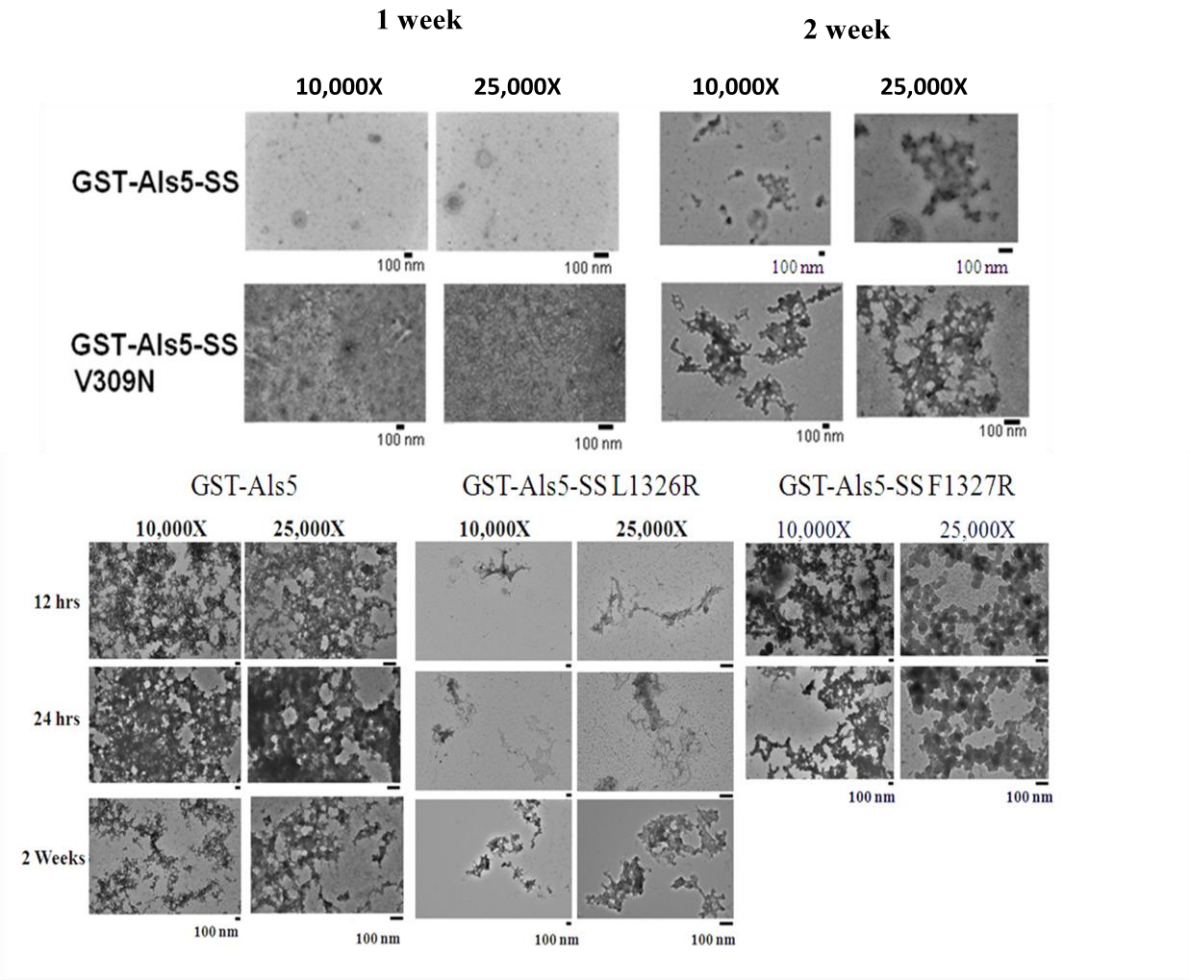


Figure III.4.2a: TEM Images showing the aggregation of proteins- GST-Als5, GST-Als5-SS, GST-Als5-SS V309N, GST-Als5-SS L1326R and GST-Als5-SS F1327R. The mutants probably modified the interactions between the amyloidogenic domain and C-terminal signal sequence (SS) thus resulted to the enhanced aggregates than GST-Als5-SS. The aggregation of GST alone was taken as control to demonstrate that the aggregation of proteins was not affected due to the GST tag (Ahmad et al., 2015). This experiment was done twice to confirm the results.

In the present study, we carried out aggregation assays for all three mutants [GST-Als5-SS V309N (within amyloidogenic patch), GST-Als5-SS L1326R and GST-Als5-SS F1327R (within signal sequence)]. The mutant proteins showed high aggregation as compared to that of wild type Als5-SS with the aggregation increasing upon increasing incubation time (Figure III.4.2a). The results suggested that the mutations abrogated the interactions between the amyloid patch and the signal sequence in Als5-SS. Thus, the regions capable of β-aggregation are exposed or accessible and the mutants become more aggregation-prone.

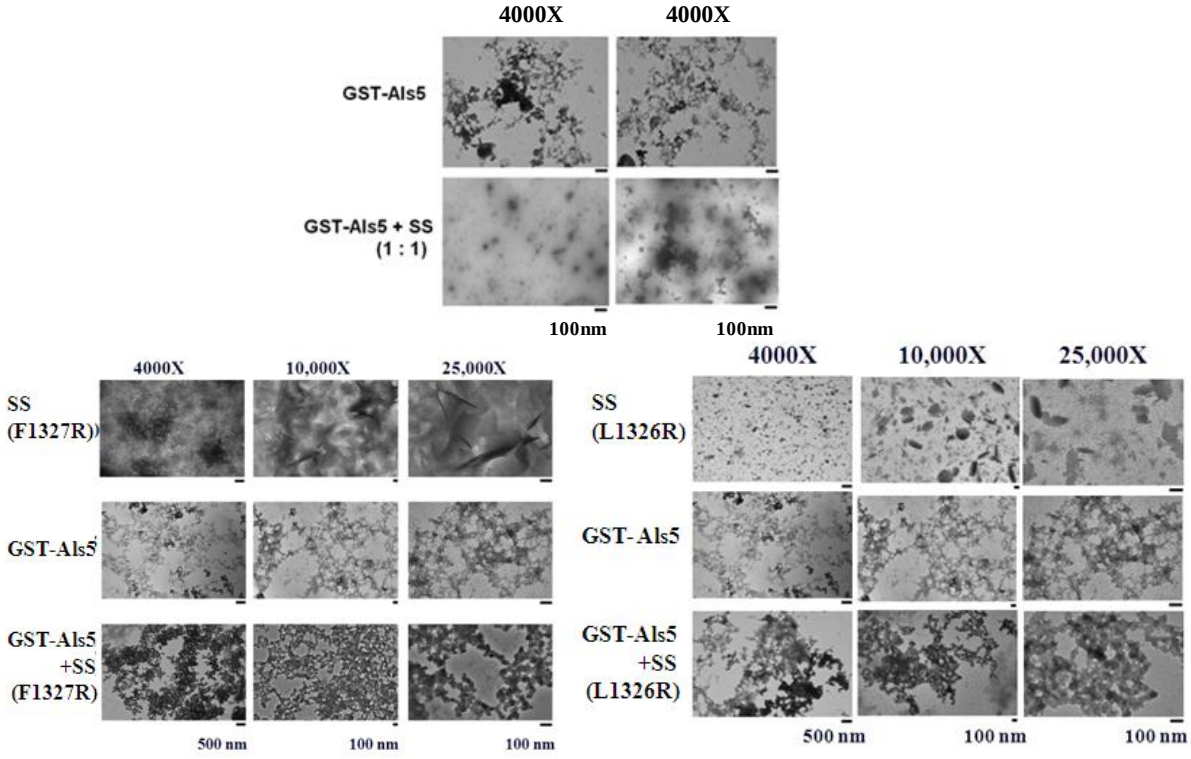


Figure III.4.2b: The aggregation of Als5 protein was inhibited in the presence of equimolar concentration of wild type synthetic SS peptide (Ahmad et al., 2012). But, the synthetic SS peptide with mutations were unable to inhibit the aggregation of GST-Als5 protein, suggested that the SS with the mutations were unable to interact with amyloidogenic patch of GST-Als5 (Ahmad et al., 2015). This experiment was done twice to confirm the results.

Further, we performed the aggregation assays using mutated synthetic signal sequence (L1326R and F1327R) acting as competitors for GST-Als5, to see whether the mutated synthetic signal sequence is able to suppressing the aggregation of GST-Als5 or not. We incubated the mutated synthetic signal sequences peptides with purified GST-Als5 protein and performed TEM analysis of the products. We observed that synthetic signal sequence with mutations were unable to suppress the aggregation of Als5 protein, while the wild type synthetic signal sequence was able to do so (Figure III.4.2b). These results suggested that the mutations affected the ability of the synthetic signal sequence to bind to the amyloid patch of Als5 protein and so, couldn't suppress the aggregation of Als5 protein.

III.5 Site directed mutations in ligand binding pocket

As discussed in the Introduction, besides carrying the amyloidogenic patch, the N-terminal domain of Als5 has another very important feature. It has the Ig-like domain consisting of the ligand binding pocket which is involved in the recognition of specific peptide ligands of the protein. This function allows the Als proteins to bind to host basal laminar proteins and establish adhesive colonies of *C. albicans* in due course. Hence, we attempted to generate mutations in the peptide binding pocket as well in order to identify amino acid residues critical for ligand binding.

As mentioned in the Introduction, the crystal structure of N-terminal domain of the Als9-2 (NtAls9-2) suggested that the Lys59 is a very critical residue for ligand binding. The author's suggested that Lys59 makes a salt-bridge with the C-terminal carboxylate group of the peptide ligand (Salgado et al., 2011). To confirm the importance of Lys59 in terms of ligand recognition, Als5 Lys59Ala site directed mutation was made in our lab and it was observed that this residue is not involved in ligand recognition. Studies from our lab suggested that Lys59 is involved in the aggregation but not in ligand binding (Faiz thesis).

A recent study on Als3 suggested that Ser170 residue is very critical for ligand binding (Lin et al., 2014). The Ser 170 residue is present at the entry of the peptide binding pocked of Als3 protein. The authors suggested that a site directed mutation (S170Y) completely blocked the entry of peptide into the peptide binding pocket. In the crystal structure of N-terminal domain of Als3, it was shown that the S170Y mutation didn't affect the surface conformation of protein but blocked the entry of peptide thus resulting in the formation of a hollow peptide binding pocket. It has been observed that this mutant had decreased adhesion to endothelial as well as epithelial cells (Lin et al., 2014).

Sequence alignment analysis showed that Ser170 is conserved in Als5 protein. To check whether the Ser170 has a role in ligand binding, the site directed mutation (S170Y)

was generated in Als5. The clone was made successfully as shown below (Figure III.5.1) and confirmed by sequencing. The protocol used for protein expression is mentioned in Methods. The clone was expressed in *E.coli* but the protein band was observed in the insoluble fraction. We tried to solubilize it by using different conditions of buffers and strains as well, but we couldn't succeed in solubilizing Als5 S170Y (Figure III.5.2). We hypothesized that the protein was probably aggregating rapidly and becoming insoluble. Later on the molecular modeling and molecular dynamics simulations studies suggested that S170Y is indeed a very unstable protein (Figure III.5.3). Due to the solubility issues, we couldn't purify Als5 S170Y and therefore, we couldn't carry out the ligand binding studies of Als5 S170Y mutant.

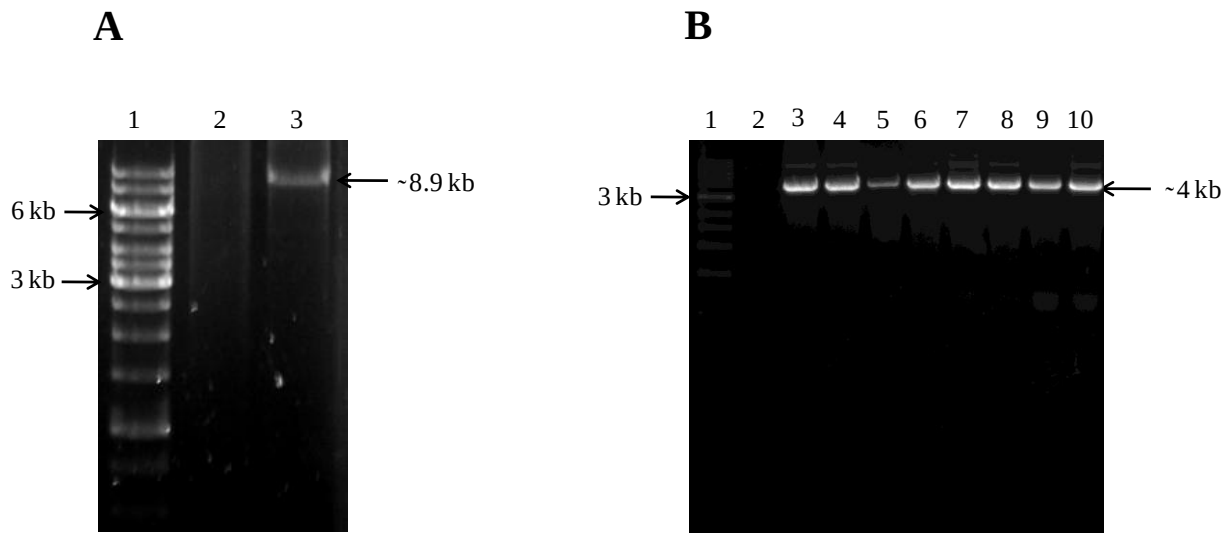


Figure III.5.1: A. PCR amplification of Als5 S170Y. Lane1: 1 kb DNA Ladder; Lane2: Negative control; lane3: Amplification of Als5 S170Y. B. Colony PCR for the obtained colonies of Als5 S170Y after transformation. Lane 1: 1 kb DNA Ladder; Lane 2: Negative control; Lane 3-10: Screening of colonies obtained after transformation.

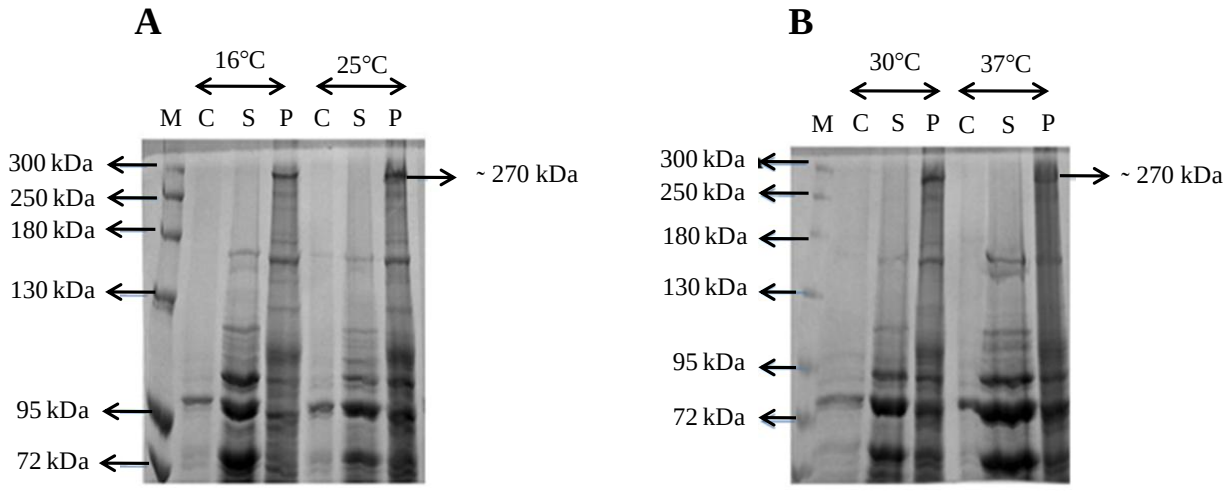


Figure III.5.2: A. Expression of Als5 S170Y at 16°C and 25°C. B. Expression of Als5 S170Y at 30°C and 37°C. M: Protein ladder; C: Control; S: Supernatant; P: Pellet. Expression was done on different temperatures, arrow indicating band of our interest approximately 270 kDa.

III.6 Molecular modelling and molecular dynamic simulations

III.6.1 Model genertaion of Als5 and the mutant

As we described in Methods, the structure of N-terminal domain of *C. albicans* Als9-2 was used to produce the model for Als5Nt. Previously, the model of Als5Nt bound with a segment of C-terminal SS was modeled using 2Y7L C-terminal end as template, which forms βG2 strand over N1 domain of Als9-2 structure. Then a model of Als5Nt-C-terminal SS complex with peptide ligand was produced by using Fg-γ peptide as template. It was observed that the C-terminal SS can fold back and interact with the N-terminal domain of Als5-SS protein and the C-terminal SS doesn't occupy the ligand binding pocket (Ahmad et al., 2012). The mutation in the Als5 was incorporated using the Rosetta Backrub protocol which uses Monte-Carlo simulated annealing using the Rosetta all-atom force field and the lowest energy model for Als5 and Als5 S170Y were obtained (Figure III.6.1).

III.6.2 Molecular dynamic simulations

Molecular dynamic simulations were performed as described in Methods. The root mean square deviation (rmsd) represents distance between the atoms of protein backbone. A change on rmsd after generating a mutation in a protein is reflective of the stability or instability of a protein. From the classical and accelerated molecular dynamic simulations, the root mean square deviation and root mean square fluctuation (rmsf) analysis showed that the structure of N-terminal domain of Als5 S170Y significantly different from that of Als5Nt [Figures III.6.2a and b (A and B)]. In other words it appears that this mutation leads to misfolding of the N-terminal domain of the protein in molecular dynamics simulations (data shown below).

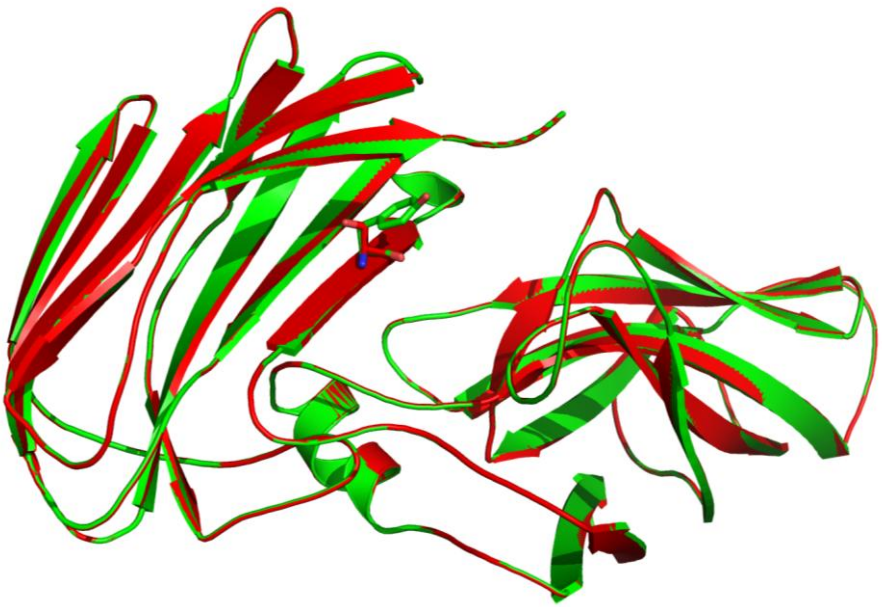


Figure III.6.1: Representation of lowest energy Rosetta model of Als5Nt and Als5 S170Y. Red color is representing the model of Als5 and green color is representing the model of Als5 S170Y. PyMOL program was used to generate the figure.

The radius of gyration (tells about the compactness of the structure of a protein) and solvent accessible surface area (SASA) analysis showed that Als5 S170Y had more radius of

gyration and more solvent accessible surface area as compare to that of wild type Als5 [Figure III.6.2a and b (C and D)]. These analyses suggest that the mutation introduces significant changes in the secondary structural of Als5 S170Y, making the protein unstable. This confirms what we observed experimentally as well. Due to the inherent instability of the Als5 S170Y mutant, it was insoluble and we couldn't purify it from solution for further studies.

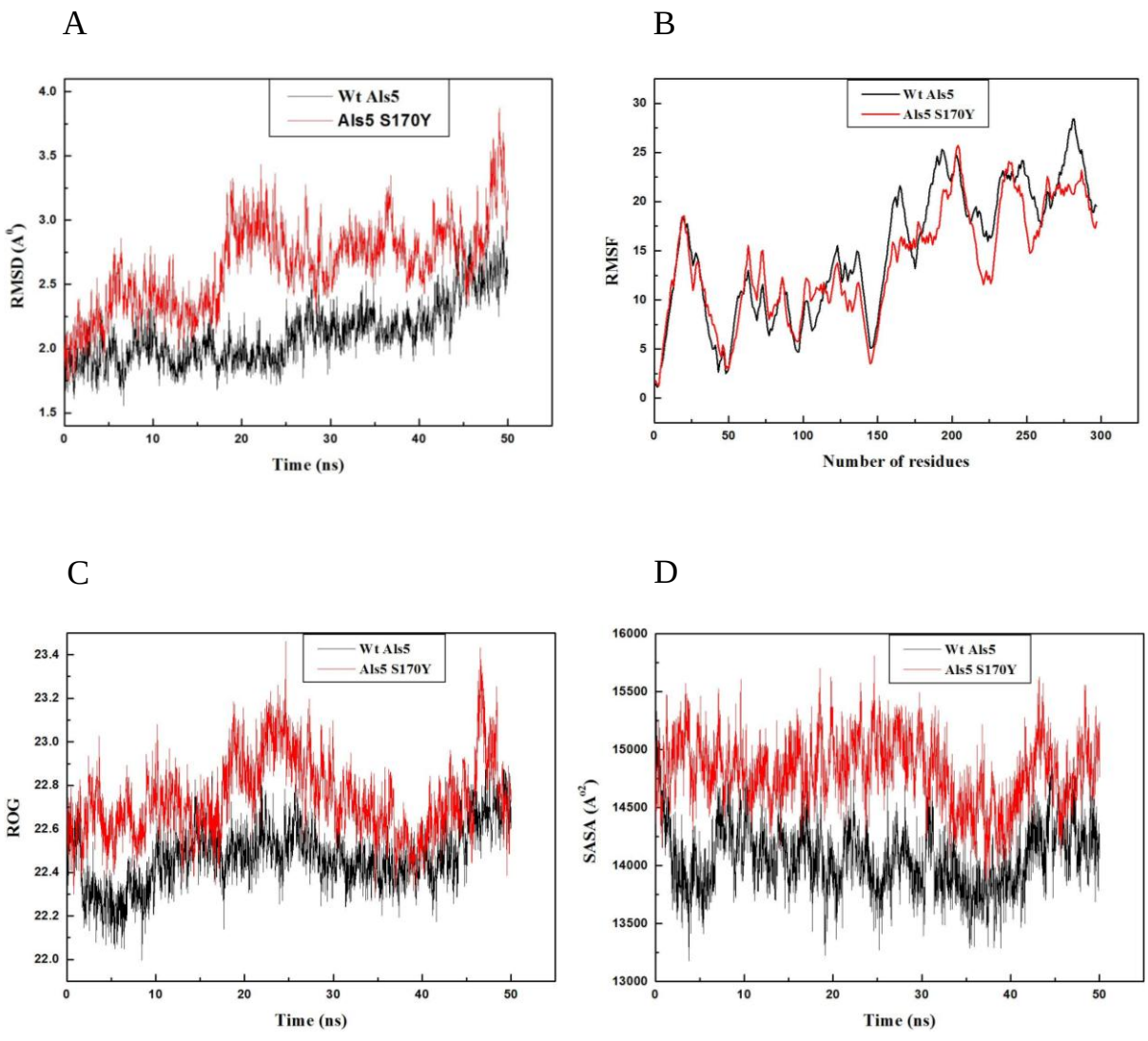


Figure III.6.2a: Classical molecular dynamic simulations of Als5 and Als5 S170Y. A. Comparison between the rmsds of wild type Als5 and Als5 S170Y from the molecular dynamic simulation runs of 50 ns for each protein. B. Simulation runs are showing the rmsf for each residue of type Als5 and Als5 S170Y. C. Simulation runs of 50 ns are showing the radius of gyration for wild type Als5 and Als5 S170Y. D. Simulation runs of 50 ns are showing solvent accessible surface area for wild type Als5 and Als5 S170Y.

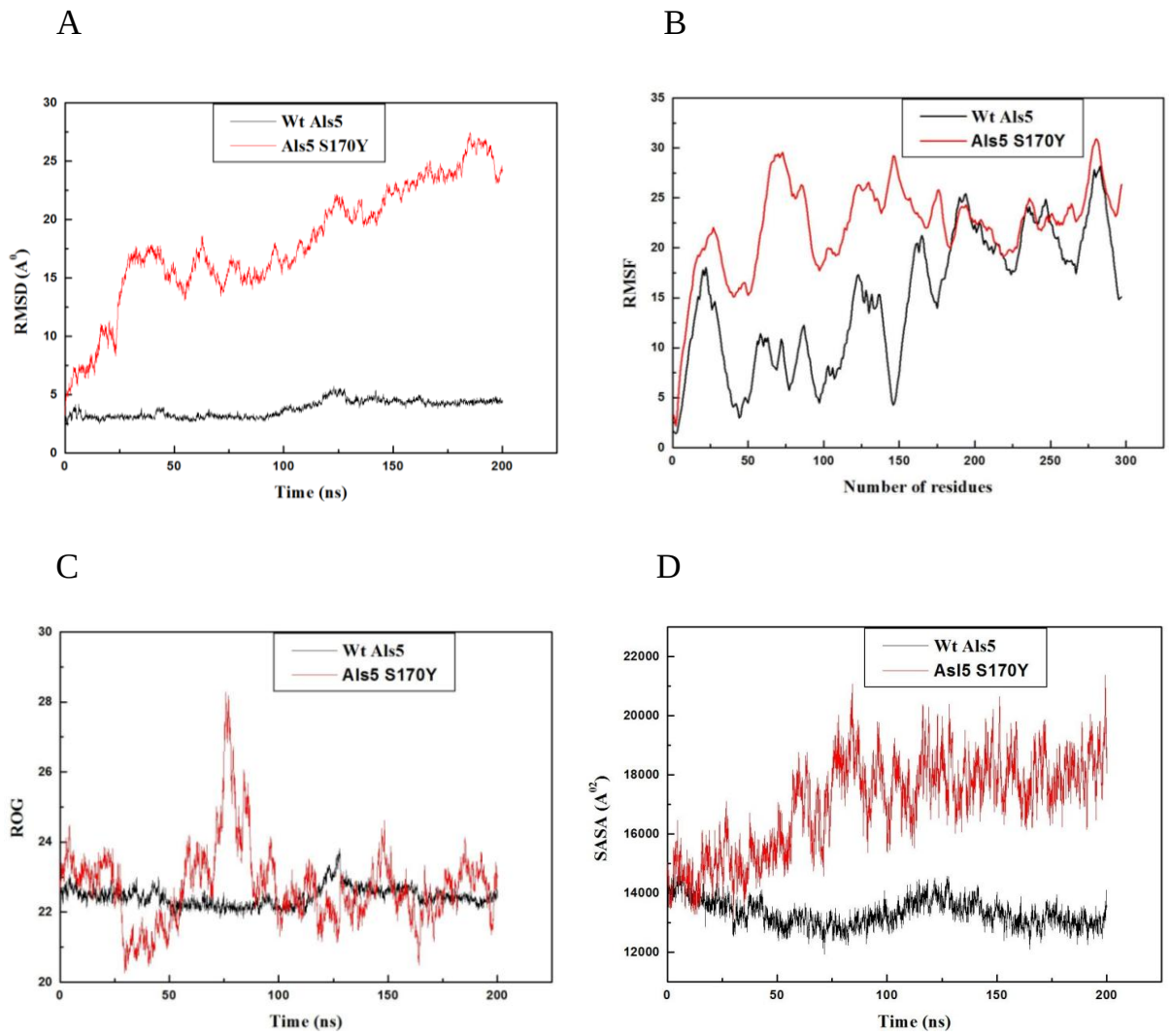


Figure III.6.2b: Accelerated molecular dynamic simulations of Als5 and Als5 S170Y. A. Comparison between the rmsds of wild type Als5 and Als5 S170Y from the molecular dynamic simulation runs of 50 ns for each protein. B. Simulation runs are showing the rmsf for each residue of type Als5 and Als5 S170Y. C. Simulation runs of 50 ns are showing the radius of gyration for wild type Als5 and Als5 S170Y. D. Simulation runs of 50 ns are showing solvent accessible surface area for wild type Als5 and Als5 S170Y.

Chapter IV

Characterization of Als5 mutants

by heterologous expression in *S. cerevisiae*

S. cerevisiae is a unicellular eukaryotic organism, approximately 5-10 µm in size and very easy to culture optimally at 30 °C. It is widely used as a model organism to study the Als proteins of *C. albicans* (Fu et al., 1998; Gaur and Klotz, 1997; Gaur et al., 1999; Sheppard et al., 2004). There are several features which make *S. cerevisiae* a model organism for our studies. The first and most important reason is that *S. cerevisiae* genome is highly similar to *C. albicans* but it doesn't have Als proteins and has no adhesive properties. Thus, we can easily examine the role of Als adhesins by heterologous expression in *S. cerevisiae*. Further, its life cycle is very simple and short and takes just 100 minutes for doubling their population (Herskowitz, 1988). The reproduction cycle of *S. cerevisiae* involves the process where two haploid cells mate with each other by fusion and forms the diploid cell, then the diploid cell (mother cell) is divided into daughter cells by budding process. Several studies have carried out characterization of full length and individual domains of heterologously expressed Als proteins in *S. cerevisiae* (Fu et al., 1998; Gaur and Klotz, 1997; Rauceo et al., 2004; Sheppard et al., 2004). These systems, for example, have been used to study ligand binding abilities and specificities of Als proteins. Deletion and insertion mutations of Als1 have shown that the ligand binding capability resides within the N-terminal domain whereas the C-terminus is essential for cell surface localization of Als1 protein (Loza et al., 2004). Protein chimeras made by swapping of N-terminals of Als5 and Als6 also suggested that the ligand binding specificity lies within the N-terminus of Als proteins (Sheppard et al., 2004).

Similarly, heterologous expression systems have enabled study of adhesion by Als proteins. Heterologous expression of *ALS5* in non adherent *S. cerevisiae* showed increased adherence to extra cellular matrix protein (fibronectin, collagen type IV and laminin) coated beads as well as cellular aggregation (Gaur and Klotz, 1997; Gaur et al., 1999). Similarly, heterologous expression of *ALS1* in *S. cerevisiae* showed increased adherence to epithelial and endothelial cells (Fu et al., 1998). Sheppard *et al*. showed that, *S. cerevisiae* expressing

different Als family members had different adherence profiles (Sheppard et al., 2004). *S. cerevisiae* expressing Als1, Als3 and Als5 proteins showed adherence to extracellular matrix proteins (gelatin, laminin and fibronectin) as well as to epithelial and endothelial cells (Rauceo et al., 2004; Sheppard et al., 2004). *S. cerevisiae* expressing Als6 showed binding to gelatin only; cells expressing Als9 alone showed poor binding with laminin while cells expressing Als7 didn't bind to any substrate (Sheppard et al., 2004). Even defects in the peptide binding cavity of the N-terminal of Als3 caused defects in adhesion (Lin et al., 2014). Strains with the mutations in the PBC (peptide binding cavity) of Als3 was non-adherent on human pharyngeal epithelial, umbilical vein endothelial cells and human buccal epithelial cells suggesting that an intact PBC in Als3 was required for adhesion of *C. albicans* to host cells (Lin et al., 2014). The functional role of Als2 and Als4 could not be studied due to problems in their amplification (Sheppard et al., 2004).

The heterologous expression system has also been successfully employed for the study of cellular aggregation mediated by the Als proteins. As already discussed, aggregation by Als proteins have characteristics similar to the amyloid aggregates and are governed by amyloid forming sequences (Otoo et al., 2008; Rauceo et al., 2004). *S. cerevisiae* strain expressing Als5 show increased fluorescence of ANS in aggregating cells suggesting that Als5 forms amyloid aggregates (Rauceo et al., 2004). Ramsook *et al* also showed that *S. cerevisiae* cells expressing Als1 and Als5 bound to amyloid specific dyes (Congo Red and Thioflavin T) (Ramsook et al., 2010). The role of amyloid forming region (IVIVATT) in this aggregation was confirmed by mutational approaches (Garcia et al., 2011). *S. cerevisiae* cells expressing $Als5^{V326N}$ (mutation in amyloid patch) mutant protein showed very small aggregates as compare to cells expressing wild type Als5 protein. Amyloid perturbing dyes Congo red and Thioflavin T inhibited the aggregation of cells expressing wild type Als5, whereas they didn't show any effect on the aggregation of cells expressing $Als5^{V326N}$ mutant

protein. *S. cerevisiae* cells expressing Als5^{V326N} incubated with the amyloid forming sequence (SNGIV^{326}IVATTRTV) formed larger aggregats similar to wild type Als5 protein, suggesting that Als proteins mediated aggregation is dependent on amyloid formation (Garcia et al., 2011; Lipke et al., 2014). A fluorescent peptide from *C. albicans* Als5 containing the amyloid forming sequence has also been developed (Garcia-Sherman et al., 2014). This peptide bound specifically to *S.cerevisiae* cells expressing Als5. This peptide also bound to the yeast and hyphae form of *C. albicans* which expresses Als1 and Als3 proteins. Whereas the null mutants of a*ls1* and *als3* showed reduced fluorescence indicating that the fluorescent peptide binds to Als1 and Als3 proteins. The authors suggested that the amyloid sequence of Als1, Als3 and Als5 are similar therefore the Als5 fluorescent peptide would bind to yeast and hyphae form of *C. albicans* (Garcia-Sherman et al., 2014). Domain analysis suggested that the number of tandem repeats also contributes to the extent of cellular aggregation. *S. cerevisiae* cells expressing a truncated mutant Als5 (Ig-T-TR) was found to be more adherent to fibronectin and mediated more cellular aggregation as compared to cells expressing Als5 (Ig-T) (Frank et al., 2010; Rauceo et al., 2006).

Using a similar heterologous expression system we too examined the role of Als5 and its mutant variants in cellular adhesion and aggregation. As explained in the previous chapter, our *in vitro* studies suggest that the site directed mutations in the amyloid patch and C-terminal SS altered the secondary structure of the Als5-SS protein. The first question to ask is whether these changes are significant enough to make a difference to recognition of the protein by the GPI-transamidase complex? If so, we expect to see reduced expression of the proteins on the cell surface as compared to the wild type protein. If they do get processed by the GPI-transamidase and the other post-translational machinery correctly, the mutant proteins will enter the secretory pathway, and appear as GPI anchored proteins on the cell

surface. Thereafter, it would become possible to examine the role of these mutations on cellular adhesion and aggregation.

Thus, we began by first examining the expression of Als5 and its mutant proteins in the heterologuos expression system using *S. cerevisiae* (YPH 501 strain).

An *in-silico* analysis with two GPI anchor prediction softwares (GPI-SOM and BIG-PI predictor) to predict the GPI anchoring of Als5-SS and its mutants (V309N, L1326R and F1327R) was first carried out to ensure that the mutations we chose did not seriously compromise the GPI anchoring ability of the proteins.

Table 4: Prediction of GPI anchor attachment by BIG-PI predictor and GPI-SOM.

Proteins	**BIG PI- Predictor**	**GPI- SOM**
Als5-SS	GPI anchored	GPI anchored
Als5-SS L1326R	Non GPI anchored	GPI anchored
Als5-SS F1327R	GPI anchored	GPI anchored
Als5-SS V309N	GPI anchored	GPI anchored

BIG-PI predictor use a data set which is designed by the collection of GPI anchored protein sequences entries from SWISS-PROT and SWISS-NEW (Eisenhaber et al., 1999). Whereas, GPI-SOM is a Self Organiging Map developed by Kohonen and it predicts the GPI anchor attachment site with high accuracy. In this analytical software, we can search, browse and visualize the information (Fankhauser and Maser, 2005; Kohonen, 2001). We observed that all the mutants were predicted to be GPI anchored except L1326R which was GPI anchored according to GPI-SOM but not by BIG PI predictor (Table IV.1).

IV.1 Construction of Als5-SS and its mutants

We already had the site directed mutants (GST-Als5-SS, GST-Als5-SS V309N, GST-Als5-SS L1326R and GST-Als5-SS F1327R in pGEX-6P-2), which we had used for the bacterial expression and *in-vitro* studies. For studies in *S. cerevisiae*, these genes were

digested with the help of BamHI and XhoI restriction enzymes and ligated them into pYES2 vector as described in Methods. The DH5α strain of *E. coli* was transformed with the ligated products. The colonies obtained after transformation were screened by colony PCR using gene specific primers (Table II.1) and amplification bands of approximately 4 kb were obtained for Als5-SS, Als5-SS-V309N, Als5-SS-L1326R and Als5-SS-F1327R as shown in Figure IV.1a (A, B, C and D), respectively.

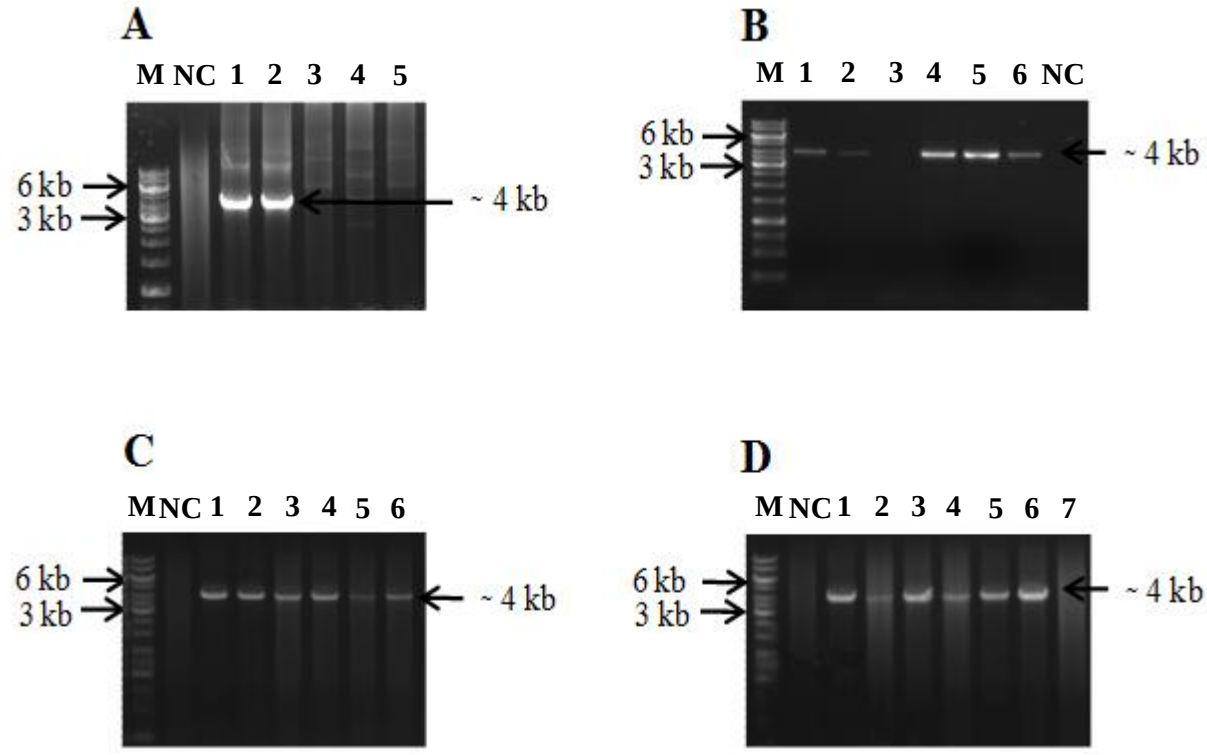


Figure IV.1a: Figure A, B, C and D representing colony PCR of Als5SS, Als5SS V309N, Als5SS L1326R and Als5SS F1327R respectively. A. Lane M: 1 kb DNA ladder; Lane NC: Negative control; Lane 1-5: number of screened colonies obtained after transformation. B. Lane M: 1 kb DNA ladder; Lane NC: Negative control; Lane 1-6: number of screened colonies obtained after transformation. C. Lane M: 1 kb DNA ladder; Lane NC: Negative control; Lane 1-6: number of screened colonies obtained after transformation. D. Lane M: 1 kb DNA ladder; Lane NC: Negative control; Lane 1-7: number of screened colonies obtained after transformation.

The plasmids from all the colonies that were positive by colony PCR were isolated and digested with BamHI and XhoI restriction enzymes. The release of approximately 4 kb

and vector backbone of approximately 5.9 kb was observed [Als5-SS, Als5-SS-V309N, Als5-SS-L1326R and Als5-SS-F1327R, Figure IV.1b (A, B, C and D), respectively].

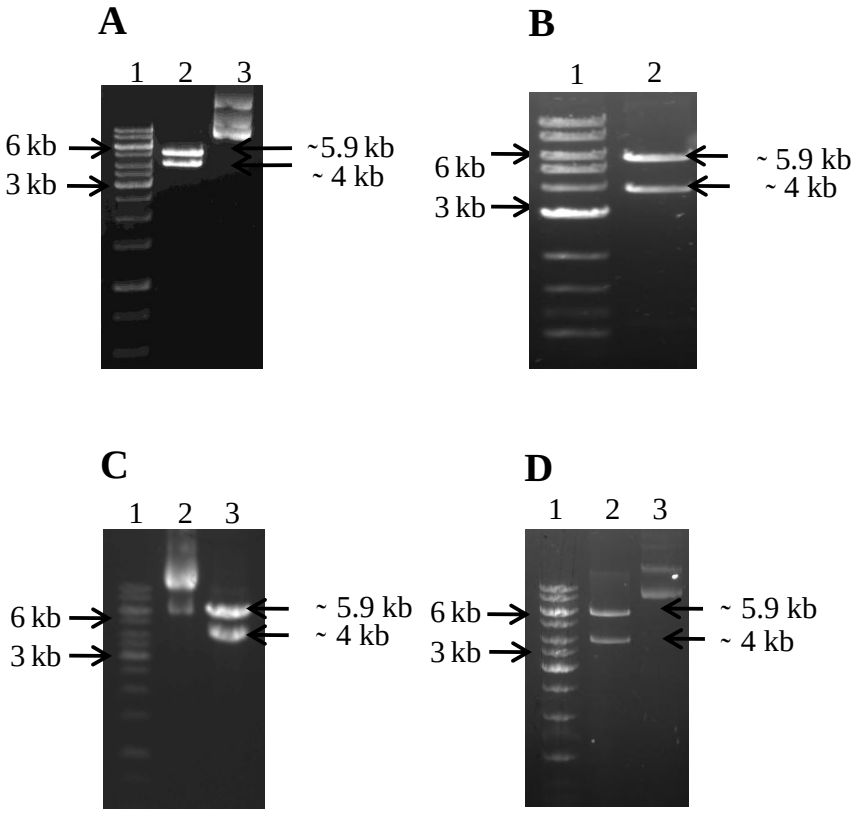


Figure IV.1b: Figure A, B, C and D representing double digestion of Als5SS, Als5SS V309N, Als5SS L1326R and Als5SS F1327R respectively. A. Lane 1: 1 kb DNA Ladder; Lane 2and 3: Double digestion and undigested plasmid respectively. B. Lane 1: 1 kb DNA Ladder; Lane 2: Double digestion. C. Lane 1: 1 kb DNA Ladder; Lane 2and 3: Undigested plasmid and double digestion respectively. D. Lane 1: 1 kb DNA Ladder; Lane 2and 3: Double digestion and undigested plasmid respectively.

The YPH501 strain of *S. cerevisiae* was transformed with plasmids incorporated with inserts of our interest (Als5-SS, Als5-SS-V309N, Als5-SS-L1326R and Als5-SS-F1327R). The pYES2 vector alone was also used to transform YPH501 as a control. Colonies were obtained on the SD Ura^- selection plates after transformation. All the constructs were confirmed by colony PCR using gene specific primers (Table II.1) where amplification bands of approximately 4 kb were observed [Figure IV.1c (B, C, D and E)]. The transformed colonies with vector control (pYES2) were confirmed by using primers specific for *URA3* (Table II.1) where an amplified band of approximately 1.1 kb was obtained (Figure IV.1c A).

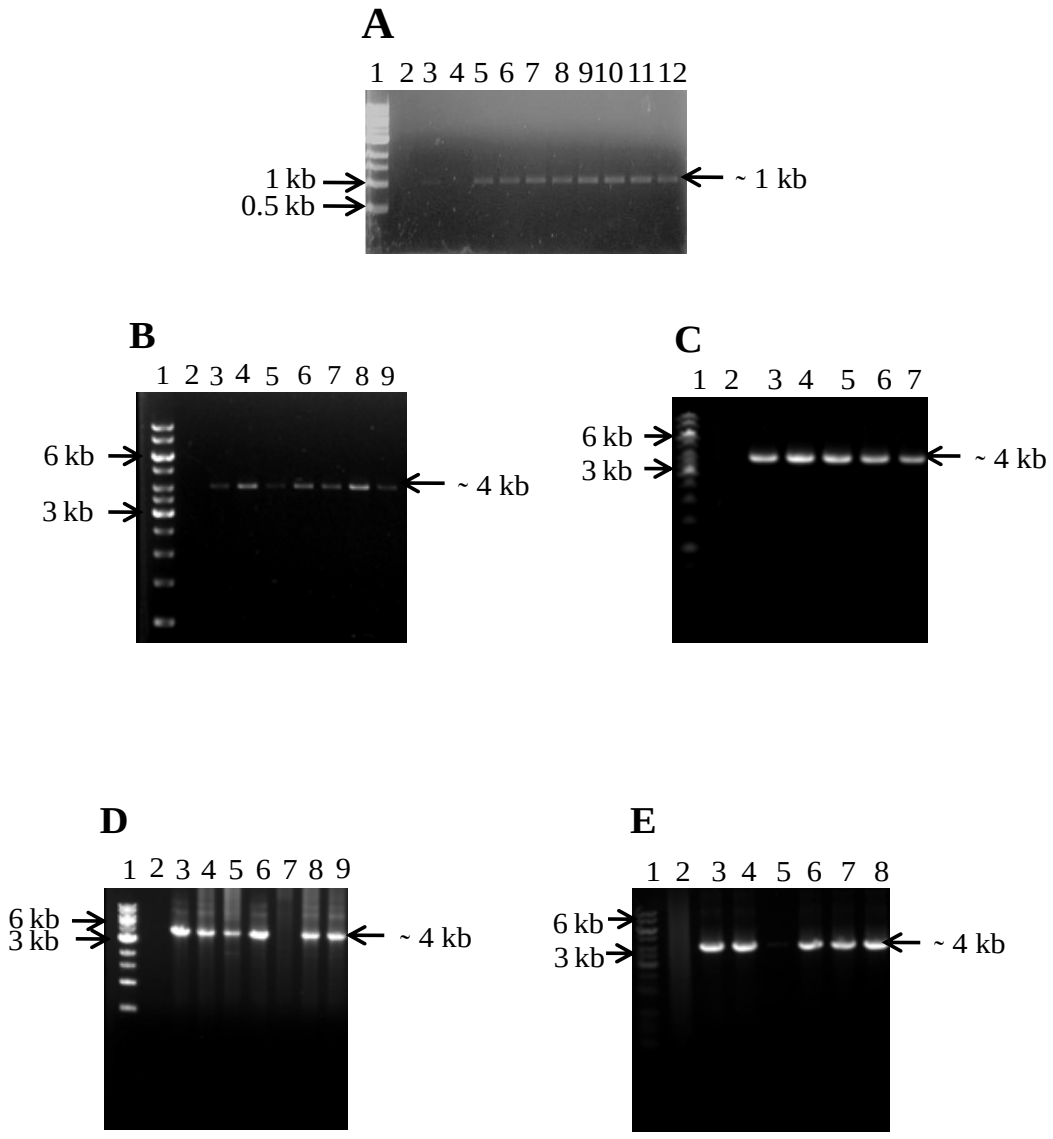


Figure IV.1c: A, B, C, D and E showing colony PCR for pYES2 (vector control), Als5SS, Als5SS V309N, Als5SS L1326R and Als5SS F1327R. A. Lane 1: 1 kb DNA Ladder; Lane 2: Negative control; Lane 1-12: Screening of colonies obtained after transformation of *S. cerevisiae* with vector only. B. Lane 1: 1 kb DNA Ladder; Lane 2: Negative control; Lane 1-9: Screening of colonies obtained after transformation of *S. cerevisiae* with vector having Als5-SS. C. Lane 1: 1 kb DNA Ladder; Lane 2: Negative control; Lane 1-7: Screening of colonies obtained after transformation of *S. cerevisiae* with vector having Als5-SS V309N. D. Lane 1: 1 kb DNA Ladder; Lane 2: Negative control; Lane 1-9: Screening of colonies obtained after transformation of *S. cerevisiae* with vector having Als5-SS L1326R. E. Lane 1: 1 kb DNA Ladder; Lane 2: Negative control; Lane 1-8: Screening of colonies obtained after transformation of *S. cerevisiae* with vector having Als5-SS F1327R.

IV.2 *In-vivo* functional characterization of Als5-SS and its mutant variants

IV.2.1 Expression of Als5-SS and its mutant variants in *S. cerevisiae*

Do the mutations in the C-terminal SS affect the final destination of Als5-SS protein or not? For this, we checked the expression of mutant proteins on the cell surface of *S. cerevisiae* and compared with wild type Als5-SS. For expression of Als5-SS constructs, we followed the procedure described in Methods. Our genes were cloned in pYES2 vector under the *PGAL1* promoter, so the genes will be switched on in the presence of galactose only. And *URA3* is the selection marker present in pYES2 vector, which will ensure that only *URA3* containing strains will grow in the minimal media. Therefore, the primary culture was grown in minimal medium (Ura^-) and the secondary culture was grown in inducible medium (Ura^- Gal^+ medium). The cells were incubated with anti-Als5 primary antibody and TRITC-labeled secondary antibody and viewed under a fluorescence microscope (TiE, Nikon). YPH 501 cells transformed with the empty vector, pYES2, were taken as a control.

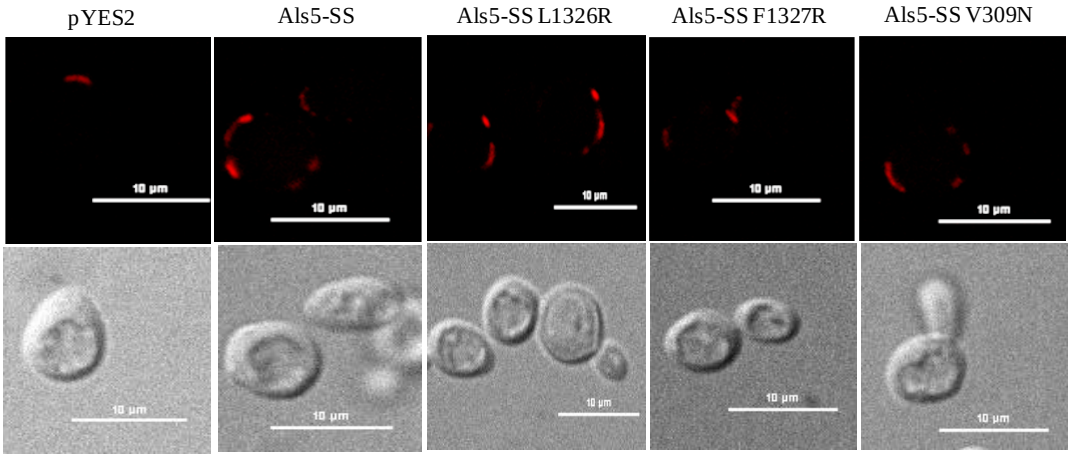


Figure IV.2.1a: Fluorescence microscopy images showing the cell surface expression of Als5SS and its mutant proteins. Cells transformed with pYES2 were taken as a vector control. This experiment was done thrice to confirm the results and here we have taken one representative fluorescence image showing expression of each protein.

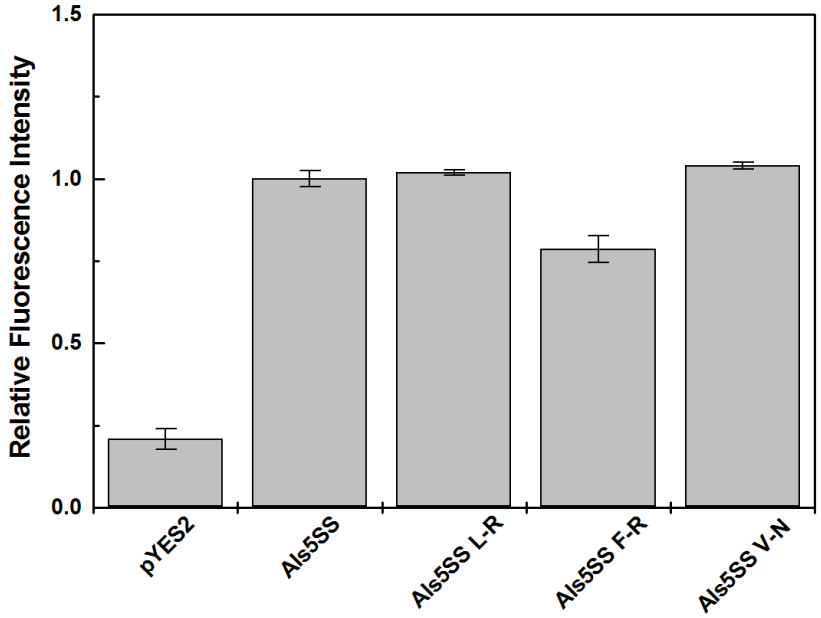


Figure IV.2.1b: Quantification of Als5-SS and its mutant variants: We did the quantification of fluorescence intensities of Als5-SS protein and its mutant proteins expressed on the cell surface were done. The wild type as well as mutant proteins showed almost equal expression, pYES2 was taken as a vector control. The average is plotted with standard deviation. The *p* value for Als5-SS L1326R is 0.58, for Als5-SS F1327R is 0.03 and for Als5-SS V309N is 0.36 with respect to Als5-SS, suggesting no significant differences between them.

The expression of wild type as well as mutant proteins in our set-up was observed to be roughly equal (Figure IV.2.1a). To compare the expression levels of Als5-SS versus its mutant proteins, quantification of fluorescence intensities was done for all the proteins. For the quantification, we used 80 cells of each strain. We observed almost equal relative fluorescence intensity in all the strains expressing Als5-SS, Als5-SS V309N, Als5-SS L1326R and Als5-SS F1327R proteins (Figure IV.2.1b). This suggested that the mutation did not affect the translocation of the protein to the cell surface that's why relative fluorescence intensity was equal in all the proteins on the cell surface. Thus, one may infer that the mutations do not interfere with the recognition of Als5-SS by the GPI-transamidase. This is in keeping with what was predicted by *in silico* analysis. Certain other inferences too may be drawn from these results. It would appear that if the mutations do indeed affect the secondary structure of the pro-protein, this structure itself is irrelevant to the processing of the protein

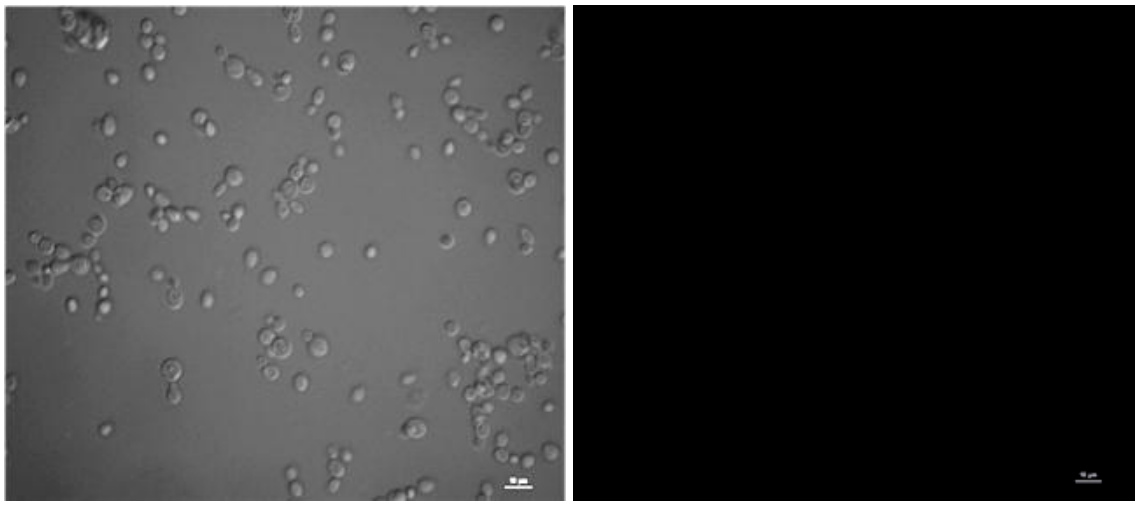
by the GPI-transamidase. It is possible that the GPI-transamidase is entirely sequence dependent, rather than conformation dependent. It is also possible that post-translational glycosylation (Mora-Montes *et al*., 2009) of Als5 in the ER of *S. cverevisiae* prevents the Als5-SS from adopting the same conformation that is observed in the unglycosylated variant purified from bacterial cells.

IV.2.2 Aggregation properties were unaffected in Als5-SS and its mutants

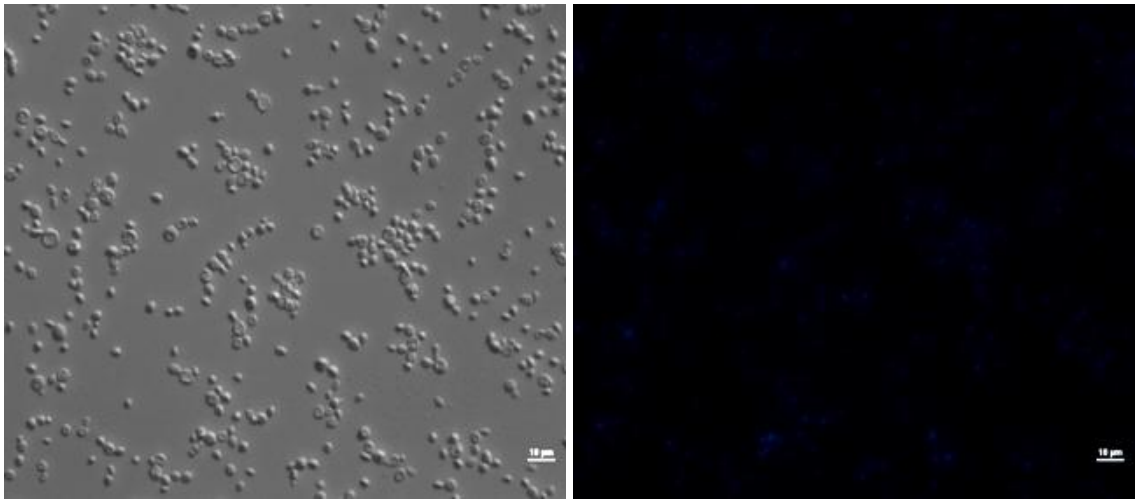
Since the cell surface expression of the mutant proteins were almost equal to that of wild type Als5 protein. We could carry out biochemical assays for these proteins in the heterologous expression system. To study cellular aggregation induced by these proteins, the cells expressing Als5 variants were grown in appropriate inducible medium and stained with ANS (8-anilino-1-naphthalene-sulfonic acid) dye. ANS is an amyloid specific dye, which binds specifically to the hydrophobic part of protein aggregates (Yu and Strobel, 1996).

As can be seen from Figure IV.2.2a, the cells expressing mutant proteins were able to form aggregates similar to the cells expressing wild type Als5 protein. As before, cells transformed with pYES2 were taken as a vector control (Figure IV.2.2a). To compare the aggregation potential of Als5-SS versus its mutant proteins, quantification of fluorescence intensities were done for the aggregates formed by the *S. cerevisiae* strains expressing all the proteins. For the quantification, we used 80 aggregated bunches of cells for each strain. We observed almost equal relative fluorescence intensity in aggregates formed by the strains expressing Als5-SS, Als5-SS V309N, Als5-SS L1326R and Als5-SS F1327R proteins (Figure IV.2.2b). Thus, it appears that the mutations did not affect the ability of Als5 protein to form aggregates.

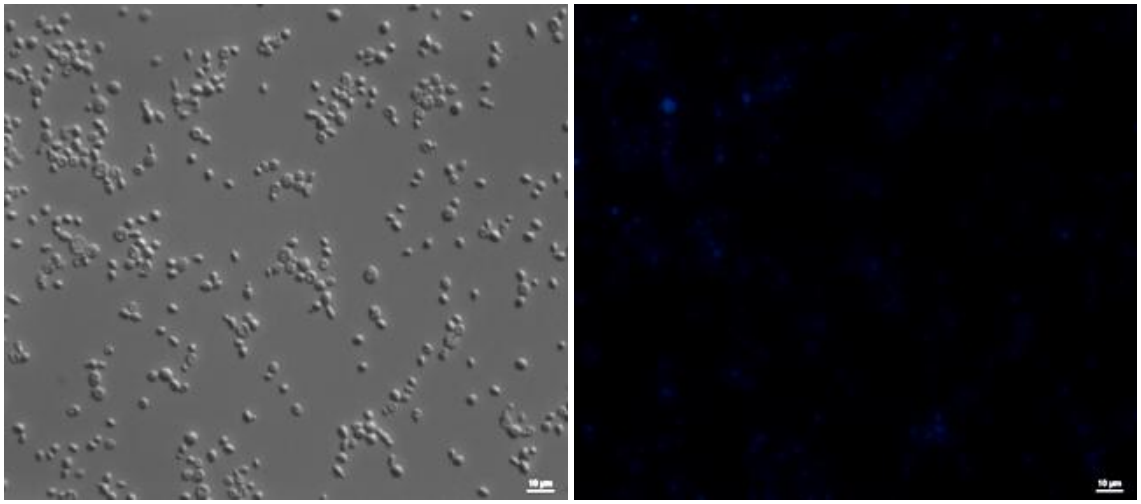
pYES2



Als5



Als5
V309N



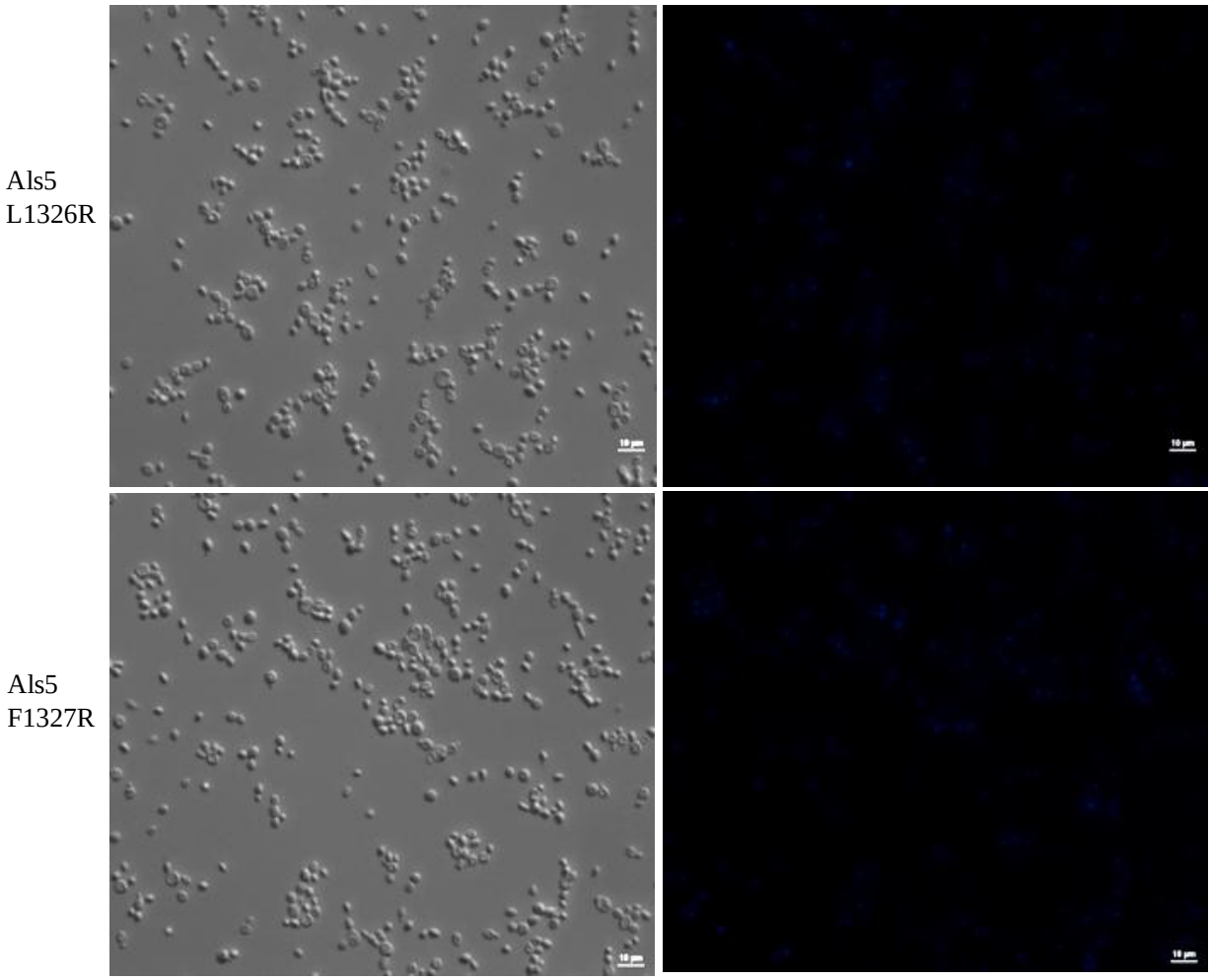


Figure IV.2.2a: Figure shows comparison of aggregation between strains expressing wild type Als5SS protein and its mutant proteins. The strains expressing mutant proteins showed almost equal extent of aggregation like the wild type Als5SS proteins, whereas pYES2 was taken as a vector control. This experiment was done thrice to confirm the results and here we have taken one representative fluorescence image for each protein.

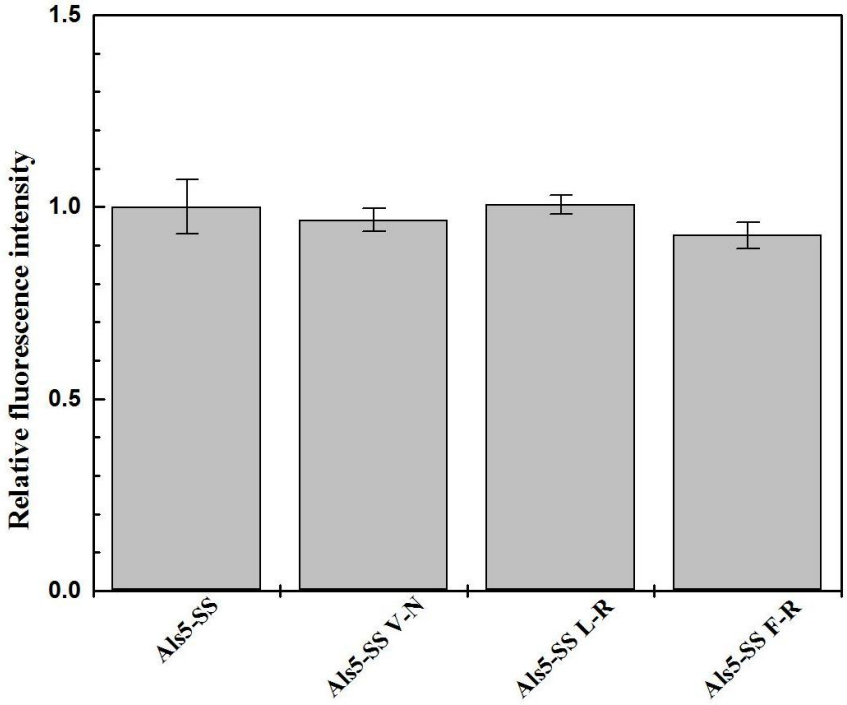


Figure IV.2.2b: Quantification of Als5-SS and its mutant variants: Quantification of fluorescence intensity in aggregates of cells expressing Als5-SS and its mutants were done. The cells expressing wild type as well as mutant Als5 proteins showed almost equal extent to form aggregates; pYES2 was taken as a vector control. The average is plotted with standard deviation. The *p* value for Als5-SS V309N is 0.72, for Als5-SS L1326R is 0.94 and for Als5-SS F1327R is 0.50 with respect to Als5-SS.

IV.2.3 Mutant variants of Als5-SS showed equivalent adherence to wild type Als5-SS protein

Adhesion to the extracellular matrix proteins (collagen, fibronectin and laminin) of host cells is another function for Als family of adhesins. We performed adhesion assays as described in Methods. In brief, we coated a 96 well plate with collagen type IV and cells were allowed to adhere to the coated wells. The adhered cells were incubated with anti-Als5 primary antibody and HRP conjugated secondary antibody. Tetramethylbenzidine solution (which is a substrate for HRP) was added to each well and OD_{650nm} was recorded by plate reader (Multiskan™ GO).

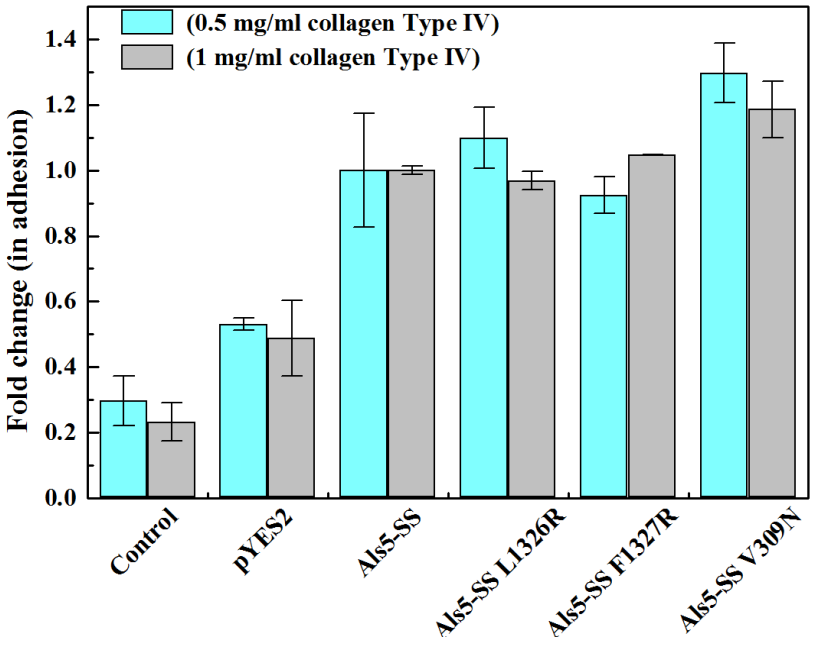


Figure IV.2.3: Adhesion assay for cells expressing Als5-SS and its mutant proteins at different concentration of collagen type IV. The cells expressing mutant proteins showed somewhat better adherence to collagen type IV as compare to wild type Als5SS protein, whereas pYES2 was taken as a vector control. This experiment was done twice and the average is plotted with standard deviation. The *p* value at 0.5 mg/ml concentration of collagen for Als5-SS L1326R is 0.97, for Als5-SS F1327R is 0.38 and for Als5-SS V309N is 0.53 with respect to Als5-SS. And the *p* value at 1.0 mg/ml concentration of collagen for Als5-SS L1326R is 0.21, for Als5-SS F1327R is 0.14 and for Als5-SS V309N is 0.20 with respect to Als5-SS.

We used two different concentrations (0.5 mg/ml and 1 mg/ml) of collagen and there was no significant difference in adhesion after increasing the concentration of collagen from 0.5 mg/mi to 1 mg/ml, suggesting that the 0.5 mg/ml collagen was sufficient for saturation of adhesion. There was no significant difference in adhesion to collagen type IV between cells expressing mutant proteins and the cells expressing wild type Als5-SS protein (Figure IV.2.3). This suggested that the mutations didn't affect the functionality of the protein with respect to aggregation either.

Chapter V

Discussion and Future perspectives

As mentioned in the Introduction, *C. albicans* is an opportunistic fungal pathogen. It causes very serious infections in immunodeficient people worldwide (Fidel and Sobel, 1996; Hostetter, 1994; Kabir et al., 2012; Powderly et al., 1999; Prasad et al., 2005). Several GPI anchored proteins are present on the cell surface of *C. albicans* and act as virulence factor for its pathogenicity. Among them, Als family of proteins are very important virulence factors which contribute to adhesion and aggregation of *C. albicans* (Hoyer, 2001; Rauceo et al., 2004).

Als proteins mediate adherence on biotic (gelatin, laminin, fibronectin, epithelial and endothelial cells) and abiotic surfaces (Aoki et al., 2012; de Groot et al., 2013; Sheppard et al., 2004). Aoki *et al* used many abiotic surfaces (polypropylene, polyvinyl chloride, borosilicate glass, polymethyl methacrylate, polyurethane, polytetrafluoroethylene and titanium) but, they found that the Als proteins binds to only polypropylene, polyvinyl chloride and borosilicate glass materials (Aoki et al., 2012). Since these materials are used in medical devices development so these findings may help to develop medical devices resistant from *C. albicans* infection. Several studies have been reported that the N-terminal domain of Als proteins consists ligand binding pocket which shows differential specificity to the substrates and involved in adhesion (Donohue et al., 2011; Salgado et al., 2011; Sheppard et al., 2004). Studies also have shown that the amyloid forming region present in the N-terminal domain of Als proteins interact with each other and form aggregates (Lin et al., 2014; Lipke et al., 2012). A recent study showed that deletion of the amyloid forming region from the N-terminal domain of Als3 protein converted it to a soluble nonaggregating monomeric form (Lin et al., 2014). The amyloid forming region mediated aggregation was confirmed by mutational approaches. In *C. albicans* the double mutation (I311S/I313S) within the amyloid forming region of Als3 blocked the aggregative potential of the protein (Lin et al., 2014). Recently, it has been observed that the amyloid forming region of Als5 protein is involved in

the commensalism of *C. elegans* and *S. cerevisiae* (Bois et al., 2013). When *S. cerevisiae* cells expressing wild type Als5 were fed to *C. elegans*, the *S. cerevisiae* cells were accumulated in the gut of *C. aligans* and improved its survival as compared to control experiment. But, when *S. cerevisiae* cells expressing Als5-V309N mutant were fed, the accumulation as well as the survival of *C. elegans* was almost equal to the control experiment (Bois et al., 2013). Als proteins are also involved in invasion into host tissues and Als3 is mainly involved in this process. A report has shown that Als3 mediated endocytosis of *C. albicans* by epithelial cells is dependent on cadherin interactions which involve the host actin cytoskeleton (Phan et al., 2007). A recent research has been reported that there are two possible ways of Als3 mediated internalization of *C. albicans* into the host, induced endocytosis and active penetration (Wächtler et al., 2012). Active penetration was observed as the main mechanism of invasion for *C. albicans* which is mainly depends on the viability of the fungal cells (Wächtler et al., 2012). By using a microfilament inhibitor (cytochalasin D) and thimerosal (block active penetration) they found that the induced endocytosis is mediated by cadherin dependent interactions although cadherin independent induced endocytosis also exists.

Previous studies from our lab have shown the significance of the C-terminal signal sequence (SS) in the conformation and function of the full length Als5 protein from *C. albicans* (Ahmad et al., 2012). It was a big challenge to characterize full length protein, since it is a 1347 amino acid long protein which consist a 20 amino acid long C-terminal GPI anchor attachment signal sequence (SS). In this study conformational as well as functional differences were observed in the Als5 protein in the presence and absence of the C-terminal SS. It was observed that the pro-protein (Als5-SS) had an α-helical conformation and was unable to perform adhesion and aggregation assays, that are essential features of Als-like adhesins. But the mature protein (Als5) adopted a pre-molten globular structure and was able

to exhibit adhesion as well as self-aggregation *in vitro*. Based on these studies, it was proposed that the C-terminal SS may be folding back and interact with the amyloidogenic region (IVIVATT) in the N-terminal domain of Als5-SS. This was perhaps creating a torsional strain in the Als5-SS protein, making it adopt a predominantly α- helical conformation. As a result of such an interaction between the amyloidogenic region and C-terminal sequence of Als5-SS, it was hypothesized that the aggregation-prone region of Als5-SS protein was masked making it unable to function as a true adhesin. On the other hand, in Als5 protein this torsion strain is released due to the absence of C-terminal signal sequence, making the protein adopt natively unfolded or pre-molten globular conformation. It also exposed the amyloidogenic patch on Als5 making it functional. In other words, the absence of rigid structure assisted the protein in gaining functionality. Other examples of such natively unfolded proteins are known in nature such as tau protein and p53 protein (Joerger and Fersht, 2010; Mandelkow and Mandelkow, 2012). Tau protein is involved in the assembly of tubulin into microtubules which is regulated by the phosphorylation of the tau protein. And the abnormal phosphorylation of tau protein leads to formation of neurofibrillary tangles, which results in a neurodegenerative disease known as Alzheimer's disease (Kolarova et al., 2012). The tumor suppressor protein p53 is a 393 amino acid long protein which is biologically active in its tetrameric form. It has folded (DNA binding domain) as well as unfolded regions (present at both N-terminus and C-terminus) in the protein but is largely unfolded and is structurally classified as a pre-molten globule (Joerger and Fersht, 2007, 2010). Premolten globular proteins are very less compact due to the lack of tertiary structure and contain less than 50 % of secondary structure as compared to natively folded proteins and these proteins show conversion from disordered to ordered structure in the presence of ligand (Uversky, 2002).

If indeed Als5 was a natively unfolded protein which functioned best in its pre-molten globular state, could Als5-SS too become functional if its folding was disrupted? Since we hypothesised that Als5-SS adopted a folded structure primarily due to the interaction between its amyloidogenic patch and its C-terminal sequence, we aimed to first determine the mutations required to disrupt this interaction. Hence, we inserted single site specific mutations in amyloidogenic patch and C-terminal signal sequence and analyzed the β-aggregation potential of the mutant proteins by TANGO software (Ahmad et al., 2015). TANGO analysis showed that the amyloidogenic patch and C-terminal signal sequence had >95% β-aggregation potential, which was drastically reduced (to nearly zero) in case of mutants (Als5-SS V309N, Als5-SS L1326R and Als5-SS F1327R) (Figure III.1). These mutations were then experimentally generated and functional studies were performed.

All three site directed mutants along with wild type Als5-SS protein were expressed and purified from *E. coli* expression system. Firstly, circular dichroism (CD) spectroscopy was used to study the secondary structure conformation for all the proteins. As expected, Als5-SS protein adopted an α-helical conformation (Ahmad et al., 2012). In the present study, we observed that the secondary structural conformations of mutant proteins (Als5-SS V309N, Als5-SS L1326R and Als5-SS F1327R) were altered as compared to Als5-SS protein (Figure III.3.1). CD analysis suggested that the site directed mutations interfered in the interactions between the N-terminal amyloid patch and C-terminal signal sequence of Als5-SS protein, although none of the protein had a pre-molten globular conformation (Figure III.3.1).

Next, we checked the effects of mutations on the function of Als5-SS protein. We observed that the mutations significantly enhanced the adhesion of the protein to collagen (Figure III.4.1). They also improved the self-aggregation (III.4.2a) of the Als5-SS proteins. Next, we used SS peptides as inhibitors of the aggregation. Wild type SS inhibits Als5 aggregation (Ahmad *et al*., 2012). We observed that mutated synthetic C-terminal SS

peptides were unable to suppress the aggregation of purified Als5 protein (Figure III. 4.1b). This suggests that wild type synthetic C-terminal signal sequence is capable of interacting with the amyloid patch of Als5 protein and suppressing its aggregation, while the mutations were not capable of similar interactions thus, couldn't suppress the aggregation of Als5. These results support the model proposed that in wild type Als5-SS protein the C-terminal signal sequence masks the amyloidogenic patch and blocks its aggregation and adhesion functions. Mutations in the C-terminal signal sequence leads to weakening the interaction with amyloid patch, opens up the structure and enables adhesion and aggregation.

The GPI transamidase enzyme complex recognizes the ω-site at the extreme C-terminal, cleaves the C-terminal signal sequence and replace it with the precursor GPI anchor, this substitution occurs in the ER lumen from where the GPI anchored protein is transported to the cell surface by COPII coated vesicles. As we discussed in *in vitro* studies, the mutations in the C-terminal signal sequence of Als5-SS protein altered the secondary structure conformations from that of wild type Als5-SS. These conformational changes may affect the GPI transamidase recognition of the ω-site and, therefore, the protein may remain stuck in the ER without being transported to the cell surface. To test this hypothesis we needed to perform *in vivo* experiments. Before attempting wet lab experiments we did an *in-silico* analysis (GPI-SOM and BIG-PI predictor) to predict the GPI anchoring of mutant proteins. These programs predict the GPI anchoring of proteins based on the conserved nature of the SS. The conformation or structure of the SS is not a parameter used in this analysis. As mentioned in Introduction, for the GPI anchoring of the proteins C-terminal signal sequence requires some consensus sequences. The ω and ω+2 sites at the C-terminal signal sequence must possessing amino acids with small side chains (Ser, Gly, Ala, Asp, Asn or Cys) and proline at ω+1 position prevent the GPI anchoring of the proteins while the amino acids (Asp, Ser, Cys, Met, Thr, Glu, Arg, Trp) doesn't affect the GPI anchoring

(Moran et al., 1991; Orlean and Menon, 2007). The amino acid residues at N-terminus to ω-site (ω-5 to ω-1) determine the plasma membrane or cell wall localization of proteins (Mao et al., 2008). The basic residues at ω-2 and ω-1 position target the proteins to plasma membrane whereas the hydrophobic residues lead to the cell wall localization (Mao et al., 2008). The Als5-SS sequence suggests that it is predominantly a cell wall protein. A hydrophobic patch present about 10-12 amino acid residue away from the ω-site is known to be critical for GPI anchoring of proteins and its length varies in different proteins (Caras, 1989; Coyne et al., 1993; Furukawa et al., 1994). All the mutants we were interested in studying were predicted to be GPI anchored except for Als5-SS L1326R which was GPI anchored according to GPI-SOM but not by BIG PI predictor (Table IV.1). In other words, if the recognition by the transamidase is based on sequence alone and not on conformation, these mutant proteins are likely to be GPI anchored, as per the *in silico* analysis.

In our *in-vivo* studies, we first examined the effect of the mutations on the expression of Als5 protein. Als5-SS and its mutant variants were expressed in *S. cerevisiae* which does not have any endogenous Als proteins. We observed that the mutations didn't affect the expression levels of Als5 protein on the cell surface of *S. cerevisiae* (Figures IV.2.1a and IV.2.1b). It suggests that the GPI transamidase enzyme recognition of the ω-site was not affected by the site directed mutation in the C-terminal signal sequence of Als5-SS protein thus the protein translocation to the cell surface was not affected. Next, we checked the effect of mutations on the functionalities of Als5 protein. We performed adhesion and aggregation assays for *S. cerevisiae* cells expressing Als5-SS and its mutant variants and observed similar results in both assays. The mutations didn't affect the functionality of Als5 protein. Neither the adhesion (Figure IV.2.3) nor the aggregation (Figures IV.2.2a and IV.2.2b) of the mutant proteins were any different from that of the wild type Als5 protein. Interestingly, even the mutation in the amyloidogenic patch (V309N) of the protein did not result in any significant

alteration in this functionality. But, previous studies showed that Als5 V326N mutant (this valine lies at position 309, when 17 amino acid residues of N-terminal signal sequence is not considered and has been normally labelled as V309 in this thesis) shows abrogation of aggregation (Alsteens et al., 2010; Garcia et al., 2011; Lipke et al., 2014). They have observed that *S. cerevisiae* cells expressing Als5 V326N mutant formed very small aggregates as compared to cells expressing wild type Als5 protein. When the Als5 V236N expressing cells were tested for staining with Thioflavin-T, they showed very less fluorescence suggesting that V326N mutation abrogates the amyloid formation (Garcia et al., 2011; Lipke et al., 2014). In our *in vitro* studies using recombinantly expressed Als5V309N from *E. coli*, we have shown that this mutation altered the secondary structure conformation (Figure III.3.1). In the biochemical assays also (*in vitro* as well as *in vivo*) we found that V309N mutation made Als5 functional (Figures III.4.1, III.4.2a, IV.2.2a, IV.2.2b and IV.2.3). Our results suggest that V309N mutation enhances the functionalities of Als5 protein.

The mechanism for ω-site recognition by GPI transamidase is not clear till now. Our results could be interpreted in multiple ways. For example, if the mutations do indeed alter conformation of the proteins *in vivo* as observed in our *in vitro* studies, then we could infer that the GPI transamidase has a sequence-specific and not a conformation-specific recognition of the SS. On the other hand, it is possible that the mutations did not alter the conformation of the proteins *in vivo.* Als proteins are known to be heavily glycosylated. And while it is well known that a large part of glycosylation remodeling on GPI anchored proteins occur in the Golgi (Martínez-Duncker et al., 2014; Mora-Montes et al., 2009), it is possible that at least a fraction of this occurs in the ER as well. In particular, O-mannosylations in the ER of *C. albicans* is widely reported (Munro et al., 2005; Prill et al., 2004; Timpel et al., 2000). Tandem repeat domain of Als proteins is enriched in Ser/Thr, which is known to be heavily glycosylated (Frank et al., 2010; Rauceo et al., 2006). In *C. albicans*, O-

mannosylation of Ser/Thr is mediated by protein mannosytransferase (PMT) gene family consisting five genes; PTM1, PTM2, PTM4, PTM5 and PTM6 (Munro et al., 2005; Prill et al., 2004). It is known that mutations in PMT's leads to defects in *C. albicans* growth, hyphal morphogenesis, sensitivity to antifungal drugs and virulence (Prill et al., 2004; Rouabhia et al., 2005; Timpel et al., 2000). It was observed that Als5 fragment consisting tandem repeat ($Als5^{1-664}$) showed structural and functional differences from that of the Als fragment lacking the tandem repeat ($Als5^{1-431}$) (Rauceo et al., 2006). The $Als5^{1-664}$ fragment showed greater binding to fibronectin and cellular interaction compared to that of $Als5^{1-431}$. The CD spectrum of $Als5^{1-664}$ was also different from that of $Als5^{1-431}$ but the secondary structure content of both Als fragment was not different. The authors suggested that differences in the conformation of both fragments of Als5 is not because of secondary structure content but due to the glycosylation of the tandem repeat domain (Rauceo et al., 2006). It is possible that these post-translational modifications result in conformations that are different from what we observed *in vitro* since the proteins in this latter case were purified from bacterial cells that lack such post-translational machinery. One way to test this would be to purify the proteins from the yeast cells and examine their conformation and functions *in vitro.* However, the protein expressions were too low for us to be able to purify or characterize them from *S. cerevisiae* cells. A small possibility also exists that the protein expressed in *E. coli* as well as in *S. cerevisiae* are different from the actual protein in *C. albicans. C. albicans* is known to have a codon bias and several studies have shown that in *C. albicans* CUG (a universal codon for leucine) codon encodes for serine instead of leucine (Ohama et al., 1993; Santos and Tuite, 1995). During evolution about 272±25 years ago, in *C. albicans* the CUG codon was decoded as serine by the addition of an adenosine in the intron of ser-$tRNA_{CGA}$ gene thus, it acquired a leucine anticodon ser-$tRNA_{CAG}$ (Massey, 2003; Miranda et al., 2006). In 1993, it was reported that several *Candida* species including *C. albicans* encodes serine instead of

leucine from the CUG codon (Ohama et al., 1993). Later, it was observed *in-vivo*, when *RBP1* (rapamycine-binding protein) gene was expressed under the control of *TEF3* promoter of *C. albicans* and CUG codon was decoded as serine (Santos and Tuite, 1995). It was also observed that the *S. cerevisiae* cells expressing *C. albicans* tRNA encoding for serine affected the gene expression and sexual reproduction of *S. cerevisiae* (Silva et al., 2007).

In addition to the above studies, we also attempted to study the peptide binding pocket of the protein and to understand its ligand specificities by introducing mutations in this pocket. A recent study on Als3 observed that Ser170 residue is very critical for ligand binding (Lin et al., 2014). The Ser 170 residue is present at the entry of the peptide binding pocked of Als3 protein and the site directed mutation (S170Y) blocks the entry of peptide into the peptide binding pocket without affecting the surface conformation of the protein. This mutant also resulted in decreased adhesion to endothelial as well as epithelial cells (Lin et al., 2014). The same mutant was generated in Als5 protein to confirm the role of Ser170 in ligand binding, but we couldn't succeed in solubilizing Als5 S170Y (Figures III.5.1 and III.5.2). From molecular modeling and molecular dynamics simulations studies, we observed that S170Y is a very unstable protein (Figures III.6.2.a and III.6.2.b), which probably explains why we couldn't purify Als5 S170Y and carry out the ligand binding studies for this mutant.

Future perspective

- Studies have shown that glycosylation of Ser/Thr domain of Als5 proteins affect the secondary structure conformation (Rauceo et al., 2006). In this study, we have shown the secondary structure conformation of full length Als5 protein and its mutant variants purified from *E. coli* (Figure III.3.1). But *in vivo* glycosylation of protein may result in different conformations from that observed *in vitro*. To test this, one can express and purify Als5 protein in *S. cerevisiae* to obtain the glycosylated protein, but it is a major challenge to purify Als proteins from *S. cerevisiae* due to the very low expression levels.
- Studies have also shown that the recombinant N-terminal of Als proteins (Als1 and Als3) could be a potential vaccine against Candida infection (Spellberg et al., 2005, 2006), so these proteins can be used in drug therapies. Thus, it is very important to characterize the role of each Als protein for a better understanding of their individual roles in the cell.
- Recent studies have been focused on the adhesion, invasion and aggregation processes of *C. albicans* on the host cell surfaces, but there is a lack in knowledge of exact mechanism of host-pathogen interactions. Future work could be focused on identifying the molecular basis of host-pathogen interactions through Als proteins and therefore identifying a potential drug to inhibit these interactions.
- Most of the proteins involved in the virulence of *C. albicans* are attached to the preformed GPI anchor posttranslationally with the help of the GPI transamidase enzyme complex. This step could be a potential drug target and the GPI anchoring of proteins could be stopped by targeting the transamidase. But, the molecular mechanism of the transamidase step is not clear. While some studies have reported on the role of Gpi8, Gpi16 and Gaa1 but the role of other two subunits (PIG-S/GPI17 and

PIG-U/GAB1) is not clear and needs to be explored (Eisenhaber et al., 2003, 2014; Ohishi et al., 2003; Vainauskas, 2003; Yi et al., 2017). As we discussed, it is also not very clear how all the subunits of GPI transamidase interact with each other. So, it is very critical to identify the role of each subunit of the complex and how they interact with each other to make a stable complex. There-after one would be able to design an effective drug against the GPI transamidase to block the GPI anchor attachment to the proteins and then it would be possible to control the virulence caused by *C. albicans*.

Appendix

In our studies we observed the differences in conformation, adhesion and aggregation of Als5 and Als5-SS protein. Further we performed ligand binding studies to see whether these functional and conformational differences are correlated with the ligand binding of Als5 and Als5-SS. We used four peptide ligands AYKSLMT, EHAHTPR, KLRIPSV and VSPIRLK (scrambled peptide of KLRIPSV) (Ahmad et al., 2012). The first three peptide ligands were previously known as the ligands for Als5 protein (Klotz et al., 2004) whereas the fourth peptide (VSPIRLK) was a scrambled peptide used to check the ligand binding specificity. It was previously observed that AYKSLMT and KLRIPSV peptide were able to bind with Als5 protein, but EHAHTPR was unable to bind with Als5 protein (Klotz et al., 2004). In the ligand binding studies, we observed that both Als5 and Als5-SS shows binding to all the peptide ligands except VSPIRLK (Ahmad et al., 2012). Als5-SS showed binding to the ligands at lower temperatures whereas Als5 bound to the peptide ligands at higher temperatures, suggesting that both proteins show some differences in their functionalities.

In the present study, we observed that the mutations in the amyloid patch and the C-terminal signal sequence altered the conformation and functions (adhesion and aggregation) of Als5-SS protein. Next, we check whether the mutations affected the ligand binding affinities of Als5-SS protein thus we performed the ligand binding studies with all three peptides (AYKSLMT, EHAHTPR and KLRIPSV) and the results have been described below.

❖ Interaction of GST-Als5-SS V309N, GST-Als5-SS L1326R and GST-Als5-SS F1327R protein with three different peptide ligands at different temperatures

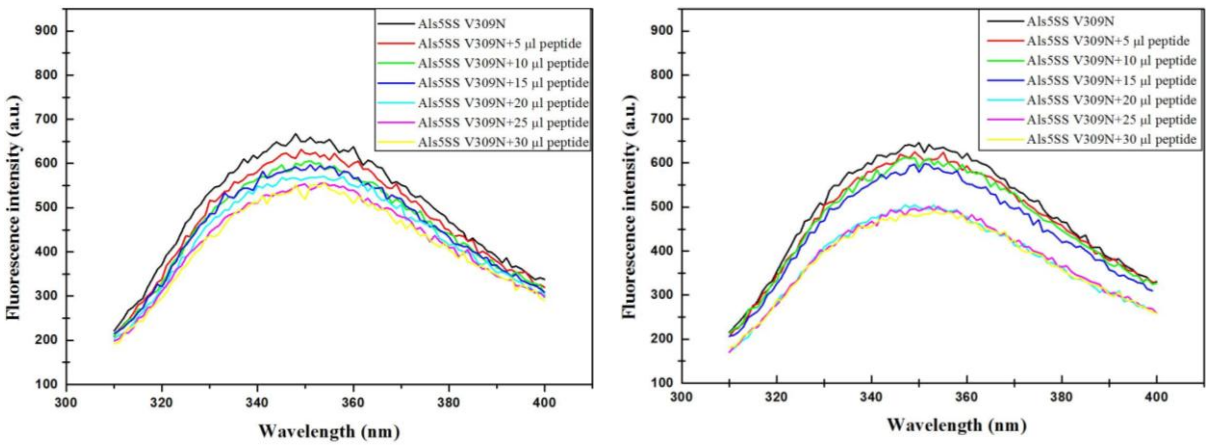
The peptide binding of GST-Als5-SS V309N, GST-Als5-SS L1326R and GST-Als5-SS F1327R was done by fluorescence emission microscopy. We used 0.05 mg/ml of each

mutant and tryptophan emission spectrum was recorded between 310 nm to 400 nm. The peptide ligands were added to the proteins and incubated for 5 minutes at a particular temperature and emission spectra were recorded after excitation at 295 nm. The bandwidth was fixed at 5 nm and the final spectra were the average of 5 repeat scans. The spectrum of buffer was taken for background correction. Previously, we observed that purified GST alone didn't show any fluorescence emission spectrum, it suggests that the GST didn't contribute to emission spectra of proteins. This is expected since GST does not have Trp residues. The binding data was analysed by one-site saturation model using SigmaPlot software and K_d values were obtained from the fit.

1. Binding studies with AYKSLMT peptide

In previous studies, we found that the wild type Als5-SS protein shows binding with AYKSLMT at different temperatures significantly (Ahmad et al., 2012)), but in the present study we found that the mutant proteins didn't show significant binding with AYKSLMT at 27° C, 37° C and 47° C (Table 1).

(1.1) At 27° C the Kd value for Als5-SS V309N binding with AYKSLMT was 6.73±6.3 x 10^{-2} μM, at 37° C was 1.49±0.35 x 10^{-2} μM and at 47° C was 9.95±1.3 x 10^{-2} μM (Table 1).



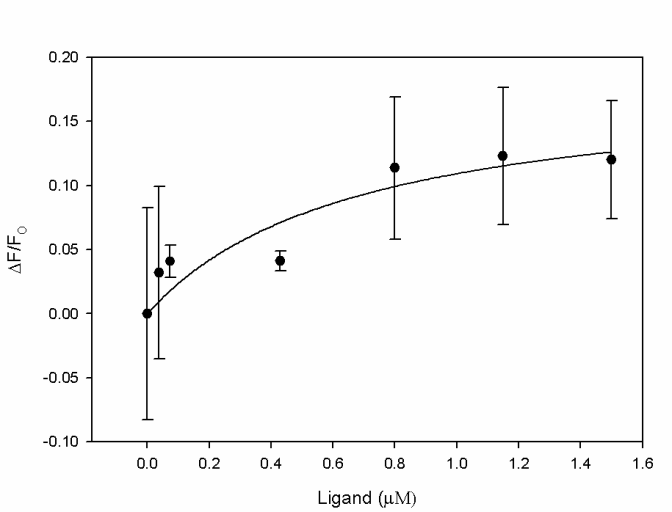


Figure 1.1a: Fluorescence spectra and peptide binding plot of Als5-SS V309N with AYKSLMT at 27° C.

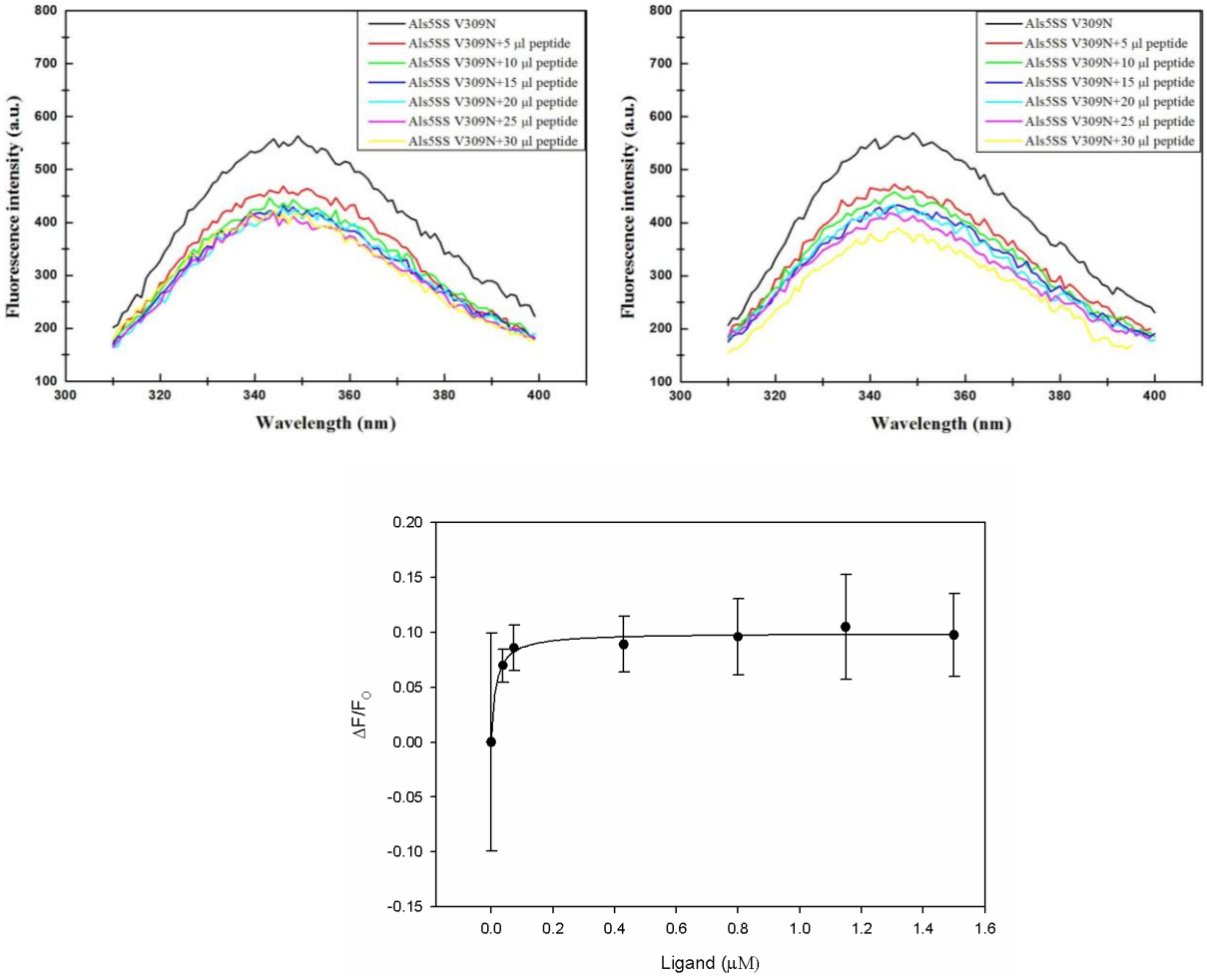


Figure 1.1b: Fluorescence spectra and peptide binding plot of Als5-SS V309N with AYKSLMT at 37° C.

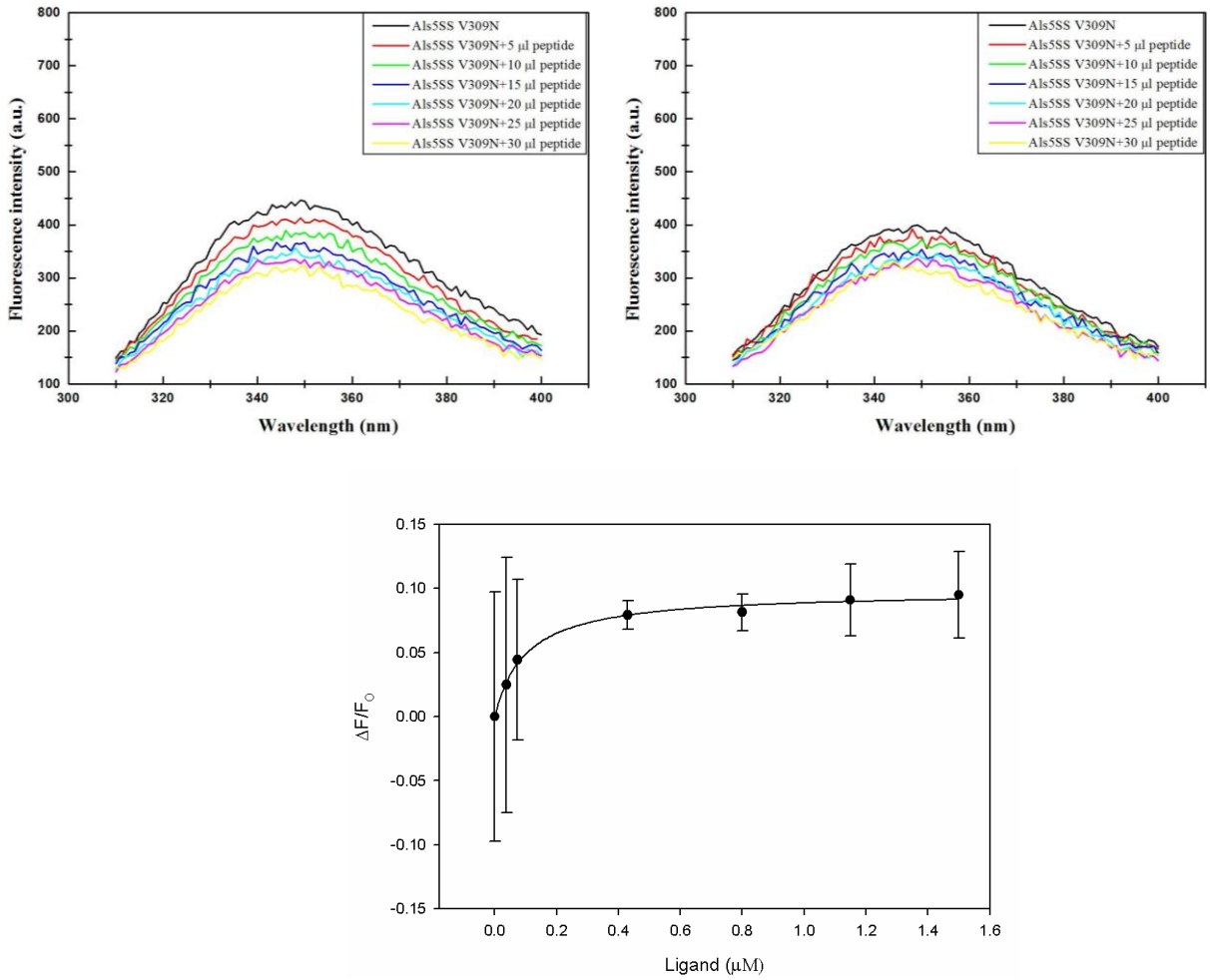
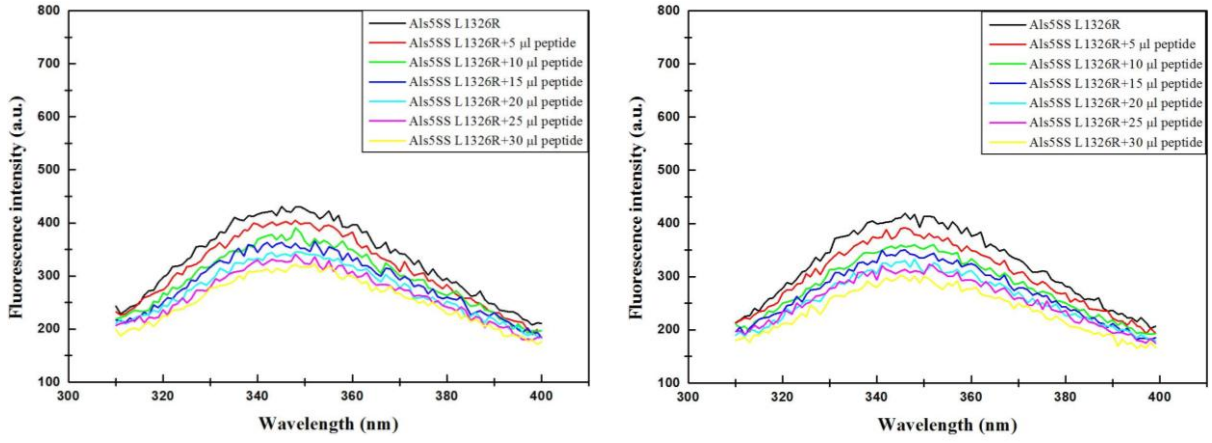


Figure 1.1c: Fluorescence spectra and peptide binding plot of Als5-SS V309N with AYKSLMT at 47° C.

(1.2) The Kd value for Als5-SS L1326R at 27° was 9.18±4.6 x 10^{-2} µM and at 37° C was 8.05±2.6 x 10^{-2} µM (Table 1).



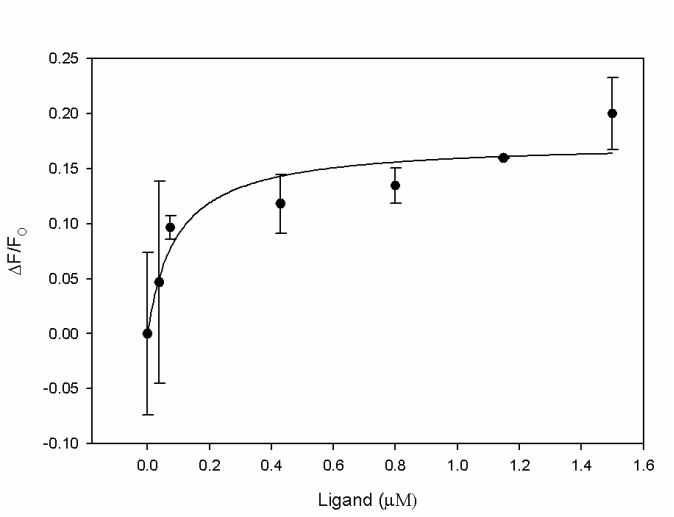
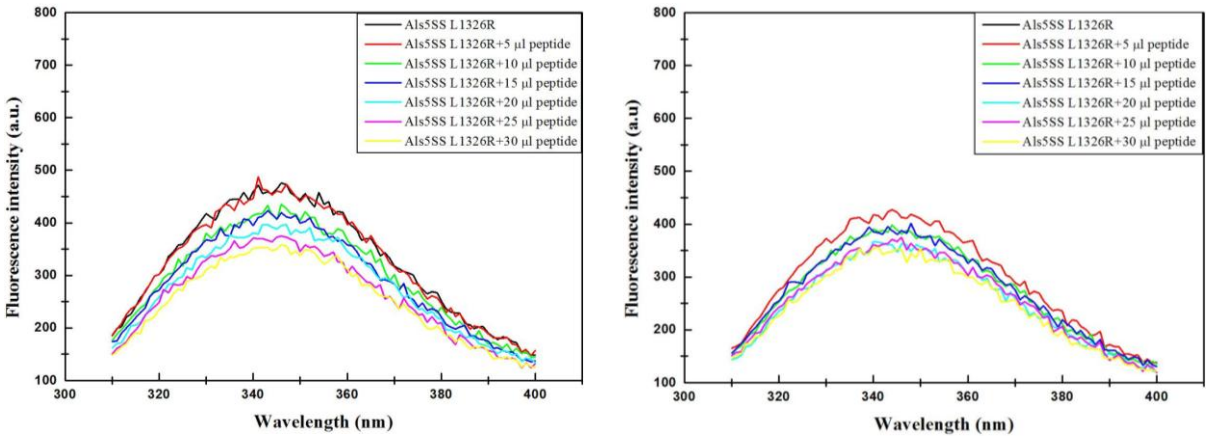


Figure 1.2a: Fluorescence spectra and peptide binding plot of Als5-SS L1326R with AYKSLMT at 27° C.



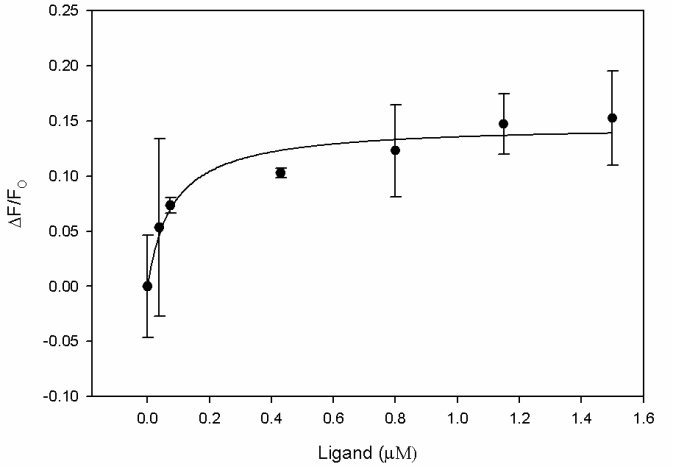


Figure 1.2b: Fluorescence spectra and peptide binding plot of Als5-SS L1326R with AYKSLMT at 37° C.

(1.3) The Kd value for Als5-SS F1327R at 27° was 4.29±1.2 x 10^{-2} μM, at 37° C was 4.78±1.2 x 10^{-2} μM and at 47° C was 8.29±4.9 x 10^{-2} μM (Table 1).

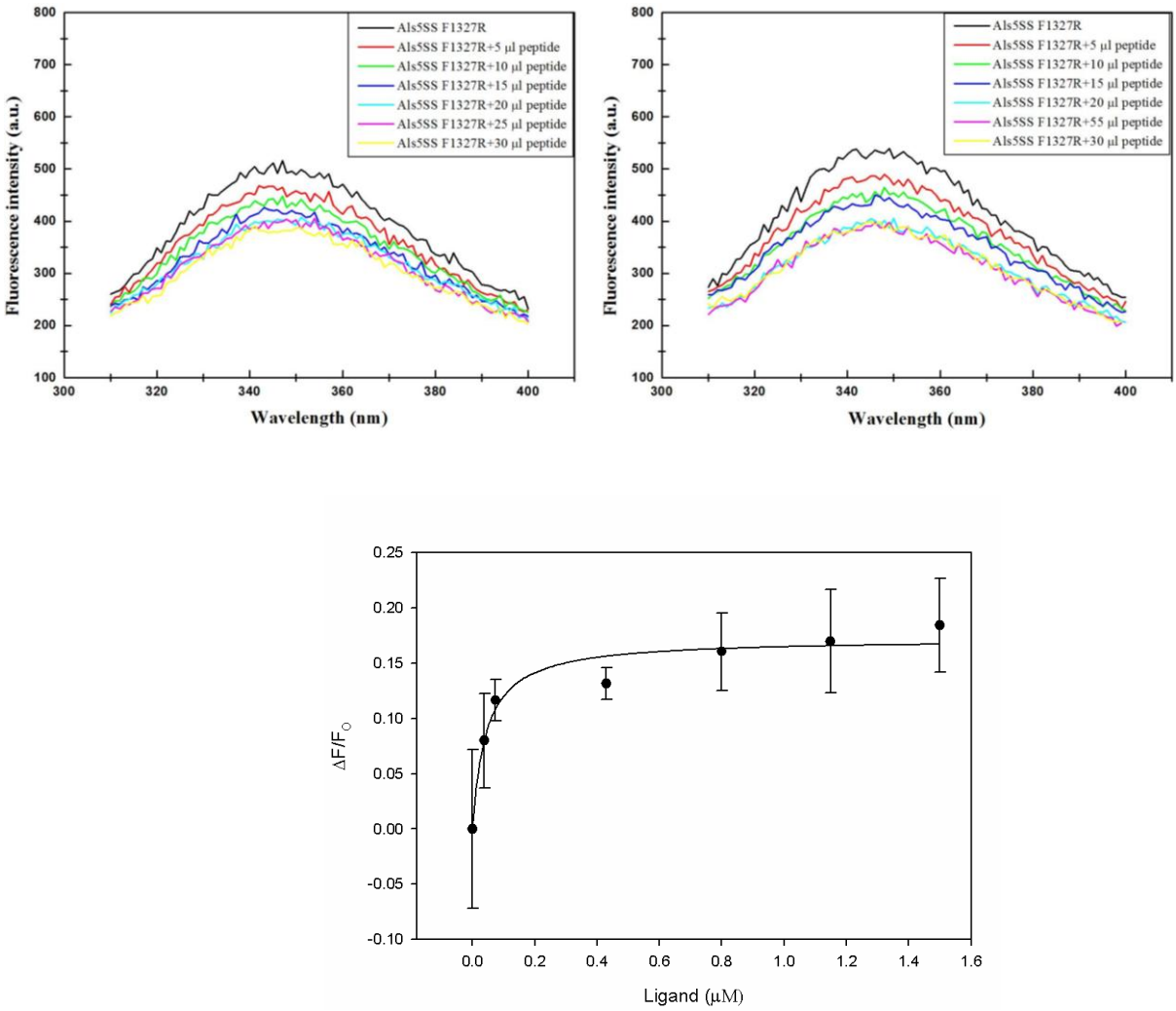


Figure 1.3a: Fluorescence spectra and peptide binding plot of Als5-SS F1327R with AYKSLMT at 27° C.

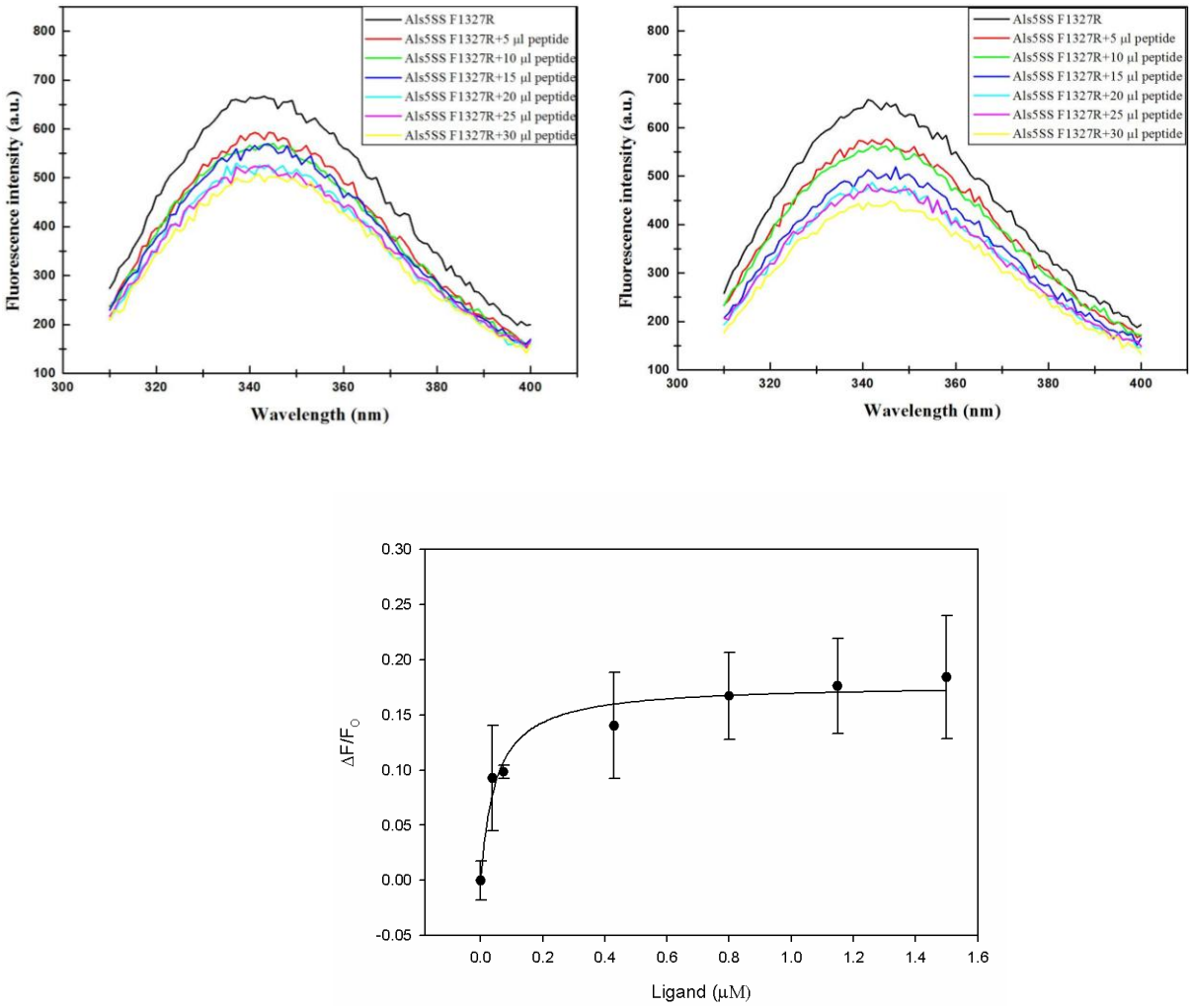
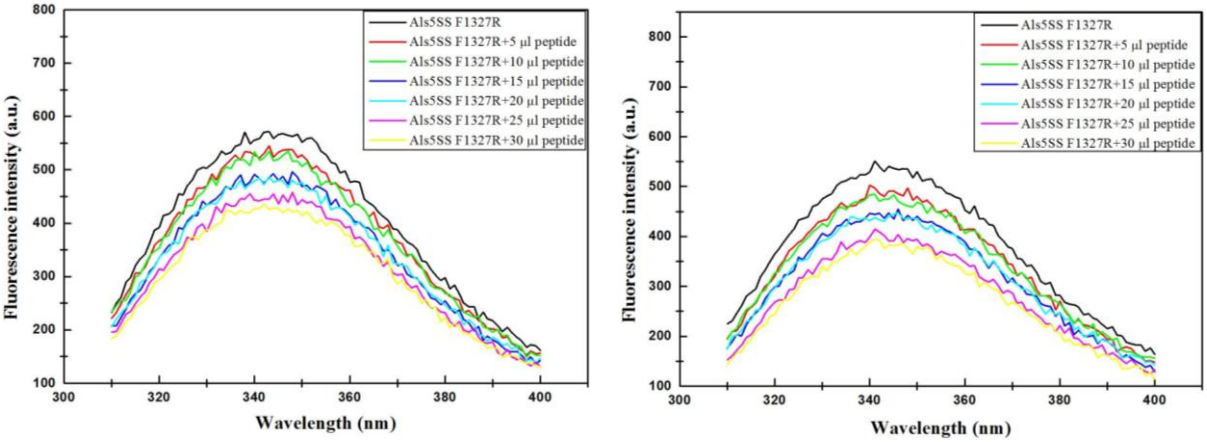


Figure 1.3b: Fluorescence spectra and peptide binding plot of Als5-SS F1327R with AYKSLMT at 37° C.



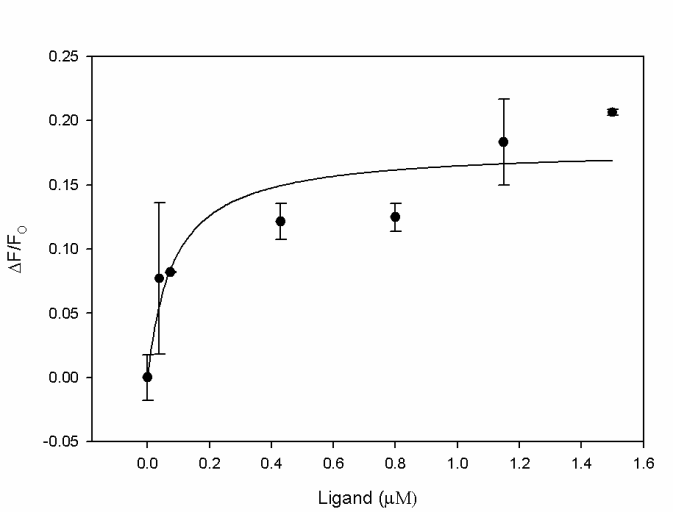
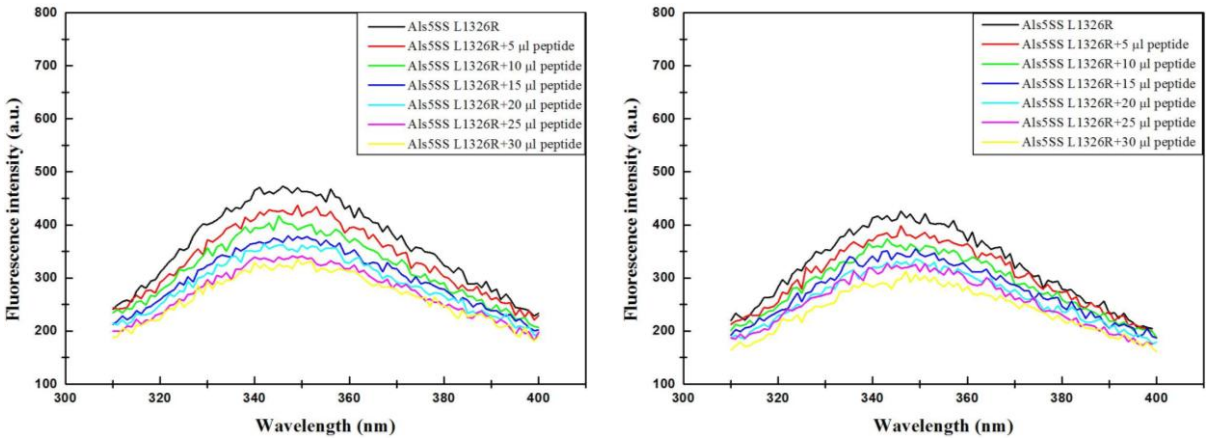


Figure 1.3c: Fluorescence spectra and peptide binding plot of Als5-SS F1327R with AYKSLMT at 47° C.

2. Binding studies with EHAHTPR peptide

We didn't find significant binding of GST-Als5-SS L1326R and GST-Als5-SS F1327R proteins with EHAHTPR at different temperature (27° C and 37° C) (Table 1). While in previous studies, we found that the wild type Als5-SS protein shows significant binding with EHAHTPR at 27° C and 37° C (Ahmad et al., 2012).

(2.1) At 27° C the Kd value for Als5-SS L1326R binding with EHAHTPR was 10.7±5.8 x 10^{-2} μM and at 37° C was 4.02±3.8 x 10^{-2} μM.



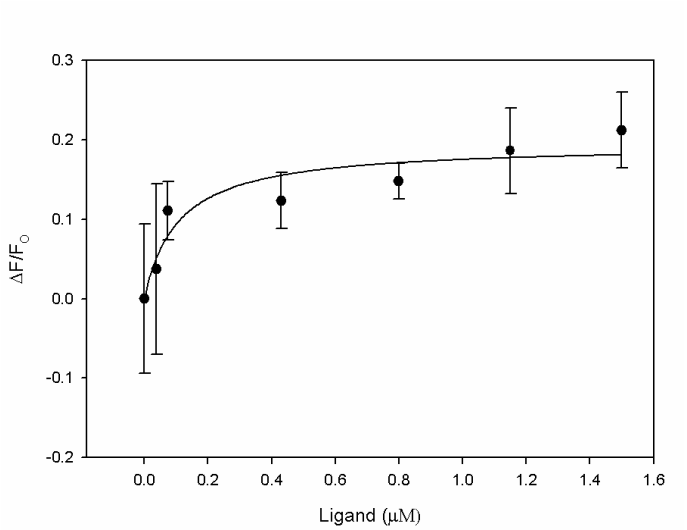
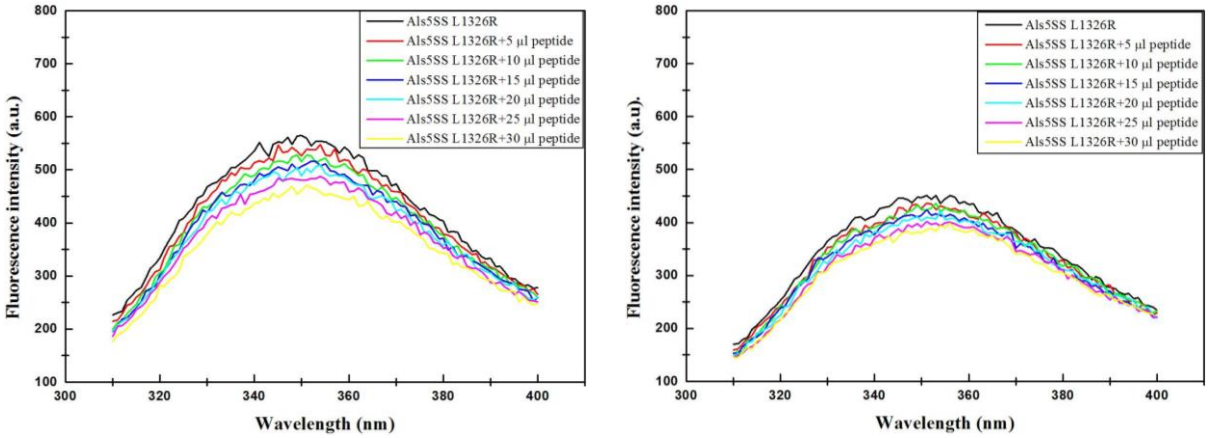


Figure 2.1a: Fluorescence spectra and peptide binding plot of Als5-SS L1326R with EHAHTPR at 27° C.



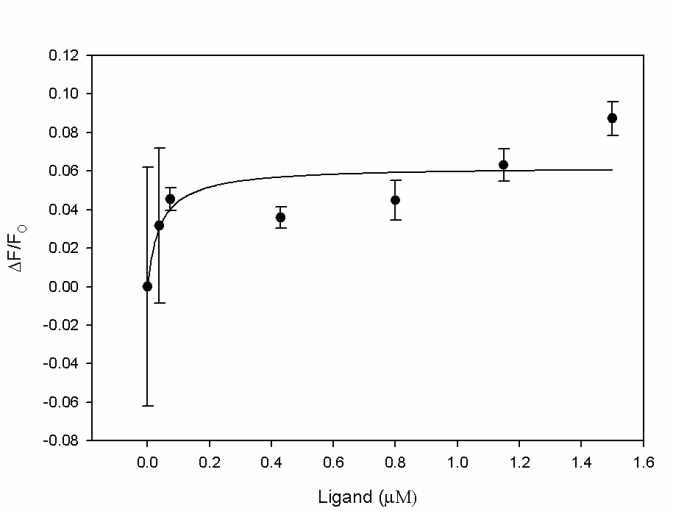


Figure 2.1b: Fluorescence spectra and peptide binding plot of Als5-SS L1326R with EHAHTPR at 37° C.

(2.2) At 27° the Kd value for Als5-SS F1327R binding with EHAHTPR was 7.7±3.4 x 10^{-2} μM, at 37° C was 3.39±0.71 x 10^{-2} μM (Table 1).

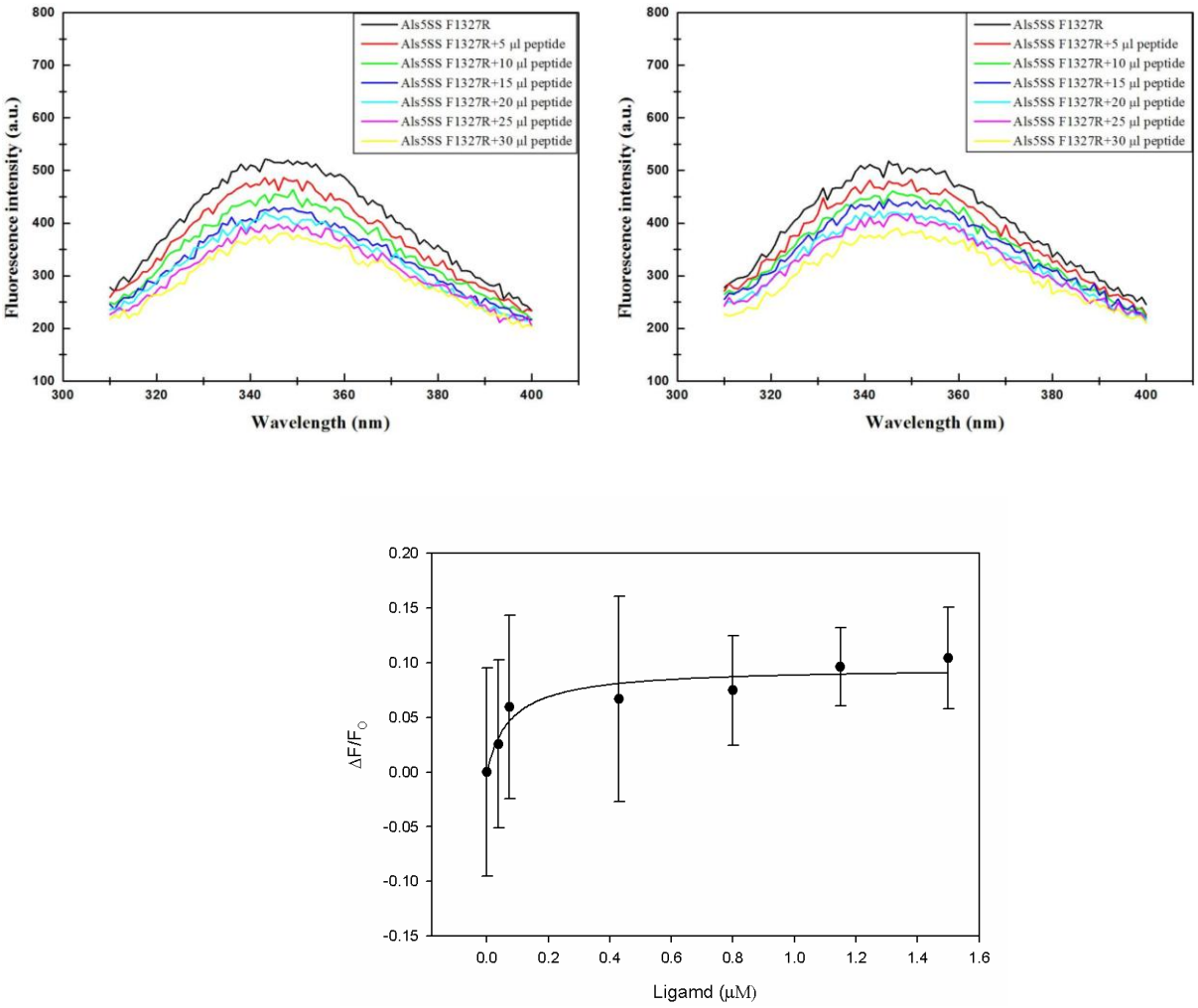


Figure 2.2a: Fluorescence spectra and peptide binding plot of Als5-SS F1327R with EHAHTPR at 27° C.

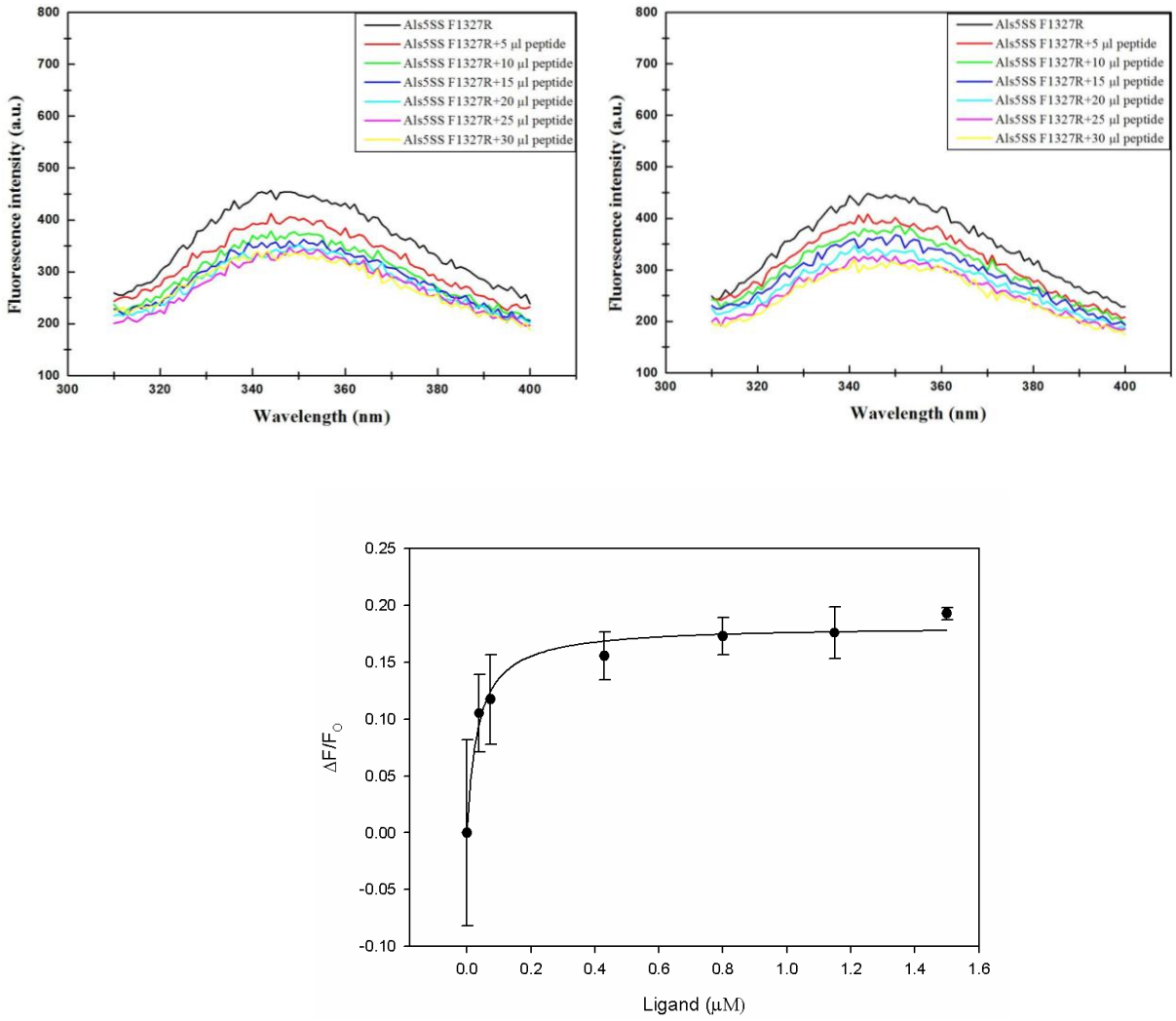


Figure 2.2b: Fluorescence spectra and peptide binding plot of Als5-SS F1327R with EHAHTPR at 37° C.

3. Binding studies with KLRIPSV peptide

The same results we showed in KLRIPSV binding studies. GST-Als5-SS L1326R and GST-Als5-SS F1327R proteins didn't show significant binding with KLRIPSV at different temperature (27° C and 37° C) (Table 1). While in previous studies, we found that the wild type GST-Als5-SS protein shows significant binding with KLRIPSV at 27° C and 37° C (Ahmad et al., 2012).

(3.1) At 27° C the Kd value for Als5-SS L1326R binding with KLRIPSV was 1.02±0.74 x 10^{-2} µM and at 37° C was 7.47±3.4 x 10^{-2} µM.

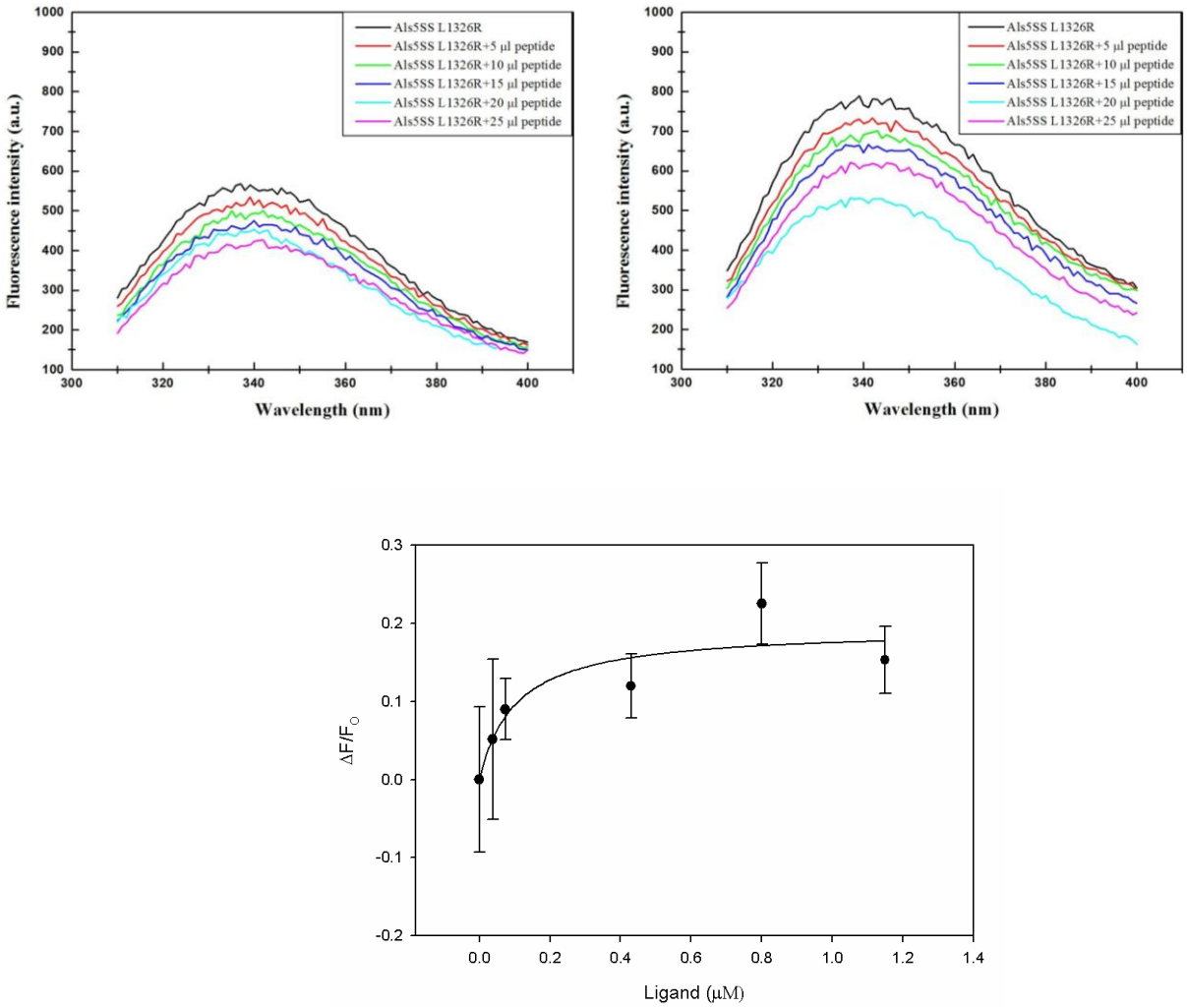
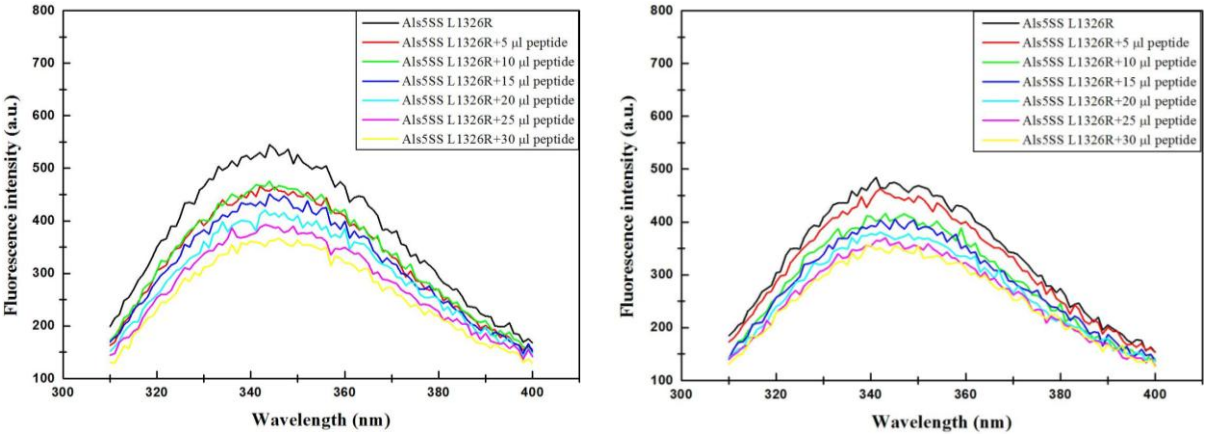


Figure 3.1a: Fluorescence spectra and peptide binding plot of Als5-SS L1326R with KLRIPSV at 27° C.



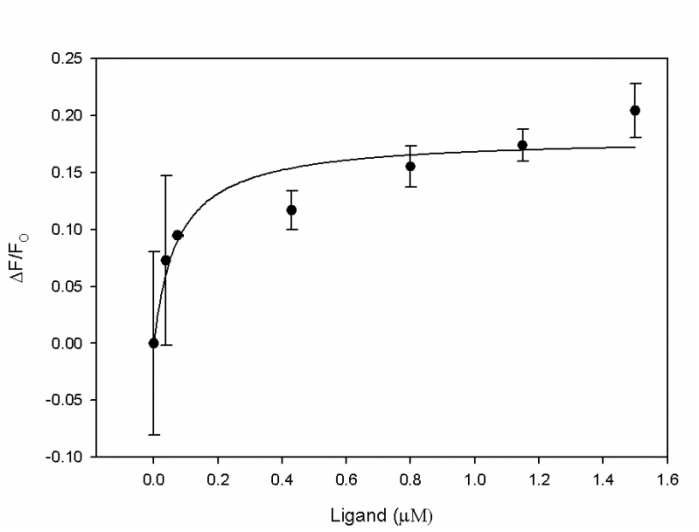
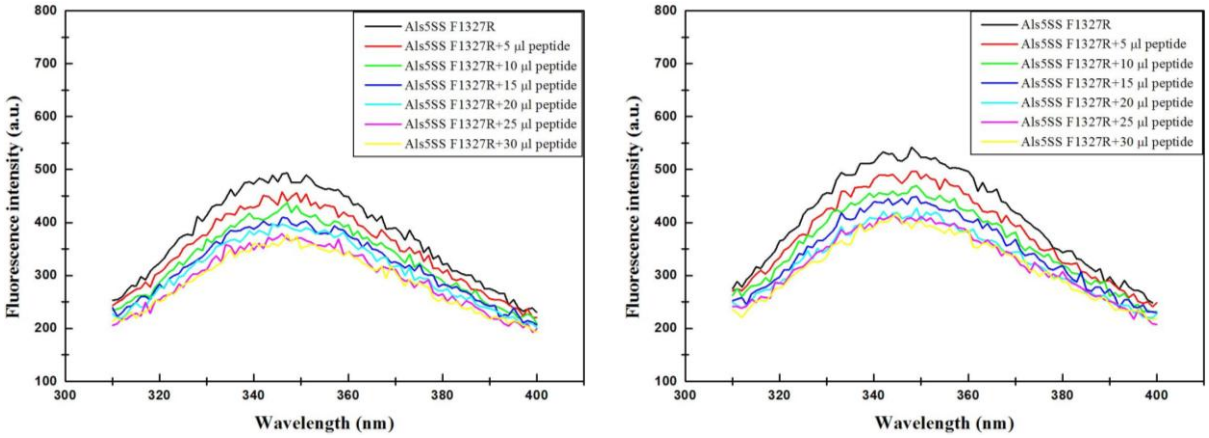


Figure 3.1b: Fluorescence spectra and peptide binding plot of Als5-SS L1326R with KLRIPSV at 37° C.

(3.2) The Kd value for Als5-SS F1327R at 27° was 6.59±2.3 x 10^{-2} μM, at 37° C was 3.0±1.0 x 10^{-2} μM (Table 1).



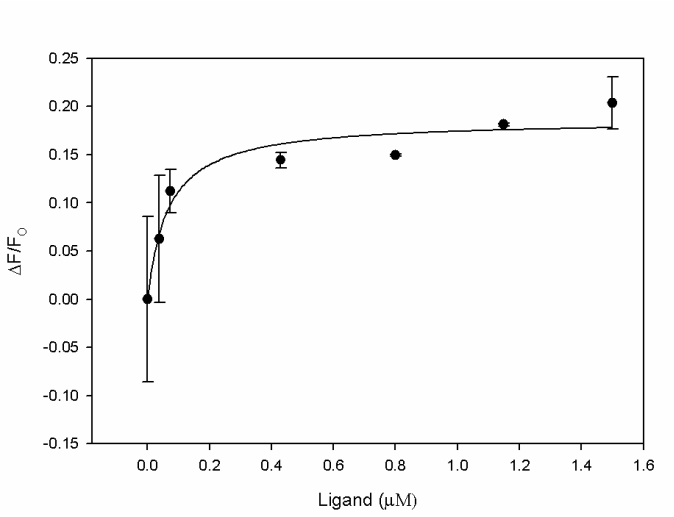
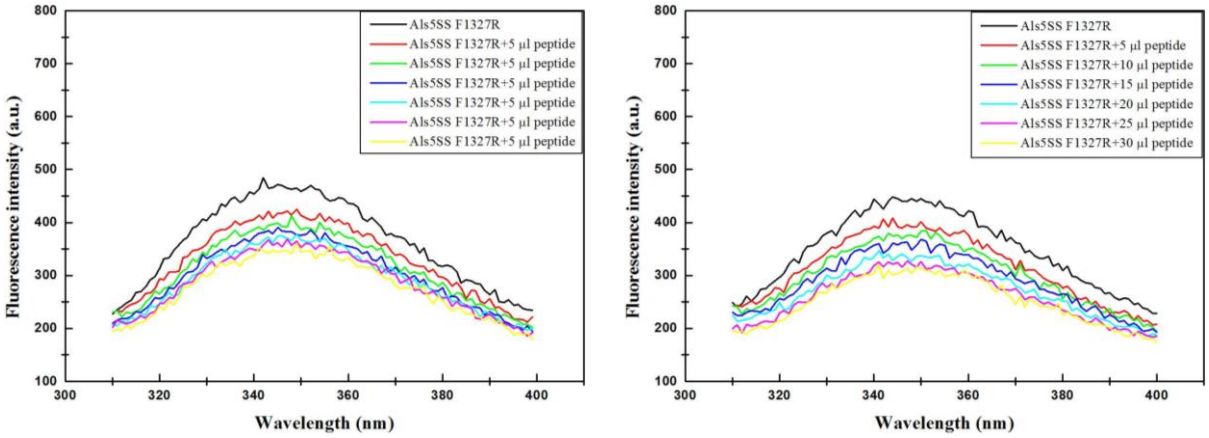


Figure 3.2a: Fluorescence spectra and peptide binding plot of Als5-SS F1327R with KLRIPSV at 27° C.



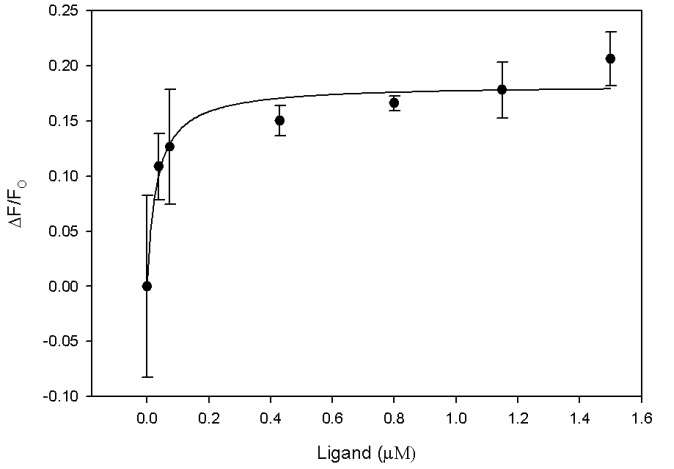


Figure 3.2b: Fluorescence spectra and peptide binding plot of Als5-SS F1327R with KLRIPSV at 37° C.

Table 5: Representing the dissociation constant (K_d) for the binding of mutant proteins with different peptides.

Peptide	**Protein**	**27° C K_d x 10^{-2} µM**	**37° C K_d x 10^{-2} µM**	**47° C K_d x 10^{-2} µM**
AYKSLMT	Als5-SS V309N	6.73±6.3	1.49±0.35	9.95±1.3
	Als5-SS L1326R	9.18±4.6	8.05±2.6	ND
	Als5-SS F1327R	4.29±1.2	4.78±1.2	8.29±4.9
EHAHTPR	Als5-SS V309N	ND	ND	ND
	Als5-SS L1326R	10.7±5.8	4.02±3.8	ND
	Als5-SS F1327R	7.7±3.4	3.39±0.71	ND
KLRIPSV	Als5-SS V309N	ND	ND	ND
	Als5-SS L1326R	1.02±0.74	7.47±3.4	ND
	Als5-SS F1327R	6.59±2.3	3.0±1.0	ND

Table 5: The table showing the dissociation constant (K_d) for each mutant protein at different temperature. For the binding, titration of the proteins with the different concentrations of peptides from a stock 30.0 µM was done. The data was plotted using SigmaPlot software and dissociation constant (K_d) was determined from the fit. Here, ND implies not determined.

In conclusion, we observed that GST-Als5-SS V309N, GST-Als5-SS L1326R and GST-Als5-SS F1327R mutant proteins showed binding with AYKSLMT peptide at 27° C, 37° C and 47 ° C, but the error bars were high suggesting the poor binding of mutant proteins. While the wild type Als5-SS showed significant binding with AYKSLMT at 27° C and 37° C. Similar results we observed in binding of GST-Als5-SS L1326R and GST-Als5-SS F1327R with EHAHTPR at 27° C and 37° C. Similarly, error bars were high in binding of GST-Als5-SS L1326R and GST-Als5-SS F1327R with KLRIPSV peptide. We were expecting the better binding of mutant proteins to the peptide ligands compared to the wild type Als5-SS. But, we observed that the mutant proteins were unable to bind significantly with peptide ligands at different temperature. As we observed that the mutations affected the

secondary structure conformation of Als5-SS protein, so it might be possible that due to the altered interactions of the protein the ligand binding pocket may not be accessible the ligand peptide in mutant proteins. Second possibility could be the concentration and the purity issues of mutant proteins. It is also possible that the peptides had degraded during storage. These experiments should be repeated using freshly synthesized peptides.

A.I.1 Media

A.I.1.1 Luria Bertani medium (LB) (g/100ml)

Tryptone (1.0 g)

Yeast extract (0.5 g)

NaCl (1.0 g)

(LB Agar plates- 1.5 % agar with suitable antibiotic)

A.I.1.2 YEPD medium (g/100 ml)

Peptone (2.0 g)

Yeast extract (1.0 g)

Glucose (2.0 g)

(YEPD Agar plates- 2.5 % agar)

A.I.1.3 SD Ura^- minimal medium (g/100 ml)

Yeast Nitrogen base (0.67g)

Glucose (2.0 g)

Ura^- amino acid dropout mix (0.2 g)

(SD Ura^- Agar plates- 2.5 % agar)

A.I.1.4 SD Ura^- Gal^+ induction medium (g/100 ml)

Yeast Nitrogen base (0.67%)

Galactose (2.0 g)

Sucrose (1.5 g)

Ura⁻ amino acid dropout mix (0.2 g)

A.I.2 Buffers and solutions

A.I.2.1 Alkaline lysis solutions for plasmid isolation

A.I.2.1a Solution I (Autoclaved and stored at 4 °C)

Glucose (50 mM)

Tris-HCl (25 mM, pH-8.0)

EDTA (10 mM, pH-8.0)

A.I.2.1b Solution II (freshly prepared)

NaOH (0.2 N)

SDS (1.0 %)

A.I.2.1c Solution III (stored at 4 °C)

5 M Potassium acetate (60 ml) autoclaved

Glacial acetic acid (11.5 ml)

Volume make up to 100 ml

A.I.2.2 1X Tris EDTA buffer (TE buffer)

EDTA (1 mM, pH 8.0)

10.0 mM Tris-HCl (pH 8.0)

A.I.2.3 10X TAE buffer

Tris-base (48.4 g)

Glacial acitic acid (11.42 ml)

20.0 ml EDTA (0.5 M, pH- 8.0)

A.I.2.4 Agarose gel electrophoresis

A.I.2.4a TAE solution

1X TAE

0.8% Agarose

A.I.2.4b DNA loading dye (6X)

0.25% (w/v) bromophenol blue

40 % (w/v) sucrose

A.I.3 Protein purification

A.I.3.1 Lysis buffer

Sodium phosphate buffer (50 mM.0, pH- 8)

NaCl (150 mM)

PMSF (10 µM)

Lysozyme (0.1 mg/ml)

Glycerol (5.0 %)

A.I.3.2 Equilibration buffer

Sodium phosphate buffer (50 mM, pH- 8)

NaCl (150 mM)

Glycerol (5.0 %)

A.I.3.3. Wash buffer for washing of beads

Tris-HCl (50 mM, pH-8.0)

NaCl (3 M)

A.I.3.4 Elution buffer

Sodium phosphate buffer (50 mM.0, pH- 8)

NaCl (150 mM)

Reduced Glutathione (10 mM)

Glycerol (20 %)

A.I.3.5 Dialysis buffer

Sodium phosphate buffer (50 mM.0, pH- 8)

NaCl (150 mM)

Glycerol (20 %)

A.I.4 SDS-PAGE

A.I.4.1 Resolving gel (6 %)

2.5 ml Tris-HCl (1.5 M, pH 8.8)

3.3 ml 30 % Acrylamide

0.1 ml 10 % SDS

0.1 ml 10 % Ammonium persulphate (APS)

0.004 ml TEMED

4 ml H_2O

A.I.4.2 Stacking gel (5 %)

0.63 ml Tris-HCl (1 M, pH 6.8)

0.83 ml 30 % Acrylamide

0.05 ml 10 % SDS

0.05 ml 10 % Ammonium persulphate (APS)

0.004 ml TEMED

3.4 ml H_2O

A.I.4.3 SDS-PAGE loading dye (4X)

200 mM Tris-HCl (pH 6.8)

400 mM Dithiothreitol

8 % SDS

0.4 % Bromophenol blue

40 % Glycerol

A.I.4.4 Staining solution

45 ml Methanol

45 ml H_2O

10 ml Glacial acitic acid

0.25 % Coomassie brilliant blue

A.I.4.5 Destaining solution

45 ml Methanol

45 ml H_2O

10 ml Glacial acitic acid

A.I.5. Western blotting

A.I.5.1 Western transfer buffer

Glycine (14.42 g)

Tris-base (3.03 g)

SDS (0.375 g)

Methanol (200 ml)

Volume makes up to 1 litre with autoclaved water

A.I.5.2 Phosphate Buffer Saline (PBS)

Nacl (8.0 g)

Na_2HPO_4 (1.44 g)

KH_2PO_4 (0.24 g)

KCl (0.2 g)

Volume make up to 1000 ml

A.I.5.3 Blocking solution

1X PBS

5 % Skimmed milk

0.05 % Tween-20

A.I.5.4 Wash buffer

1X PBS

0.05 % Tween-20

A.I.5.5 Antibody buffer (in PBS) used in western blotting

1 g Skimmed milk

0.025 % Tween-20

A.I.6 Tables

A.I.6.1 PCR reaction mixture

Components	Volume
Template	2 µl
10 X Buffer (XT-20 buffer)	2.5 µl
Forward primer (100 µM)	2.5 µl
Reverse primer (100 µM)	2.5 µl
$MgCl_2$ (25 mg/ml)	4 µl
dNTP	1 µl
Enzyme (XT-20 Polymerase)	0.3 µl
dH_2O	10.2 µl
Total	25 µl

A.I.6.2 PCR conditions

Initial denaturetion	94 ˚C for 5 min
Denaturation	94 ˚C for 1min
Annealing	65 ˚C for 1 min
Extension	72 ˚C for 5 min
Final Extension	72 ˚C for 10 min
Number of cycles	25

A.I.6.3 Double digestion reaction mixture

Components	Volume
Plasmid	3 µl
CutSmart® Buffer (10X)	2 µl
BamHI	1.0 µl
XhoI	1.0 µl
dH_2O	13 µl

Total	**20 µl**

A.I.6.4 Ligation reaction mixture

Components	**Volume**
Vector (pYES2)	1 µl
Insert	5 µl
T4 DNA ligase buffer (10 X)	1.0 µl
Enzyme (T4 DNA ligase)	1.0 µl
dH_2O	2 µl
Total	10 µl

References

Abeijon, C., and Hirschberg, C.B. (1992). Topography of glycosylation reactions in the endoplasmic reticulum. Trends Biochem. Sci. *17*, 32–36.

Adam, B., Baillie, G.S., and Douglas, L.J. (2002). Mixed species biofilms of *Candida albicans* and Staphylococcus epidermidis. J. Med. Microbiol. *51*, 344–349.

Aguilera-Romero, A., Kaminska, J., Spang, A., Riezman, H., and Muñiz, M. (2008). The yeast p24 complex is required for the formation of COPI retrograde transport vesicles from the Golgi apparatus. J. Cell Biol. *180*, 713–720.

Ahmad, M.F., Yadav, B., Kumar, P., Puri, A., Mazumder, M., Ali, A., Gourinath, S., Muthuswami, R., and Komath, S.S. (2012). The GPI Anchor Signal Sequence Dictates the Folding and Functionality of the Als5 Adhesin from *Candida albicans*. PLoS ONE *7*, e35305.

Ahmad, M.F., Mann, P.G., and Komath, S.S. (2015). A Signal with a Difference: The Role of GPI Anchor Signal Sequence in Dictating Conformation and Function of the Als5 Adhesin in *Candida albicans*. In Biochemical Roles of Eukaryotic Cell Surface Macromolecules, A. Chakrabarti, and A. Surolia, eds. (Cham: Springer International Publishing), pp. 147–163.

Almeida, A., Layton, M., and Karadimitris, A. (2009). Inherited glycosylphosphatidyl inositol deficiency: A treatable CDG. Biochim. Biophys. Acta BBA - Mol. Basis Dis. *1792*, 874–880.

Almeida, A.M., Murakami, Y., Layton, D.M., Hillmen, P., Sellick, G.S., Maeda, Y., Richards, S., Patterson, S., Kotsianidis, I., Mollica, L., et al. (2006). Hypomorphic promoter mutation in PIGM causes inherited glycosylphosphatidylinositol deficiency. Nat. Med. *12*, 846–851.

Almeida, R.S., Brunke, S., Albrecht, A., Thewes, S., Laue, M., Edwards, J.E., Filler, S.G., and Hube, B. (2008). The Hyphal-Associated Adhesin and Invasin Als3 of *Candida albicans* Mediates Iron Acquisition from Host Ferritin. PLoS Pathog. *4*, e1000217.

Alsteens, D., Garcia, M.C., Lipke, P.N., and Dufrêne, Y.F. (2010). Force-induced formation and propagation of adhesion nanodomains in living fungal cells. Proc. Natl. Acad. Sci. U. S. A. *107*, 20744–20749.

Aoki, W., Kitahara, N., Miura, N., Morisaka, H., Kuroda, K., and Ueda, M. (2012). Profiling of adhesive properties of the agglutinin-like sequence (*ALS*) protein family, a virulent attribute of *Candida albicans*. FEMS Immunol. Med. Microbiol. *65*, 121–124.

Ashida, H. (2005). Mammalian PIG-X and Yeast Pbn1p Are the Essential Components of Glycosylphosphatidylinositol-Mannosyltransferase I. Mol. Biol. Cell *16*, 1439–1448.

Ashraf, M., Yadav, B., Perinthottathil, S., Kumar, K.S., Vats, D., Muthuswami, R., and Komath, S.S. (2011). *N*-Acetyl-*D*-glucosaminylphosphatidylinositol De-*N*-acetylase from Entamoeba histolytica: METAL ALTERS CATALYTIC RATES BUT NOT SUBSTRATE AFFINITY. J. Biol. Chem. *286*, 2543–2549.

Ashraf, M., Sreejith, P., Yadav, U., and Komath, S.S. (2013). Catalysis by N-Acetyl-D-glucosaminylphosphatidylinositol De-N-acetylase (PIG-L) from Entamoeba histolytica: NEW ROLES FOR CONSERVED RESIDUES. J. Biol. Chem. *288*, 7590–7595.

Bailey, D.A., Feldmann, P.J., Bovey, M., Gow, N.A., and Brown, A.J. (1996). The *Candida albicans HYR1* gene, which is activated in response to hyphal development, belongs to a gene family encoding yeast cell wall proteins. J. Bacteriol. *178*, 5353–5360.

Bamford, C.V., Nobbs, A.H., Barbour, M.E., Lamont, R.J., and Jenkinson, H.F. (2015). Functional regions of *Candida albicans* hyphal cell wall protein Als3 that determine interaction with the oral bacterium Streptococcus gordonii. Microbiology *161*, 18–29.

Barboni, E., Rivero, B.P., George, A.J., Martin, S.R., Renoup, D.V., Hounsell, E.F., Barber, P.C., and Morris, R.J. (1995). The glycophosphatidylinositol anchor affects the conformation of Thy-1 protein. J. Cell Sci. *108 (Pt 2)*, 487–497.

Bates, S., de la Rosa, J.M., MacCallum, D.M., Brown, A.J.P., Gow, N.A.R., and Odds, F.C. (2007). *Candida albicans* Iff11, a Secreted Protein Required for Cell Wall Structure and Virulence. Infect. Immun. *75*, 2922–2928.

Benachour, A., Sipos, G., Flury, I., Reggiori, F., Canivenc-Gansel, E., Vionnet, C., Conzelmann, A., and Benghezal, M. (1999). Deletion of GPI7, a Yeast Gene Required for Addition of a Side Chain to the Glycosylphosphatidylinositol (GPI) Core Structure, Affects GPI Protein Transport, Remodeling, and Cell Wall Integrity. J. Biol. Chem. *274*, 15251–15261.

Benghezal, M., Benachour, A., Rusconi, S., Aebi, M., and Conzelmann, A. (1996). Yeast Gpi8p is essential for GPI anchor attachment onto proteins. EMBO J. *15*, 6575–6583.

Berger, J., Howard, A.D., Brink, L., Gerber, L., Hauber, J., Cullen, B.R., and Udenfriend, S. (1988). COOH-terminal requirements for the correct processing of a phosphatidylinositol-glycan anchored membrane protein. J. Biol. Chem. *263*, 10016–10021.

Bi, X., Corpina, R.A., and Goldberg, J. (2002). Structure of the Sec23/24–Sar1 pre-budding complex of the COPII vesicle coat. Nature *419*, 271–277.

Bois, M., Singh, S., Samlalsingh, A., Lipke, P.N., and Garcia, M.C. (2013). Does *Candida albicans* Als5p Amyloid Play a Role in Commensalism in Caenorhabditis elegans? Eukaryot. Cell *12*, 703–711.

Bosson, R., Jaquenoud, M., and Conzelmann, A. (2006). GUP1 of *Saccharomyces cerevisiae* encodes an O-acyltransferase involved in remodeling of the GPI anchor. Mol. Biol. Cell *17*, 2636–2645.

Bradway, S.D., and Levine, M.J. (1993). Do proline-rich proteins modulate a transglutaminase catalyzed mechanism of candidal adhesion? Crit. Rev. Oral Biol. Med. Off. Publ. Am. Assoc. Oral Biol. *4*, 293–299.

Brodsky, R.A. (2006). New Insights into Paroxysmal Nocturnal Hemoglobinuria. Hematology *2006*, 24–28.

Brown, G.D., and Gordon, S. (2001). Immune recognition: A new receptor for β-glucans. Nature *413*, 36–37.

Brown, G.D., Denning, D.W., and Levitz, S.M. (2012). Tackling Human Fungal Infections. Science *336*, 647–647.

Busscher, H.J., van Hoogmoed, C.G., Geertsema-Doornbusch, G.I., van der Kuijl-Booij, M., and van der Mei, H.C. (1997). *Streptococcus thermophilus* and its biosurfactants inhibit adhesion by *Candida* spp. on silicone rubber. Appl. Environ. Microbiol. *63*, 3810–3817.

Calderone, R.A., and Fonzi, W.A. (2001). Virulence factors of *Candida albicans*. Trends Microbiol. *9*, 327–335.

Cannon, R.D., Lamping, E., Holmes, A.R., Niimi, K., Baret, P.V., Keniya, M.V., Tanabe, K., Niimi, M., Goffeau, A., and Monk, B.C. (2009). Efflux-Mediated Antifungal Drug Resistance. Clin. Microbiol. Rev. *22*, 291–321.

Cao, X., Surma, M.A., and Simons, K. (2012). Polarized sorting and trafficking in epithelial cells. Cell Res. *22*, 793–805.

Caras, I.W. (1989). Analysis of the signal for attachment of a glycophospholipid membrane anchor. J. Cell Biol. *108*, 1387–1396.

Caras, I.W. (1991). An internally positioned signal can direct attachment of a glycophospholipid membrane anchor. J. Cell Biol. *113*, 77–85.

Caras, I.W., and Weddell, G.N. (1989). Signal peptide for protein secretion directing glycophospholipid membrane anchor attachment. Science *243*, 1196–1198.

Caro, L.H.P., Tettelin, H., Vossen, J.H., Ram, A.F.J., Van Den Ende, H., and Klis, F.M. (1997). In silicio identification of glycosyl-phosphatidylinositol-anchored plasma-membrane and cell wall proteins of *Saccharomyces cerevisiae*. Yeast *13*, 1477–1489.

Carrington, M., Miller, N., Blum, M., Roditi, I., Wiley, D., and Turner, M. (1991). Variant specific glycoprotein of Trypanosoma brucei consists of two domains each having an independently conserved pattern of cysteine residues. J. Mol. Biol. *221*, 823–835.

Castillon, G.A., Aguilera-Romero, A., Manzano-Lopez, J., Epstein, S., Kajiwara, K., Funato, K., Watanabe, R., Riezman, H., and Muniz, M. (2011). The yeast p24 complex regulates GPI-anchored protein transport and quality control by monitoring anchor remodeling. Mol. Biol. Cell *22*, 2924–2936.

Chaffin, W.L. (2008). *Candida albicans* Cell Wall Proteins. Microbiol. Mol. Biol. Rev. *72*, 495–544.

Chaffin, W.L., López-Ribot, J.L., Casanova, M., Gozalbo, D., and Martínez, J.P. (1998). Cell wall and secreted proteins of *Candida albicans*: identification, function, and expression. Microbiol. Mol. Biol. Rev. MMBR *62*, 130–180.

Chakrabarti, A., Chatterjee, S.S., and Shivaprakash, M.R. (2008). Overview of opportunistic fungal infections in India. Nihon Ishinkin Gakkai Zasshi Jpn. J. Med. Mycol. *49*, 165–172.

Chang, T., Milne, K.G., Güther, M.L.S., Smith, T.K., and Ferguson, M.A.J. (2002). Cloning of *Trypanosoma brucei* and *Leishmania* major genes encoding the GlcNAc-phosphatidylinositol de-*N*-acetylase of glycosylphosphatidylinositol biosynthesis that is essential to the African sleeping sickness parasite. J. Biol. Chem. *277*, 50176–50182.

Cheng, G., Wozniak, K., Wallig, M.A., Fidel, P.L., Trupin, S.R., and Hoyer, L.L. (2005). Comparison between *Candida albicans* agglutinin-like sequence gene expression patterns in human clinical specimens and models of vaginal candidiasis. Infect. Immun. *73*, 1656–1663.

Chuang, J.Z., and Sung, C.H. (1998). The cytoplasmic tail of rhodopsin acts as a novel apical sorting signal in polarized MDCK cells. J. Cell Biol. *142*, 1245–1256.

Coleman, D.A., Oh, S.-H., Zhao, X., Zhao, H., Hutchins, J.T., Vernachio, J.H., Patti, J.M., and Hoyer, L.L. (2009). Monoclonal antibodies specific for *Candida albicans* Als3 that immunolabel fungal cells in vitro and in vivo and block adhesion to host surfaces. J. Microbiol. Methods *78*, 71–78.

Coleman, D.A., Oh, S.-H., Zhao, X., and Hoyer, L.L. (2010). Heterogeneous distribution of *Candida albicans* cell-surface antigens demonstrated with an Als1-specific monoclonal antibody. Microbiology *156*, 3645–3659.

Coleman, D.A., Oh, S.-H., Manfra-Maretta, S.L., and Hoyer, L.L. (2012). A monoclonal antibody specific for *Candida albicans* Als4 demonstrates overlapping localization of Als family proteins on the fungal cell surface and highlights differences between Als localization *in vitro* and *in vivo*. FEMS Immunol. Med. Microbiol. *64*, 321–333.

Cormack, B., and Zordan, R. (2012). Adhesins in Opportunistic Fungal Pathogens. In *Candida* and Candidiasis, Second Edition, R.A. Calderone, and C.J. Clancy, eds. (American Society of Microbiology), pp. 243–259.

Costello, L.C., and Orlean, P. (1992). Inositol acylation of a potential glycosyl phosphoinositol anchor precursor from yeast requires acyl coenzyme A. J. Biol. Chem. *267*, 8599–8603.

Cousin, X., Bon, S., Massoulié, J., and Bon, C. (1998). Identification of a novel type of alternatively spliced exon from the acetylcholinesterase gene of Bungarus fasciatus. Molecular forms of acetylcholinesterase in the snake liver and muscle. J. Biol. Chem. *273*, 9812–9820.

Coyne, K.E., Crisci, A., and Lublin, D.M. (1993). Construction of synthetic signals for glycosyl-phosphatidylinositol anchor attachment. Analysis of amino acid sequence requirements for anchoring. J. Biol. Chem. *268*, 6689–6693.

Csank, C., Schröppel, K., Leberer, E., Harcus, D., Mohamed, O., Meloche, S., Thomas, D.Y., and Whiteway, M. (1998). Roles of the *Candida albicans* mitogen-activated protein kinase homolog, Cek1p, in hyphal development and systemic candidiasis. Infect. Immun. *66*, 2713–2721.

Davis, D.A. (2009). How human pathogenic fungi sense and adapt to pH: the link to virulence. Curr. Opin. Microbiol. *12*, 365–370.

De Bernardis, F., Mühlschlegel, F.A., Cassone, A., and Fonzi, W.A. (1998). The pH of the host niche controls gene expression in and virulence of *Candida albicans*. Infect. Immun. *66*, 3317–3325.

De Groot, P.W.J., Hellingwerf, K.J., and Klis, F.M. (2003). Genome-wide identification of fungal GPI proteins. Yeast Chichester Engl. *20*, 781–796.

Deorukhkar, S.C., Saini, S., and Mathew, S. (2014). Virulence Factors Contributing to Pathogenicity of *Candida tropicalis* and Its Antifungal Susceptibility Profile. Int. J. Microbiol. *2014*, 1–6.

Desai, J.V., and Mitchell, A.P. (2015). Candida albicans Biofilm Development and Its Genetic Control. In Microbial Biofilms, 2nd Edition, P.K. Mukherjee, M. Ghannoum, M. Whiteley, and M. Parsek, eds. (American Society of Microbiology), pp. 99–114.

Doering, T.L., and Schekman, R. (1996). GPI anchor attachment is required for Gas1p transport from the endoplasmic reticulum in COP II vesicles. EMBO J. *15*, 182–191.

Doering, T.L., Masterson, W.J., Hart, G.W., and Englund, P.T. (1990). Biosynthesis of glycosyl phosphatidylinositol membrane anchors. J. Biol. Chem. *265*, 611–614.

Donohue, D.S., Ielasi, F.S., Goossens, K.V.Y., and Willaert, R.G. (2011). The N-terminal part of Als1 protein from *Candida albicans* specifically binds fucose-containing glycans: Als1p-N from *Candida albicans* binds to fucose glycans. Mol. Microbiol. *80*, 1667–1679.

Dranginis, A.M., Rauceo, J.M., Coronado, J.E., and Lipke, P.N. (2007). A biochemical guide to yeast adhesins: glycoproteins for social and antisocial occasions. Microbiol. Mol. Biol. Rev. MMBR *71*, 282–294.

Eisenhaber, B., Bork, P., and Eisenhaber, F. (1999). Prediction of Potential GPI-modification Sites in Proprotein Sequences. J. Mol. Biol. *292*, 741–758.

Eisenhaber, B., Maurer-Stroh, S., Novatchkova, M., Schneider, G., and Eisenhaber, F. (2003). Enzymes and auxiliary factors for GPI lipid anchor biosynthesis and post-translational transfer to proteins. BioEssays *25*, 367–385.

Eisenhaber, B., Eisenhaber, S., Kwang, T.Y., Grüber, G., and Eisenhaber, F. (2014). Transamidase subunit GAA1/GPAA1 is a M28 family metallo-peptide-synthetase that catalyzes the peptide bond formation between the substrate protein's omega-site and the GPI lipid anchor's phosphoethanolamine. Cell Cycle *13*, 1912–1917.

Ellgaard, L., and Helenius, A. (2003). Quality control in the endoplasmic reticulum. Nat. Rev. Mol. Cell Biol. *4*, 181–191.

Fabre, A.-L., Orlean, P., and Taron, C.H. (2005). *Saccharomyces cerevisiae* Ybr004c and its human homologue are required for addition of the second mannose during glycosylphosphatidylinositol precursor assembly. FEBS J. *272*, 1160–1168.

Fankhauser, N., and Maser, P. (2005). Identification of GPI anchor attachment signals by a Kohonen self-organizing map. Bioinformatics *21*, 1846–1852.

Fanning, S., and Mitchell, A.P. (2012). Fungal Biofilms. PLoS Pathog. *8*, e1002585.

Felk, A., Kretschmar, M., Albrecht, A., Schaller, M., Beinhauer, S., Nichterlein, T., Sanglard, D., Korting, H.C., Schafer, W., and Hube, B. (2002). *Candida albicans* Hyphal Formation and the Expression of the Efg1-Regulated Proteinases Sap4 to Sap6 Are Required for the Invasion of Parenchymal Organs. Infect. Immun. *70*, 3689–3700.

Ferguson, M.A. (1999). The structure, biosynthesis and functions of glycosylphosphatidylinositol anchors, and the contributions of trypanosome research. J. Cell Sci. *112 (Pt 17)*, 2799–2809.

Ferguson, M.A.J., and Williams, A.F. (1988). Cell-Surface Anchoring of Proteins via Glycosyl-Phosphatidylinositol Structures. Annu. Rev. Biochem. *57*, 285–320.

Ferguson, M.A., Haldar, K., and Cross, G.A. (1985). *Trypanosoma brucei* variant surface glycoprotein has a sn-1,2-dimyristyl glycerol membrane anchor at its COOH terminus. J. Biol. Chem. *260*, 4963–4968.

Ferguson, M.A., Homans, S.W., Dwek, R.A., and Rademacher, T.W. (1988). Glycosyl-phosphatidylinositol moiety that anchors *Trypanosoma brucei* variant surface glycoprotein to the membrane. Science *239*, 753–759.

Ferrando-Miguel, R., Cheon, M.S., and Lubec, G. (2004). Protein levels of genes encoded on chromosome 21 in fetal Down Syndrome brain (Part V): Overexpression of phosphatidyl-inositol-glycan class P protein (DSCR5). Amino Acids *26*.

Fidel, P.L., and Sobel, J.D. (1996). Immunopathogenesis of recurrent vulvovaginal candidiasis. Clin. Microbiol. Rev. *9*, 335–348.

Finkel, J.S., and Mitchell, A.P. (2011). Genetic control of *Candida albicans* biofilm development. Nat. Rev. Microbiol. *9*, 109–118.

Fleck, C.B., Schöbel, F., and Brock, M. (2011). Nutrient acquisition by pathogenic fungi: Nutrient availability, pathway regulation, and differences in substrate utilization. Int. J. Med. Microbiol. *301*, 400–407.

Fraering, P., Imhof, I., Meyer, U., Strub, J.M., van Dorsselaer, A., Vionnet, C., and Conzelmann, A. (2001). The GPI transamidase complex of *Saccharomyces cerevisiae* contains Gaa1p, Gpi8p, and Gpi16p. Mol. Biol. Cell *12*, 3295–3306.

Frank, A.T., Ramsook, C.B., Otoo, H.N., Tan, C., Soybelman, G., Rauceo, J.M., Gaur, N.K., Klotz, S.A., and Lipke, P.N. (2010). Structure and function of glycosylated tandem repeats from *Candida albicans* Als adhesins. Eukaryot. Cell *9*, 405–414.

Fu, Y., Rieg, G., Fonzi, W.A., Belanger, P.H., Edwards, J.E., and Filler, S.G. (1998). Expression of the *Candida albicans* gene *ALS1* in *Saccharomyces cerevisiae* induces adherence to endothelial and epithelial cells. Infect. Immun. *66*, 1783–1786.

Fu, Y., Ibrahim, A.S., Sheppard, D.C., Chen, Y.-C., French, S.W., Cutler, J.E., Filler, S.G., and Edwards, J.E. (2002). *Candida albicans* Als1p: an adhesin that is a downstream effector of the EFG1 filamentation pathway. Mol. Microbiol. *44*, 61–72.

Fujita, M. (2005). Inositol Deacylation by Bst1p Is Required for the Quality Control of Glycosylphosphatidylinositol-anchored Proteins. Mol. Biol. Cell *17*, 834–850.

Fujita, M., Umemura, M., Yoko-o, T., and Jigami, Y. (2006). *PER1* Is Required for GPI-Phospholipase A2 Activity and Involved in Lipid Remodeling of GPI-anchored Proteins. Mol. Biol. Cell *17*, 5253–5264.

Fujita, M., Maeda, Y., Ra, M., Yamaguchi, Y., Taguchi, R., and Kinoshita, T. (2009). GPI glycan remodeling by PGAP5 regulates transport of GPI-anchored proteins from the ER to the Golgi. Cell *139*, 352–365.

Fujita, M., Watanabe, R., Jaensch, N., Romanova-Michaelides, M., Satoh, T., Kato, M., Riezman, H., Yamaguchi, Y., Maeda, Y., and Kinoshita, T. (2011). Sorting of GPI-anchored proteins into ER exit sites by p24 proteins is dependent on remodeled GPI. J. Cell Biol. *194*, 61–75.

Furukawa, Y., Tamura, H., and Ikezawa, H. (1994). Mutational analysis of the COOH-terminal hydrophobic domain of bovine liver 5'-nucleotidase as a signal for glycosylphosphatidylinositol (GPI) anchor attachment. Biochim. Biophys. Acta BBA - Biomembr. *1190*, 273–278.

Garcia, M.C., Lee, J.T., Ramsook, C.B., Alsteens, D., Dufrêne, Y.F., and Lipke, P.N. (2011). A Role for Amyloid in Cell Aggregation and Biofilm Formation. PLoS ONE *6*, e17632.

García-Sánchez, S., Aubert, S., Iraqui, I., Janbon, G., Ghigo, J.-M., and d'Enfert, C. (2004). *Candida albicans* biofilms: a developmental state associated with specific and stable gene expression patterns. Eukaryot. Cell *3*, 536–545.

Garcia-Sherman, M.C., Lysak, N., Filonenko, A., Richards, H., Sobonya, R.E., Klotz, S.A., and Lipke, P.N. (2014). Peptide Detection of Fungal Functional Amyloids in Infected Tissue. PLoS ONE *9*, e86067.

Gaur, N.K., and Klotz, S.A. (1997). Expression, cloning, and characterization of a *Candida albicans* gene, *ALA1*, that confers adherence properties upon *Saccharomyces cerevisiae* for extracellular matrix proteins. Infect. Immun. *65*, 5289–5294.

Gaur, N.K., Klotz, S.A., and Henderson, R.L. (1999). Overexpression of the Candida albicans *ALA1* gene in Saccharomyces cerevisiae results in aggregation following attachment of yeast cells to extracellular matrix proteins, adherence properties similar to those of *Candida albicans*. Infect. Immun. *67*, 6040–6047.

Gaynor, E.C., Mondésert, G., Grimme, S.J., Reed, S.I., Orlean, P., and Emr, S.D. (1999). MCD4 encodes a conserved endoplasmic reticulum membrane protein essential for glycosylphosphatidylinositol anchor synthesis in yeast. Mol. Biol. Cell *10*, 627–648.

Gerber, L.D., Kodukula, K., and Udenfriend, S. (1992). Phosphatidylinositol glycan (PI-G) anchored membrane proteins. Amino acid requirements adjacent to the site of cleavage and PI-G attachment in the COOH-terminal signal peptide. J. Biol. Chem. *267*, 12168–12173.

Ghugtyal, V., Vionnet, C., Roubaty, C., and Conzelmann, A. (2007). *CWH43* is required for the introduction of ceramides into GPI anchors in Saccharomyces cerevisiae. Mol. Microbiol. *65*, 1493–1502.

Gietz, R.D., Schiestl, R.H., Willems, A.R., and Woods, R.A. (1995). Studies on the transformation of intact yeast cells by the LiAc/SS-DNA/PEG procedure. Yeast *11*, 355–360.

Gil-Bona, A., Reales-Calderon, J.A., Parra-Giraldo, C.M., Martinez-Lopez, R., Monteoliva, L., and Gil, C. (2016). The Cell Wall Protein Ecm33 of *Candida albicans* is Involved in Chronological Life Span, Morphogenesis, Cell Wall Regeneration, Stress Tolerance, and Host–Cell Interaction. Front. Microbiol. *7*.

Granger, B.L. (2005). Yeast wall protein 1 of *Candida albicans*. Microbiology *151*, 1631–1644.

Granger, B.L. (2012). Insight into the Antiadhesive Effect of Yeast Wall Protein 1 of *Candida albicans*. Eukaryot. Cell *11*, 795–805.

Green, C.B. (2004). RT-PCR detection of *Candida albicans ALS* gene expression in the reconstituted human epithelium (RHE) model of oral candidiasis and in model biofilms. Microbiology *150*, 267–275.

Green, C.B., Zhao, X., and Hoyer, L.L. (2005). Use of Green Fluorescent Protein and Reverse Transcription-PCR To Monitor Candida albicans Agglutinin-Like Sequence Gene Expression in a Murine Model of Disseminated Candidiasis. Infect. Immun. *73*, 1852–1855.

Grimme, S.J. (2004). Deficiencies in the essential Smp3 mannosyltransferase block glycosylphosphatidylinositol assembly and lead to defects in growth and cell wall biogenesis in *Candida albicans*. Microbiology *150*, 3115–3128.

Grimme, S.J., Westfall, B.A., Wiedman, J.M., Taron, C.H., and Orlean, P. (2001). The Essential Smp3 Protein Is Required for Addition of the Side-branching Fourth Mannose during Assembly of Yeast Glycosylphosphatidylinositols. J. Biol. Chem. *276*, 27731–27739.

de Groot, P.W.J., Bader, O., de Boer, A.D., Weig, M., and Chauhan, N. (2013). Adhesins in Human Fungal Pathogens: Glue with Plenty of Stick. Eukaryot. Cell *12*, 470–481.

Grover, N. (2010). Echinocandins: A ray of hope in antifungal drug therapy. Indian J. Pharmacol. *42*, 9.

Gugnani, H.C., Nzelibe, F.K., Gini, P.C., Chukudebelu, W.O., and Njoku-Obi, A.N.U. (2009). Incidence of Yeasts in Pregnant and Non-Pregnant Women in Nigeria: Hefen bei Schwangeren und Nichtschwangeren in Nigeria. Mycoses *32*, 131–135.

Güther, M.L., and Ferguson, M.A. (1995). The role of inositol acylation and inositol deacylation in GPI biosynthesis in *Trypanosoma brucei*. EMBO J. *14*, 3080–3093.

Hamada, K., Terashima, H., Arisawa, M., and Kitada, K. (1998). Amino acid sequence requirement for efficient incorporation of glycosylphosphatidylinositol-associated proteins into the cell wall of *Saccharomyces cerevisiae*. J. Biol. Chem. *273*, 26946–26953.

Hamada, K., Terashima, H., Arisawa, M., Yabuki, N., and Kitada, K. (1999). Amino acid residues in the omega-minus region participate in cellular localization of yeast glycosylphosphatidylinositol-attached proteins. J. Bacteriol. *181*, 3886–3889.

Hamburger, D., Egerton, M., and Riezman, H. (1995). Yeast Gaa1p is required for attachment of a completed GPI anchor onto proteins. J. Cell Biol. *129*, 629–639.

Harder, T., and Simons, K. (1997). Caveolae, DIGs, and the dynamics of sphingolipid-cholesterol microdomains. Curr. Opin. Cell Biol. *9*, 534–542.

Hayek, P., Dib, L., Yazbeck, P., Beyrouthy, B., and Khalaf, R.A. (2010). Characterization of Hwp2, a *Candida albicans* putative GPI-anchored cell wall protein necessary for invasive growth. Microbiol. Res. *165*, 250–258.

Herskowitz, I. (1988). Life cycle of the budding yeast *Saccharomyces cerevisiae*. Microbiol. Rev. *52*, 536–553.

Hof, H. (2006). A new, broad-spectrum azole antifungal: posaconazole-mechanisms of action and resistance, spectrum of activity. Mycoses *49*, 2–6.

Hoffman, C.S., and Winston, F. (1987). A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for transformation of *Escherichia coli*. Gene *57*, 267–272.

Hogan, D.A., and Kolter, R. (2002). *Pseudomonas-Candida* interactions: an ecological role for virulence factors. Science *296*, 2229–2232.

Hong, Y., and Kinoshita, T. (2009). *Trypanosome* Glycosylphosphatidylinositol Biosynthesis. Korean J. Parasitol. *47*, 197.

Hong, Y., Maeda, Y., Watanabe, R., Ohishi, K., Mishkind, M., Riezman, H., and Kinoshita, T. (1999). Pig-n, a Mammalian Homologue of Yeast Mcd4p, Is Involved in Transferring Phosphoethanolamine to the First Mannose of the Glycosylphosphatidylinositol. J. Biol. Chem. *274*, 35099–35106.

Hong, Y., Maeda, Y., Watanabe, R., Inoue, N., Ohishi, K., and Kinoshita, T. (2000). Requirement of PIG-F and PIG-O for Transferring Phosphoethanolamine to the Third Mannose in Glycosylphosphatidylinositol. J. Biol. Chem. *275*, 20911–20919.

Hong, Y., Nagamune, K., Ohishi, K., Morita, Y.S., Ashida, H., Maeda, Y., and Kinoshita, T. (2006). TbGPI16 is an essential component of GPI transamidase in *Trypanosoma brucei*. FEBS Lett. *580*, 603–606.

Hostetter, M.K. (1994). Adhesins and ligands involved in the interaction of *Candida* spp. with epithelial and endothelial surfaces. Clin. Microbiol. Rev. *7*, 29–42.

Howell, S., Lanctôt, C., Boileau, G., and Crine, P. (1994). A cleavable N-terminal signal peptide is not a prerequisite for the biosynthesis of glycosylphosphatidylinositol-anchored proteins. J. Biol. Chem. *269*, 16993–16996.

Hoyer, L.L. (2001). The *ALS* gene family of *Candida albicans*. Trends Microbiol. *9*, 176–180.

Hoyer, L.L., and Hecht, J.E. (2000). The *ALS6* and *ALS7* genes of *Candida albicans*. Yeast Chichester Engl. *16*, 847–855.

Hoyer, L.L., and Hecht, J.E. (2001). The *ALS5* gene of *Candida albicans* and analysis of the Als5p N-terminal domain. Yeast *18*, 49–60.

Hoyer, L.L., Scherer, S., Shatzman, A.R., and Livi, G.P. (1995). *Candida albicans ALS1*: domains related to a *Saccharomyces cerevisiae* sexual agglutinin separated by a repeating motif. Mol. Microbiol. *15*, 39–54.

Hoyer, L.L., Payne, T.L., and Hecht, J.E. (1998a). Identification of *Candida albicans ALS2* and *ALS4* and localization of als proteins to the fungal cell surface. J. Bacteriol. *180*, 5334–5343.

Hoyer, L.L., Payne, T.L., Bell, M., Myers, A.M., and Scherer, S. (1998b). *Candida albicans ALS3* and insights into the nature of the *ALS* gene family. Curr. Genet. *33*, 451–459.

Hoyer, L.L., Green, C.B., Oh, S.-H., and Zhao, X. (2008). Discovering the secrets of the *Candida albicans* agglutinin-like sequence (*ALS*) gene family-a sticky pursuit. Med. Mycol. *46*, 1–15.

Hoyer, L.L., Oh, S.-H., Jones, R., and Cota, E. (2014). A proposed mechanism for the interaction between the *Candida albicans* Als3 adhesin and *streptococcal* cell wall proteins. Front. Microbiol. *5*.

Hu, R., Mukhina, G.L., Lee, S.H., Jones, R.J., Englund, P.T., Brown, P., Sharkis, S.J., Buckley, J.T., and Brodsky, R.A. (2009). Silencing of genes required for glycosylphosphatidylinositol anchor biosynthesis in Burkitt lymphoma. Exp. Hematol. *37*, 423–434.e2.

Hube, B., Monod, M., Schofield, D.A., Brown, A.J., and Gow, N.A. (1994). Expression of seven members of the gene family encoding secretory aspartyl proteinases in *Candida albicans*. Mol. Microbiol. *14*, 87–99.

Hube, B., Sanglard, D., Odds, F.C., Hess, D., Monod, M., Schäfer, W., Brown, A.J., and Gow, N.A. (1997). Disruption of each of the secreted aspartyl proteinase genes *SAP1*, *SAP2*, and *SAP3* of *Candida albicans* attenuates virulence. Infect. Immun. *65*, 3529–3538.

Hughson, F.M. (2008). Both Layers of the COPII Coat Come into View. Cell *134*, 384–385.

Ibrahim, A.S., Spellberg, B.J., Avenissian, V., Fu, Y., Filler, S.G., and Edwards, J.E. (2005). Vaccination with Recombinant N-Terminal Domain of Als1p Improves Survival during Murine Disseminated Candidiasis by Enhancing Cell-Mediated, Not Humoral, Immunity. Infect. Immun. *73*, 999–1005.

Ikezawa, H., Yamanegi, M., Taguchi, R., Miyashita, T., and Ohyabu, T. (1976). Studies on phosphatidylinositol phosphodiesterase (phospholipase C type) of *Bacillus cereus*. I. purification, properties and phosphatase-releasing activity. Biochim. Biophys. Acta *450*, 154–164.

Imhof, I., Flury, I., Vionnet, C., Roubaty, C., Egger, D., and Conzelmann, A. (2004). Glycosylphosphatidylinositol (GPI) Proteins of *Saccharomyces cerevisiae* Contain Ethanolamine Phosphate Groups on the α1,4-linked Mannose of the GPI Anchor. J. Biol. Chem. *279*, 19614–19627.

Inoue, H., Nojima, H., and Okayama, H. (1990). High efficiency transformation of *Escherichia coli* with plasmids. Gene *96*, 23–28.

Inoue, N., Kinoshita, T., Orii, T., and Takeda, J. (1993). Cloning of a human gene, PIG-F, a component of glycosylphosphatidylinositol anchor biosynthesis, by a novel expression cloning strategy. J. Biol. Chem. *268*, 6882–6885.

Inoue, N., Watanabe, R., Takeda, J., and Kinoshita, T. (1996). PIG-C, One of the Three Human Genes Involved in the First Step of Glycosylphosphatidylinositol Biosynthesis Is a Homologue of *Saccharomyces cerevisiae GPI2*. Biochem. Biophys. Res. Commun. *226*, 193–199.

Jiang, W.-W., Zahurak, M., Zhou, Z.-T., Park, H., Guo, Z.-M., Wu, G.-J., Sidransky, D., Trink, B., and Califano, J.A. (2007). Alterations of GPI transamidase subunits in head and neck squamous carcinoma. Mol. Cancer *6*, 74.

Joerger, A.C., and Fersht, A.R. (2007). Structure–function–rescue: the diverse nature of common p53 cancer mutants. Oncogene *26*, 2226–2242.

Joerger, A.C., and Fersht, A.R. (2010). The Tumor Suppressor p53: From Structures to Drug Discovery. Cold Spring Harb. Perspect. Biol. *2*, a000919–a000919.

Johnson, E.M., Warnock, D.W., Luker, J., Porter, S.R., and Scully, C. (1995). Emergence of azole drug resistance in *Candida* species from HIV-infected patients receivingprolonged fluconazole therapy for oral candidosis. J. Antimicrob. Chemother. *35*, 103–114.

Johnston, J., Gropman, A., Sapp, J., Teer, J., Martin, J., Liu, C., Yuan, X., Ye, Z., Cheng, L., Brodsky, R., et al. (2012). The Phenotype of a Germline Mutation in PIGA: The Gene Somatically Mutated in Paroxysmal Nocturnal Hemoglobinuria. Am. J. Hum. Genet. *90*, 295–300.

Kabir, M.A., Hussain, M.A., and Ahmad, Z. (2012). *Candida albicans* : A Model Organism for Studying Fungal Pathogens. ISRN Microbiol. *2012*, 1–15.

Kang, J.Y., Hong, Y., Ashida, H., Shishioh, N., Murakami, Y., Morita, Y.S., Maeda, Y., and Kinoshita, T. (2005). PIG-V involved in transferring the second mannose in glycosylphosphatidylinositol. J. Biol. Chem. *280*, 9489–9497.

Kapteyn, J.C., Van Egmond, P., Sievi, E., Van Den Ende, H., Makarow, M., and Klis, F.M. (1999). The contribution of the O-glycosylated protein Pir2p/Hsp150 to the construction of the yeast cell wall in wild-type cells and beta1,6-glucan-deficient mutants. Mol. Microbiol. *31*, 1835–1844.

Kapteyn, J.C., Hoyer, L.L., Hecht, J.E., Müller, W.H., Andel, A., Verkleij, A.J., Makarow, M., Van Den Ende, H., and Klis, F.M. (2000). The cell wall architecture of *Candida albicans* wild-type cells and cell wall-defective mutants. Mol. Microbiol. *35*, 601–611.

Kawagoe, K., Takeda, J., Endo, Y., and Kinoshita, T. (1994). Molecular Cloning of Murine Pig-a, a Gene for GPI-Anchor Biosynthesis, and Demonstration of Interspecies Conservation of Its Structure, Function, and Genetic Locus. Genomics *23*, 566–574.

Kawagoe, K., Kitamura, D., Okabe, M., Taniuchi, I., Ikawa, M., Watanabe, T., Kinoshita, T., and Takeda, J. (1996). Glycosylphosphatidylinositol-anchor-deficient mice: implications for clonal dominance of mutant cells in paroxysmal nocturnal hemoglobinuria. Blood *87*, 3600–3606.

Kett, D.H., Azoulay, E., Echeverria, P.M., and Vincent, J.-L. (2011). *Candida* bloodstream infections in intensive care units: Analysis of the extended prevalence of infection in intensive care unit study*: Crit. Care Med. *39*, 665–670.

Kim, J., and Sudbery, P. (2011). *Candida albicans*, a major human fungal pathogen. J. Microbiol. *49*, 171–177.

Kim, Y.U., Ashida, H., Mori, K., Maeda, Y., Hong, Y., and Kinoshita, T. (2007). Both Mammalian PIG-M and PIG-X are Required for Growth of GPI14-Disrupted Yeast. J. Biochem. (Tokyo) *142*, 123–129.

Kinoshita, T. (2014). Enzymatic mechanism of GPI anchor attachment clarified. Cell Cycle *13*, 1838–1839.

Kinoshita, T., and Takeda, J. (1994). GPI-anchor synthesis. Parasitol. Today Pers. Ed *10*, 139–143.

Klotz, S.A., Smith, R.L., and Stewart, B.W. (1992). Effect of an arginine-glycine-aspartic acid-containing peptide on hematogenous candidal infections in rabbits. Antimicrob. Agents Chemother. *36*, 132–136.

Klotz, S.A., Gaur, N.K., Lake, D.F., Chan, V., Rauceo, J., and Lipke, P.N. (2004). Degenerate Peptide Recognition by *Candida albicans* Adhesins Als5p and Als1p. Infect. Immun. *72*, 2029–2034.

Kodukula, K., Gerber, L.D., Amthauer, R., Brink, L., and Udenfriend, S. (1993). Biosynthesis of glycosylphosphatidylinositol (GPI)-anchored membrane proteins in intact cells: specific amino acid requirements adjacent to the site of cleavage and GPI attachment. J. Cell Biol. *120*, 657–664.

Kohonen, T. (2001). Self-Organizing Maps (Berlin, Heidelberg: Springer Berlin Heidelberg).

Kolarova, M., García-Sierra, F., Bartos, A., Ricny, J., and Ripova, D. (2012). Structure and Pathology of Tau Protein in Alzheimer Disease. Int. J. Alzheimers Dis. *2012*, 1–13.

Kollár, R., Petráková, E., Ashwell, G., Robbins, P.W., and Cabib, E. (1995). Architecture of the yeast cell wall. The linkage between chitin and beta(1-->3)-glucan. J. Biol. Chem. *270*, 1170–1178.

Kollar, R., Reinhold, B.B., Petrakova, E., Yeh, H.J.C., Ashwell, G., Drgonova, J., Kapteyn, J.C., Klis, F.M., and Cabib, E. (1997). Architecture of the Yeast Cell Wall: (1->6)-GLUCAN INTERCONNECTS MANNOPROTEIN, (1->3)-GLUCAN, AND CHITIN. J. Biol. Chem. *272*, 17762–17775.

Kondo, K., and Inouye, M. (1991). TIP 1, a cold shock-inducible gene of *Saccharomyces cerevisiae*. J. Biol. Chem. *266*, 17537–17544.

Koo, E.H., Lansbury, P.T., and Kelly, J.W. (1999). Amyloid diseases: Abnormal protein aggregation in neurodegeneration. Proc. Natl. Acad. Sci. *96*, 9989–9990.

Kostova, Z., Rancour, D.M., Menon, A.K., and Orlean, P. (2000). Photoaffinity labelling with P3-(4-azidoanilido)uridine 5’-triphosphate identifies gpi3p as the UDP-GlcNAc-binding

subunit of the enzyme that catalyses formation of GlcNAc-phosphatidylinositol, the first glycolipid intermediate in glycosylphosphatidylinositol synthesis. Biochem. J. *350 Pt 3*, 815–822.

Kowalski, L.R., Kondo, K., and Inouye, M. (1995). Cold-shock induction of a family of TIP1-related proteins associated with the membrane in Saccharomyces cerevisiae. Mol. Microbiol. *15*, 341–353.

Krawitz, P., Murakami, Y., Hecht, J., Kruger, U., Holder, S., Mortier, G., Delle Chiaie, B., De Baere, E., Thompson, M., Roscioli, T., et al. (2012). Mutations in PIG-O, a Member of the GPI-Anchor-Synthesis Pathway, Cause Hyperphosphatasia with Mental Retardation. Am. J. Hum. Genet. *91*, 146–151.

Kvarnung, M., Nilsson, D., Lindstrand, A., Korenke, G.C., Chiang, S.C.C., Blennow, E., Bergmann, M., Stodberg, T., Makitie, O., Anderlid, B.-M., et al. (2013). A novel intellectual disability syndrome caused by GPI anchor deficiency due to homozygous mutations in *PIGT*. J. Med. Genet. *50*, 521–528.

Lauck, F., Smith, C.A., Friedland, G.F., Humphris, E.L., and Kortemme, T. (2010). RosettaBackrub--a web server for flexible backbone protein structure modeling and design. Nucleic Acids Res. *38*, W569–W575.

Le Bivic, A., Sambuy, Y., Patzak, A., Patil, N., Chao, M., and Rodriguez-Boulan, E. (1991). An internal deletion in the cytoplasmic tail reverses the apical localization of human NGF receptor in transfected MDCK cells. J. Cell Biol. *115*, 607–618.

Lee, C.H., Marekov, L.N., Kim, S., Brahim, J.S., Park, M.H., and Steinert, P.M. (2000). Small proline-rich protein 1 is the major component of the cell envelope of normal human oral keratinocytes. FEBS Lett. *477*, 268–272.

Leidich, S.D., and Orlean, P. (1996). Gpi1, a Saccharomyces cerevisiae protein that participates in the first step in glycosylphosphatidylinositol anchor synthesis. J. Biol. Chem. *271*, 27829–27837.

Leidich, S.D., Drapp, D.A., and Orlean, P. (1994). A conditionally lethal yeast mutant blocked at the first step in glycosyl phosphatidylinositol anchor synthesis. J. Biol. Chem. *269*, 10193–10196.

Leidich, S.D., Kostova, Z., Latek, R.R., Costello, L.C., Drapp, D.A., Gray, W., Fassler, J.S., and Orlean, P. (1995). Temperature-sensitive Yeast GPI Anchoring Mutants *gpi2* and *gpi3* Are Defective in the Synthesis of *N* -Acetylglucosaminyl Phosphatidylinositol.: CLONING OF THE *GPI2* GENE. J. Biol. Chem. *270*, 13029–13035.

Li, F., and Palecek, S.P. (2003). EAP1, a *Candida albicans* Gene Involved in Binding Human Epithelial Cells. Eukaryot. Cell *2*, 1266–1273.

Li, F., Svarovsky, M.J., Karlsson, A.J., Wagner, J.P., Marchillo, K., Oshel, P., Andes, D., and Palecek, S.P. (2007). Eap1p, an Adhesin That Mediates *Candida albicans* Biofilm Formation *In Vitro* and *In Vivo*. Eukaryot. Cell *6*, 931–939.

Li, M., Martin, S.J., Bruno, V.M., Mitchell, A.P., and Davis, D.A. (2004). *Candida albicans* Rim13p, a Protease Required for Rim101p Processing at Acidic and Alkaline pHs. Eukaryot. Cell *3*, 741–751.

Lin, J., Oh, S.-H., Jones, R., Garnett, J.A., Salgado, P.S., Rusnakova, S., Matthews, S.J., Hoyer, L.L., and Cota, E. (2014). The Peptide-binding Cavity Is Essential for Als3-mediated Adhesion of *Candida albicans* to Human Cells. J. Biol. Chem. *289*, 18401–18412.

Lin, S., Naim, H.Y., Rodriguez, A.C., and Roth, M.G. (1998). Mutations in the middle of the transmembrane domain reverse the polarity of transport of the influenza virus hemagglutinin in MDCK epithelial cells. J. Cell Biol. *142*, 51–57.

Lipardi, C., Nitsch, L., and Zurzolo, C. (2000). Detergent-insoluble GPI-anchored proteins are apically sorted in fischer rat thyroid cells, but interference with cholesterol or sphingolipids differentially affects detergent insolubility and apical sorting. Mol. Biol. Cell *11*, 531–542.

Lipke, P.N., Wojciechowicz, D., and Kurjan, J. (1989). AG alpha 1 is the structural gene for the *Saccharomyces cerevisiae* alpha-agglutinin, a cell surface glycoprotein involved in cell-cell interactions during mating. Mol. Cell. Biol. *9*, 3155–3165.

Lipke, P.N., Garcia, M.C., Alsteens, D., Ramsook, C.B., Klotz, S.A., and Dufrêne, Y.F. (2012). Strengthening relationships: amyloids create adhesion nanodomains in yeasts. Trends Microbiol. *20*, 59–65.

Lipke, P.N., Ramsook, C., Garcia-Sherman, M.C., Jackson, D.N., Chan, C.X.J., Bois, M., and Klotz, S.A. (2014). Between Amyloids and Aggregation Lies a Connection with Strength and Adhesion. New J. Sci. *2014*, 1–12.

Lisanti, M.P., Sargiacomo, M., Graeve, L., Saltiel, A.R., and Rodriguez-Boulan, E. (1988). Polarized apical distribution of glycosyl-phosphatidylinositol-anchored proteins in a renal epithelial cell line. Proc. Natl. Acad. Sci. U. S. A. *85*, 9557–9561.

Lisanti, M.P., Caras, I.W., Davitz, M.A., and Rodriguez-Boulan, E. (1989). A glycophospholipid membrane anchor acts as an apical targeting signal in polarized epithelial cells. J. Cell Biol. *109*, 2145–2156.

Liu, H., Styles, C.A., and Fink, G.R. (1993). Elements of the yeast pheromone response pathway required for filamentous growth of diploids. Science *262*, 1741–1744.

Liu, H., Köhler, J., and Fink, G.R. (1994). Suppression of hyphal formation in *Candida albicans* by mutation of a STE12 homolog. Science *266*, 1723–1726.

Lo, H.J., Köhler, J.R., DiDomenico, B., Loebenberg, D., Cacciapuoti, A., and Fink, G.R. (1997). Nonfilamentous *C. albicans* mutants are avirulent. Cell *90*, 939–949.

Lorenz, M.C., Bender, J.A., and Fink, G.R. (2004). Transcriptional response of *Candida albicans* upon internalization by macrophages. Eukaryot. Cell *3*, 1076–1087.

Low, M.G. (1989). The glycosyl-phosphatidylinositol anchor of membrane proteins. Biochim. Biophys. Acta BBA - Rev. Biomembr. *988*, 427–454.

Low, M.G., and Finean, J.B. (1977). Non-lytic release of acetylcholinesterase from erythrocytes by a phosphatidylinositol-specific phospholipase C. FEBS Lett. *82*, 143–146.

Low, M.G., and Saltiel, A.R. (1988). Structural and functional roles of glycosyl-phosphatidylinositol in membranes. Science *239*, 268–275.

Loza, L., Fu, Y., Ibrahim, A.S., Sheppard, D.C., Filler, S.G., and Edwards, J.E. (2004). Functional analysis of the *Candida albicans ALS1* gene product. Yeast *21*, 473–482.

Lu, C.F., Montijn, R.C., Brown, J.L., Klis, F., Kurjan, J., Bussey, H., and Lipke, P.N. (1995). Glycosyl phosphatidylinositol-dependent cross-linking of alpha-agglutinin and beta 1,6-glucan in the *Saccharomyces cerevisiae* cell wall. J. Cell Biol. *128*, 333–340.

Maeda, Y. (2001). PIG-M transfers the first mannose to glycosylphosphatidylinositol on the lumenal side of the ER. EMBO J. *20*, 250–261.

Maeda, Y., and Kinoshita, T. (2011). Structural remodeling, trafficking and functions of glycosylphosphatidylinositol-anchored proteins. Prog. Lipid Res. *50*, 411–424.

el-Maghrabi, E.A., Dixon, D.M., and Burnett, J.W. (1990). Characterization of Candida albicans epidermolytic proteases and their role in yeast-cell adherence to keratinocytes. Clin. Exp. Dermatol. *15*, 183–191.

Mandelkow, E.-M., and Mandelkow, E. (2012). Biochemistry and Cell Biology of Tau Protein in Neurofibrillary Degeneration. Cold Spring Harb. Perspect. Med. *2*, a006247–a006247.

Mao, Y., Zhang, Z., Gast, C., and Wong, B. (2008). C-Terminal Signals Regulate Targeting of Glycosylphosphatidylinositol-Anchored Proteins to the Cell Wall or Plasma Membrane in *Candida albicans*. Eukaryot. Cell *7*, 1906–1915.

Martínez-Duncker, I., Díaz-Jímenez, D.F., and Mora-Montes, H.M. (2014). Comparative Analysis of Protein Glycosylation Pathways in Humans and the Fungal Pathogen *Candida albicans*. Int. J. Microbiol. *2014*, 1–16.

Martinez-Lopez, R. (2004). The GPI-anchored protein CaEcm33p is required for cell wall integrity, morphogenesis and virulence in *Candida albicans*. Microbiology *150*, 3341–3354.

Massey, S.E. (2003). Comparative Evolutionary Genomics Unveils the Molecular Mechanism of Reassignment of the CTG Codon in Candida spp. Genome Res. *13*, 544–557.

Massoulie, J. (2002). The Origin of the Molecular Diversity and Functional Anchoring of Cholinesterases. Neurosignals *11*, 130–143.

Masterson, W.J., Doering, T.L., Hart, G.W., and Englund, P.T. (1989). A novel pathway for glycan assembly: biosynthesis of the glycosyl-phosphatidylinositol anchor of the trypanosome variant surface glycoprotein. Cell *56*, 793–800.

Maxwell, S.E., Ramalingam, S., Gerber, L.D., and Udenfriend, S. (1995). Cleavage without anchor addition accompanies the processing of a nascent protein to its glycosylphosphatidylinositol-anchored form. Proc. Natl. Acad. Sci. U. S. A. *92*, 1550–1554.

Maydan, G., Noyman, I., Har-Zahav, A., Neriah, Z.B., Pasmanik-Chor, M., Yeheskel, A., Albin-Kaplanski, A., Maya, I., Magal, N., Birk, E., et al. (2011). Multiple congenital anomalies-hypotonia-seizures syndrome is caused by a mutation in PIGN. J. Med. Genet. *48*, 383–389.

Mayer, F.L., Wilson, D., and Hube, B. (2013). *Candida albicans* pathogenicity mechanisms. Virulence *4*, 119–128.

Mayor, S., and Riezman, H. (2004). Sorting GPI-anchored proteins. Nat. Rev. Mol. Cell Biol. *5*, 110–120.

Mayor, S., Menon, A.K., and Cross, G.A. (1991). Transfer of glycosyl-phosphatidylinositol membrane anchors to polypeptide acceptors in a cell-free system. J. Cell Biol. *114*, 61–71.

Medina-Acosta, E., Karess, R.E., Schwartz, H., and Russell, D.G. (1989). The promastigote surface protease (gp63) of *Leishmania* is expressed but differentially processed and localized in the amastigote stage. Mol. Biochem. Parasitol. *37*, 263–273.

Menon, A.K., and Stevens, V.L. (1992). Phosphatidylethanolamine is the donor of the ethanolamine residue linking a glycosylphosphatidylinositol anchor to protein. J. Biol. Chem. *267*, 15277–15280.

Meyer, U., Benghezal, M., Imhof, I., and Conzelmann, A. (2000). Active Site Determination of Gpi8p, a Caspase-Related Enzyme Required for Glycosylphosphatidylinositol Anchor Addition to Proteins †. Biochemistry (Mosc.) *39*, 3461–3471.

Micanovic, R., Gerber, L.D., Berger, J., Kodukula, K., and Udenfriend, S. (1990). Selectivity of the cleavage/attachment site of phosphatidylinositol-glycan-anchored membrane proteins determined by site-specific mutagenesis at Asp-484 of placental alkaline phosphatase. Proc. Natl. Acad. Sci. U. S. A. *87*, 157–161.

Millan, J.L. (2006). Alkaline Phosphatases: Structure, substrate specificity and functional relatedness to other members of a large superfamily of enzymes. Purinergic Signal. *2*, 335–341.

Miranda, I., Silva, R., and Santos, M.A.S. (2006). Evolution of the genetic code in yeasts. Yeast *23*, 203–213.

Miranda, K.C., Khromykh, T., Christy, P., Le, T.L., Gottardi, C.J., Yap, A.S., Stow, J.L., and Teasdale, R.D. (2001). A Dileucine Motif Targets E-cadherin to the Basolateral Cell Surface in Madin-Darby Canine Kidney and LLC-PK1 Epithelial Cells. J. Biol. Chem. *276*, 22565–22572.

Miyata, T., Takeda, J., Iida, Y., Yamada, N., Inoue, N., Takahashi, M., Maeda, K., Kitani, T., and Kinoshita, T. (1993). The cloning of PIG-A, a component in the early step of GPI-anchor biosynthesis. Science *259*, 1318–1320.

Monniot, C., Boisramé, A., Da Costa, G., Chauvel, M., Sautour, M., Bougnoux, M.-E., Bellon-Fontaine, M.-N., Dalle, F., d'Enfert, C., and Richard, M.L. (2013). Rbt1 Protein Domains Analysis in Candida albicans Brings Insights into Hyphal Surface Modifications and Rbt1 Potential Role during Adhesion and Biofilm Formation. PLoS ONE *8*, e82395.

Mora-Montes, H.M., Ponce-Noyola, P., Villagómez-Castro, J.C., Gow, N.A., Flores-Carreón, A., and López-Romero, E. (2009). Protein glycosylation in *Candida*. Future Microbiol. *4*, 1167–1183.

Moran, P., and Caras, I.W. (1991). Fusion of sequence elements from non-anchored proteins to generate a fully functional signal for glycophosphatidylinositol membrane anchor attachment. J. Cell Biol. *115*, 1595–1600.

Moran, P., Raab, H., Kohr, W.J., and Caras, I.W. (1991). Glycophospholipid membrane anchor attachment. Molecular analysis of the cleavage/attachment site. J. Biol. Chem. *266*, 1250–1257.

Moukadiri, I., Armero, J., Abad, A., Sentandreu, R., and Zueco, J. (1997). Identification of a mannoprotein present in the inner layer of the cell wall of *Saccharomyces cerevisiae*. J. Bacteriol. *179*, 2154–2162.

Mühlschlegel, F.A., and Fonzi, W.A. (1997). PHR2 of *Candida albicans* encodes a functional homolog of the pH-regulated gene *PHR1* with an inverted pattern of pH-dependent expression. Mol. Cell. Biol. *17*, 5960–5967.

Muñiz, M., and Zurzolo, C. (2014). Sorting of GPI-anchored proteins from yeast to mammals--common pathways at different sites? J. Cell Sci. *127*, 2793–2801.

Munro, C.A., Bates, S., Buurman, E.T., Hughes, H.B., MacCallum, D.M., Bertram, G., Atrih, A., Ferguson, M.A.J., Bain, J.M., Brand, A., et al. (2005). Mnt1p and Mnt2p of Candida albicans Are Partially Redundant -1,2-Mannosyltransferases That Participate in O-Linked Mannosylation and Are Required for Adhesion and Virulence. J. Biol. Chem. *280*, 1051–1060.

Murakami, Y., Siripanyapinyo, U., Hong, Y., Kang, J.Y., Ishihara, S., Nakakuma, H., Maeda, Y., and Kinoshita, T. (2003). PIG-W is critical for inositol acylation but not for flipping of glycosylphosphatidylinositol-anchor. Mol. Biol. Cell *14*, 4285–4295.

Murciano, C., Moyes, D.L., Runglall, M., Tobouti, P., Islam, A., Hoyer, L.L., and Naglik, J.R. (2012). Evaluation of the Role of *Candida albicans* Agglutinin-Like Sequence (Als) Proteins in Human Oral Epithelial Cell Interactions. PLoS ONE *7*, e33362.

Nadeem, S.G., Shafiq, A., Hakim, S.T., Anjum, Y., and U. Kazm, S. (2013). Effect of Growth Media, pH and Temperature on Yeast to Hyphal Transition in <i>*Candida albicans*</i> Open J. Med. Microbiol. *03*, 185–192.

Nagamune, K., Ohishi, K., Ashida, H., Hong, Y., Hino, J., Kangawa, K., Inoue, N., Maeda, Y., and Kinoshita, T. (2003). GPI transamidase of *Trypanosoma brucei* has two previously uncharacterized (trypanosomatid transamidase 1 and 2) and three common subunits. Proc. Natl. Acad. Sci. U. S. A. *100*, 10682–10687.

Naglik, J.R., Rodgers, C.A., Shirlaw, P.J., Dobbie, J.L., Fernandes-Naglik, L.L., Greenspan, D., Agabian, N., and Challacombe, S.J. (2003). Differential expression of *Candida albicans* secreted aspartyl proteinase and phospholipase B genes in humans correlates with active oral and vaginal infections. J. Infect. Dis. *188*, 469–479.

Nakamura, N., Inoue, N., Watanabe, R., Takahashi, M., Takeda, J., Stevens, V.L., and Kinoshita, T. (1997). Expression cloning of PIG-L, a candidate *N*-acetylglucosaminyl-phosphatidylinositol deacetylase. J. Biol. Chem. *272*, 15834–15840.

Nett, J.E., Lepak, A.J., Marchillo, K., and Andes, D.R. (2009). Time Course Global Gene Expression Analysis of an *In Vivo Candida* Biofilm. J. Infect. Dis. *200*, 307–313.

Ng, B.G., Hackmann, K., Jones, M.A., Eroshkin, A.M., He, P., Wiliams, R., Bhide, S., Cantagrel, V., Gleeson, J.G., Paller, A.S., et al. (2012). Mutations in the Glycosylphosphatidylinositol Gene *PIGL* Cause CHIME Syndrome. Am. J. Hum. Genet. *90*, 685–688.

Nobile, C.J., and Mitchell, A.P. (2005). Regulation of Cell-Surface Genes and Biofilm Formation by the C. albicans Transcription Factor Bcr1p. Curr. Biol. *15*, 1150–1155.

Nobile, C.J., Nett, J.E., Andes, D.R., and Mitchell, A.P. (2006). Function of *Candida albicans* Adhesin Hwp1 in Biofilm Formation. Eukaryot. Cell *5*, 1604–1610.

Nobile, C.J., Schneider, H.A., Nett, J.E., Sheppard, D.C., Filler, S.G., Andes, D.R., and Mitchell, A.P. (2008). Complementary Adhesin Function in C. albicans Biofilm Formation. Curr. Biol. *18*, 1017–1024.

Noel, T. (2012). The cellular and molecular defense mechanisms of the *Candida* yeasts against azole antifungal drugs. J. Mycol. Mdicale J. Med. Mycol. *22*, 173–178.

Nosjean, O., Briolay, A., and Roux, B. (1997). Mammalian GPI proteins: sorting, membrane residence and functions. Biochim. Biophys. Acta *1331*, 153–186.

Nozaki, M., Ohishi, K., Yamada, N., Kinoshita, T., Nagy, A., and Takeda, J. (1999). Developmental abnormalities of glycosylphosphatidylinositol-anchor-deficient embryos revealed by Cre/loxP system. Lab. Investig. J. Tech. Methods Pathol. *79*, 293–299.

Nuoffer, C., Horvath, A., and Riezman, H. (1993). Analysis of the sequence requirements for glycosylphosphatidylinositol anchoring of Saccharomyces cerevisiae Gas1 protein. J. Biol. Chem. *268*, 10558–10563.

Oberoi, J.K., Wattal, C., Goel, N., Raveendran, R., Datta, S., and Prasad, K. (2012). Non-*albicans Candida* species in blood stream infections in a tertiary care hospital at New Delhi, India. Indian J. Med. Res. *136*, 997–1003.

Odds, F.C. (1993). Resistance of yeasts to azole-derivative antifungals. J. Antimicrob. Chemother. *31*, 463–471.

Odds, F.C., Webster, C.E., Mayuranathan, P., and Simmons, P.D. (1988). *Candida* concentrations in the vagina and their association with signs and symptoms of vaginal candidosis. Med. Mycol. *26*, 277–283.

Ohama, T., Suzuki, T., Mori, M., Osawa, S., Ueda, T., Watanabe, K., and Nakase, T. (1993). Non-universal decoding of the leucine codon CUG in several Candida species. Nucleic Acids Res. *21*, 4039–4045.

Ohba, C., Okamoto, N., Murakami, Y., Suzuki, Y., Tsurusaki, Y., Nakashima, M., Miyake, N., Tanaka, F., Kinoshita, T., Matsumoto, N., et al. (2014). PIGN mutations cause congenital anomalies, developmental delay, hypotonia, epilepsy, and progressive cerebellar atrophy. Neurogenetics *15*, 85–92.

Ohishi, K., Inoue, N., Maeda, Y., Takeda, J., Riezman, H., and Kinoshita, T. (2000). Gaa1p and gpi8p are components of a glycosylphosphatidylinositol (GPI) transamidase that mediates attachment of GPI to proteins. Mol. Biol. Cell *11*, 1523–1533.

Ohishi, K., Nagamune, K., Maeda, Y., and Kinoshita, T. (2003). Two Subunits of Glycosylphosphatidylinositol Transamidase, *GPI8* and PIG-T, Form a Functionally Important Intermolecular Disulfide Bridge. J. Biol. Chem. *278*, 13959–13967.

Ollert, M.W., Söhnchen, R., Korting, H.C., Ollert, U., Bräutigam, S., and Bräutigam, W. (1993). Mechanisms of adherence of Candida albicans to cultured human epidermal keratinocytes. Infect. Immun. *61*, 4560–4568.

Omar, A.A. (2001). Gram stain versus culture in the diagnosis of vulvovaginal candidiasis. East. Mediterr. Health J. Rev. Sante Mediterr. Orient. Al-Majallah Al-Sihhiyah Li-Sharq Al-Mutawassit *7*, 925–934.

Orlean, P., and Menon, A.K. (2007). Thematic review series: lipid posttranslational modifications. GPI anchoring of protein in yeast and mammalian cells, or: how we learned to stop worrying and love glycophospholipids. J. Lipid Res. *48*, 993–1011.

Otoo, H.N., Lee, K.G., Qiu, W., and Lipke, P.N. (2008). *Candida albicans* Als Adhesins Have Conserved Amyloid-Forming Sequences. Eukaryot. Cell *7*, 776–782.

Paladino, S., Sarnataro, D., Pillich, R., Tivodar, S., Nitsch, L., and Zurzolo, C. (2004). Protein oligomerization modulates raft partitioning and apical sorting of GPI-anchored proteins. J. Cell Biol. *167*, 699–709.

Paladino, S., Pocard, T., Catino, M.A., and Zurzolo, C. (2006). GPI-anchored proteins are directly targeted to the apical surface in fully polarized MDCK cells. J. Cell Biol. *172*, 1023–1034.

Paladino, S., Sarnataro, D., Tivodar, S., and Zurzolo, C. (2007). Oligomerization Is a Specific Requirement for Apical Sorting of Glycosyl-Phosphatidylinositol-Anchored Proteins but Not for Non-Raft-Associated Apical Proteins. Traffic *8*, 251–258.

Paladino, S., Lebreton, S., Tivodar, S., Formiggini, F., Ossato, G., Gratton, E., Tramier, M., Coppey-Moisan, M., and Zurzolo, C. (2014). Golgi sorting regulates organization and activity of GPI proteins at apical membranes. Nat. Chem. Biol. *10*, 350–357.

Paulick, M.G., and Bertozzi, C.R. (2008). The glycosylphosphatidylinositol anchor: a complex membrane-anchoring structure for proteins. Biochemistry (Mosc.) *47*, 6991–7000.

Pfaller, M.A., and Diekema, D.J. (2010). Epidemiology of Invasive Mycoses in North America. Crit. Rev. Microbiol. *36*, 1–53.

Phan, Q.T., Myers, C.L., Fu, Y., Sheppard, D.C., Yeaman, M.R., Welch, W.H., Ibrahim, A.S., Edwards, J.E., and Filler, S.G. (2007). Als3 Is a *Candida albicans* Invasin That Binds to Cadherins and Induces Endocytosis by Host Cells. PLoS Biol. *5*, e64.

Pike, L.J. (2003). Lipid rafts: bringing order to chaos. J. Lipid Res. *44*, 655–667.

Pomorski, T., and Menon, A.K. (2006). Lipid flippases and their biological functions. Cell. Mol. Life Sci. *63*, 2908–2921.

Pottekat, A., and Menon, A.K. (2004). Subcellular Localization and Targeting of N-Acetylglucosaminyl Phosphatidylinositol De-*N*-acetylase, the Second Enzyme in the Glycosylphosphatidylinositol Biosynthetic Pathway. J. Biol. Chem. *279*, 15743–15751.

Powderly, W.G., Gallant, J.E., Ghannoum, M.A., Mayer, K.H., Navarro, E.E., and Perfect, A.J.R. (1999). Clinical Perspective Oropharyngeal Candidiasis in Patients with HIV: Suggested Guidelines for Therapy. AIDS Res. Hum. Retroviruses *15*, 1619–1623.

Prasad, J.H., Abraham, S., Kurz, K.M., George, V., Lalitha, M.K., John, R., Jayapaul, M.N.R., Shetty, N., and Joseph, A. (2005). Reproductive tract infections among young married women in Tamil Nadu, India. Int. Fam. Plan. Perspect. *31*, 73–82.

Prasad, R.R., Shree, V., Sagar, S., Kumar, S., and Kumar, P. (2016). Prevalence and Antifungal Susceptibility of *Candida albicans* in Patna, India. Int. J. Curr. Microbiol. Appl. Sci. *5*, 957–961.

Prill, S.K.-H., Klinkert, B., Timpel, C., Gale, C.A., Schröppel, K., and Ernst, J.F. (2004). *PMT* family of *Candida albicans*: five protein mannosyltransferase isoforms affect growth, morphogenesis and antifungal resistance: Pmt family. Mol. Microbiol. *55*, 546–560.

Ramsook, C.B., Tan, C., Garcia, M.C., Fung, R., Soybelman, G., Henry, R., Litewka, A., O'Meally, S., Otoo, H.N., Khalaf, R.A., et al. (2010). Yeast cell adhesion molecules have functional amyloid-forming sequences. Eukaryot. Cell *9*, 393–404.

Rauceo, J.M., Gaur, N.K., Lee, K.-G., Edwards, J.E., Klotz, S.A., and Lipke, P.N. (2004). Global Cell Surface Conformational Shift Mediated by a *Candida albicans* Adhesin. Infect. Immun. *72*, 4948–4955.

Rauceo, J.M., De Armond, R., Otoo, H., Kahn, P.C., Klotz, S.A., Gaur, N.K., and Lipke, P.N. (2006). Threonine-Rich Repeats Increase Fibronectin Binding in the *Candida albicans* Adhesin Als5p. Eukaryot. Cell *5*, 1664–1673.

Richard, M.L., and Plaine, A. (2007). Comprehensive Analysis of Glycosylphosphatidylinositol-Anchored Proteins in *Candida albicans*. Eukaryot. Cell *6*, 119–133.

Roberts, W.L., and Rosenberry, T.L. (1985). Identification of covalently attached fatty acids in the hydrophobic membrane-binding domain of human erythrocyte acetylcholinesterase. Biochem. Biophys. Res. Commun. *133*, 621–627.

Roberts, W.L., Santikarn, S., Reinhold, V.N., and Rosenberry, T.L. (1988). Structural characterization of the glycoinositol phospholipid membrane anchor of human erythrocyte

acetylcholinesterase by fast atom bombardment mass spectrometry. J. Biol. Chem. *263*, 18776–18784.

Rouabhia, M., Schaller, M., Corbucci, C., Vecchiarelli, A., Prill, S.K.-H., Giasson, L., and Ernst, J.F. (2005). Virulence of the Fungal Pathogen *Candida albicans* Requires the Five Isoforms of Protein Mannosyltransferases. Infect. Immun. *73*, 4571–4580.

Roudbarmohammadi, S., Roudbary, M., Bakhshi, B., Katiraee, F., Mohammadi, R., and Falahati, M. (2016). *ALS1* and *ALS3* gene expression and biofilm formation in *Candida albicans* isolated from vulvovaginal candidiasis. Adv. Biomed. Res. *5*, 105.

Roy, A., Lu, C.F., Marykwas, D.L., Lipke, P.N., and Kurjan, J. (1991). The *AGA1* product is involved in cell surface attachment of the *Saccharomyces cerevisiae* cell adhesion glycoprotein a-agglutinin. Mol. Cell. Biol. *11*, 4196–4206.

Saha, S., Anilkumar, A.A., and Mayor, S. (2015). GPI-anchored protein organization and dynamics at the cell surface. J. Lipid Res. jlr.R062885.

Salgado, P.S., Yan, R., Taylor, J.D., Burchell, L., Jones, R., Hoyer, L.L., Matthews, S.J., Simpson, P.J., and Cota, E. (2011). Structural basis for the broad specificity to host-cell ligands by the pathogenic fungus *Candida albicans*. Proc. Natl. Acad. Sci. *108*, 15775–15779.

Sambrook, J., and Russell, D.W. (2001). Molecular cloning: a laboratory manual (Cold Spring Harbor, N.Y: Cold Spring Harbor Laboratory Press).

Sangiorgio, V., Pitto, M., Palestini, P., and Masserini, M. (2004). GPI-anchored proteins and lipid rafts. Ital. J. Biochem. *53*, 98–111.

Santos, M.A., and Tuite, M.F. (1995). The CUG codon is decoded in vivo as serine and not leucine in *Candida albicans*. Nucleic Acids Res. *23*, 1481–1486.

Saporito-Irwin, S.M., Birse, C.E., Sypherd, P.S., and Fonzi, W.A. (1995). *PHR1*, a pH-regulated gene of *Candida albicans*, is required for morphogenesis. Mol. Cell. Biol. *15*, 601–613.

Schneider, P., Ferguson, M.A., McConville, M.J., Mehlert, A., Homans, S.W., and Bordier, C. (1990). Structure of the glycosyl-phosphatidylinositol membrane anchor of the *Leishmania* major promastigote surface protease. J. Biol. Chem. *265*, 16955–16964.

Shapiro, R.S., Uppuluri, P., Zaas, A.K., Collins, C., Senn, H., Perfect, J.R., Heitman, J., and Cowen, L.E. (2009). Hsp90 Orchestrates Temperature-Dependent *Candida albicans* Morphogenesis via Ras1-PKA Signaling. Curr. Biol. *19*, 621–629.

Sharma, D.K., Vidugiriene, J., Bangs, J.D., and Menon, A.K. (1999). A cell-free assay for glycosylphosphatidylinositol anchoring in African trypanosomes. Demonstration of a transamidation reaction mechanism. J. Biol. Chem. *274*, 16479–16486.

Shen, H., Chen, S.M., Liu, W., Zhu, F., He, L.J., Zhang, J.D., Zhang, S.Q., Yan, L., Xu, Z., Xu, G.T., et al. (2015). Abolishing Cell Wall Glycosylphosphatidylinositol-Anchored Proteins in *Candida albicans* Enhances Recognition by Host Dectin-1. Infect. Immun. *83*, 2694–2704.

Shenoy-Scaria, A.M., Kwong, J., Fujita, T., Olszowy, M.W., Shaw, A.S., and Lublin, D.M. (1992). Signal transduction through decay-accelerating factor. Interaction of glycosyl-phosphatidylinositol anchor and protein tyrosine kinases p56lck and p59fyn 1. J. Immunol. Baltim. Md 1950 *149*, 3535–3541.

Sheppard, D.C., Yeaman, M.R., Welch, W.H., Phan, Q.T., Fu, Y., Ibrahim, A.S., Filler, S.G., Zhang, M., Waring, A.J., and Edwards, J.E. (2004). Functional and Structural Diversity in the Als Protein Family of *Candida albicans*. J. Biol. Chem. *279*, 30480–30489.

Shimoi, H., Iimura, Y., and Obata, T. (1995). Molecular Cloning of CWP1: A Gene Encoding a *Saccharomyces cerevisiae* Cell Wall Protein Solubilized with Rarobacter faecitabidus Protease I1. J. Biochem. (Tokyo) *118*, 302–311.

Shimoi, H., Kitagaki, H., Ohmori, H., Iimura, Y., and Ito, K. (1998). Sed1p is a major cell wall protein of *Saccharomyces cerevisiae* in the stationary phase and is involved in lytic enzyme resistance. J. Bacteriol. *180*, 3381–3387.

Shishioh, N., Hong, Y., Ohishi, K., Ashida, H., Maeda, Y., and Kinoshita, T. (2005). GPI7 Is the Second Partner of PIG-F and Involved in Modification of Glycosylphosphatidylinositol. J. Biol. Chem. *280*, 9728–9734.

Silva, R.M., Paredes, J.A., Moura, G.R., Manadas, B., Lima-Costa, T., Rocha, R., Miranda, I., Gomes, A.C., Koerkamp, M.J.G., Perrot, M., et al. (2007). Critical roles for a genetic code alteration in the evolution of the genus Candida. EMBO J. *26*, 4555–4565.

Silverman, R.J., Nobbs, A.H., Vickerman, M.M., Barbour, M.E., and Jenkinson, H.F. (2010). Interaction of Candida albicans Cell Wall Als3 Protein with *Streptococcus gordonii* SspB Adhesin Promotes Development of Mixed-Species Communities. Infect. Immun. *78*, 4644–4652.

Simons, K., and Ikonen, E. (1997). Functional rafts in cell membranes. Nature *387*, 569–572.

Singh, S.L., Rai, R.C., Sah, S.K., and Komath, S.S. (2016). The catalytic subunit of the first mannosyltransferase in the GPI biosynthetic pathway affects growth, cell wall integrity and hyphal morphogenesis in *Candida albicans*: *CaGPI14* alters growth, morphogenesis and wall integrity of *C. albicans*. Yeast *33*, 365–383.

Sipos, G., Reggiori, F., Vionnet, C., and Conzelmann, A. (1997). Alternative lipid remodelling pathways for glycosylphosphatidylinositol membrane anchors in Saccharomyces cerevisiae. EMBO J. *16*, 3494–3505.

Smith, T.K. (2001). Specificity of GlcNAc-PI de-N-acetylase of GPI biosynthesis and synthesis of parasite-specific suicide substrate inhibitors. EMBO J. *20*, 3322–3332.

Smith, T.K., Sharma, D.K., Crossman, A., Brimacombe, J.S., and Ferguson, M.A. (1999). Selective inhibitors of the glycosylphosphatidylinositol biosynthetic pathway of *Trypanosoma brucei*. EMBO J. *18*, 5922–5930.

Sobel, J.D. (2007). Vulvovaginal candidosis. The Lancet *369*, 1961–1971.

Sobel, J.D., Faro, S., Force, R.W., Foxman, B., Ledger, W.J., Nyirjesy, P.R., Reed, B.D., and Summers, P.R. (1998). Vulvovaginal candidiasis: epidemiologic, diagnostic, and therapeutic considerations. Am. J. Obstet. Gynecol. *178*, 203–211.

Sobering, A.K., Watanabe, R., Romeo, M.J., Yan, B.C., Specht, C.A., Orlean, P., Riezman, H., and Levin, D.E. (2004). Yeast Ras Regulates the Complex that Catalyzes the First Step in GPI-Anchor Biosynthesis at the ER. Cell *117*, 637–648.

Spampinato, C., and Leonardi, D. (2013). *Candida* Infections, Causes, Targets, and Resistance Mechanisms: Traditional and Alternative Antifungal Agents. BioMed Res. Int. *2013*, 1–13.

Spellberg, B.J., Ibrahim, A.S., Avenissian, V., Filler, S.G., Myers, C.L., Fu, Y., and Edwards, J.E. (2005). The Anti-*Candida albicans* Vaccine Composed of the Recombinant N Terminus of Als1p Reduces Fungal Burden and Improves Survival in Both Immunocompetent and Immunocompromised Mice. Infect. Immun. *73*, 6191–6193.

Spellberg, B.J., Ibrahim, A.S., Avanesian, V., Fu, Y., Myers, C., Phan, Q.T., Filler, S.G., Yeaman, M.R., and Edwards, Jr., J.E. (2006). Efficacy of the Anti- *Candida* rAls3p-N or rAls1p-N Vaccines against Disseminated and Mucosal Candidiasis. J. Infect. Dis. *194*, 256–260.

Staab, J.F., Bradway, S.D., Fidel, P.L., and Sundstrom, P. (1999). Adhesive and mammalian transglutaminase substrate properties of Candida albicans Hwp1. Science *283*, 1535–1538.

Staab, J.F., Bahn, Y.-S., Tai, C.-H., Cook, P.F., and Sundstrom, P. (2004). Expression of Transglutaminase Substrate Activity on *Candida albicans* Germ Tubes through a Coiled, Disulfide-bonded N-terminal Domain of Hwp1 Requires C-terminal Glycosylphosphatidylinositol Modification. J. Biol. Chem. *279*, 40737–40747.

Stefanová, I., Horejsí, V., Ansotegui, I.J., Knapp, W., and Stockinger, H. (1991). GPI-anchored cell-surface molecules complexed to protein tyrosine kinases. Science *254*, 1016–1019.

Stehr, F., Felk, A., Kretschmar, M., Schaller, M., Schäfer, W., and Hube, B. (2000). [Extracellular hydrolytic enzymes and their relevance during *Candida albicans* infections]. Mycoses *43 Suppl 2*, 17–21.

Stoldt, V.R., Sonneborn, A., Leuker, C.E., and Ernst, J.F. (1997). Efg1p, an essential regulator of morphogenesis of the human pathogen *Candida albicans*, is a member of a conserved class of bHLH proteins regulating morphogenetic processes in fungi. EMBO J. *16*, 1982–1991.

Stoops, E.H., and Caplan, M.J. (2014). Trafficking to the Apical and Basolateral Membranes in Polarized Epithelial Cells. J. Am. Soc. Nephrol. *25*, 1375–1386.

Sudbery, P., Gow, N., and Berman, J. (2004). The distinct morphogenic states of *Candida albicans*. Trends Microbiol. *12*, 317–324.

Sun, N., Li, D., Fonzi, W., Li, X., Zhang, L., and Calderone, R. (2013). Multidrug-Resistant Transporter Mdr1p-Mediated Uptake of a Novel Antifungal Compound. Antimicrob. Agents Chemother. *57*, 5931–5939.

Sundstrom, P. (1999). Adhesins in Candida albicans. Curr. Opin. Microbiol. *2*, 353–357.

Sütterlin, C., Escribano, M.V., Gerold, P., Maeda, Y., Mazon, M.J., Kinoshita, T., Schwarz, R.T., and Riezman, H. (1998). *Saccharomyces cerevisiae GPI10*, the functional homologue of human PIG-B, is required for glycosylphosphatidylinositol-anchor synthesis. Biochem. J. *332 (Pt 1)*, 153–159.

Takahashi, M., Inoue, N., Ohishi, K., Maeda, Y., Nakamura, N., Endo, Y., Fujita, T., Takeda, J., and Kinoshita, T. (1996). PIG-B, a membrane protein of the endoplasmic reticulum with a large lumenal domain, is involved in transferring the third mannose of the GPI anchor. EMBO J. *15*, 4254–4261.

Tanaka, S., Maeda, Y., Tashima, Y., and Kinoshita, T. (2004). Inositol deacylation of glycosylphosphatidylinositol-anchored proteins is mediated by mammalian PGAP1 and yeast Bst1p. J. Biol. Chem. *279*, 14256–14263.

Tang, X., Nakata, Y., Li, H.O., Zhang, M., Gao, H., Fujita, A., Sakatsume, O., Ohta, T., and Yokoyama, K. (1994). The optimization of preparations of competent cells for transformation of *E. coli*. Nucleic Acids Res. *22*, 2857–2858.

Taron, B.W., Colussi, P.A., Wiedman, J.M., Orlean, P., and Taron, C.H. (2004). Human Smp3p Adds a Fourth Mannose to Yeast and Human Glycosylphosphatidylinositol Precursors in Vivo. J. Biol. Chem. *279*, 36083–36092.

Taron, C.H., Wiedman, J.M., Grimme, S.J., and Orlean, P. (2000). Glycosylphosphatidylinositol biosynthesis defects in Gpi11p- and Gpi13p-deficient yeast suggest a branched pathway and implicate gpi13p in phosphoethanolamine transfer to the third mannose. Mol. Biol. Cell *11*, 1611–1630.

Tashima, Y., Taguchi, R., Murata, C., Ashida, H., Kinoshita, T., and Maeda, Y. (2006). PGAP2 is essential for correct processing and stable expression of GPI-anchored proteins. Mol. Biol. Cell *17*, 1410–1420.

Taylor, D.R., and Hooper, N.M. (2006). The prion protein and lipid rafts (Review). Mol. Membr. Biol. *23*, 89–99.

Taylor, D.R., and Hooper, N.M. (2011). GPI-Anchored Proteins in Health and Disease. In Post-Translational Modifications in Health and Disease, C.J. Vidal, ed. (New York, NY: Springer New York), pp. 39–55.

Timpel, C., Zink, S., Strahl-Bolsinger, S., Schroppel, K., and Ernst, J. (2000). Morphogenesis, Adhesive Properties, and Antifungal Resistance Depend on the Pmt6 Protein Mannosyltransferase in the Fungal Pathogen *Candida albicans*. J. Bacteriol. *182*, 3063–3071.

Tse, A.G., Barclay, A.N., Watts, A., and Williams, A.F. (1985). A glycophospholipid tail at the carboxyl terminus of the Thy-1 glycoprotein of neurons and thymocytes. Science *230*, 1003–1008.

Umemura, M., Okamoto, M., Nakayama, K., Sagane, K., Tsukahara, K., Hata, K., and Jigami, Y. (2003). *GWT1* gene is required for inositol acylation of glycosylphosphatidylinositol anchors in yeast. J. Biol. Chem. *278*, 23639–23647.

Urbaniak, M.D., Yashunsky, D.V., Crossman, A., Nikolaev, A.V., and Ferguson, M.A.J. (2008). Probing Enzymes Late in the Trypanosomal Glycosylphosphatidylinositol Biosynthetic Pathway with Synthetic Glycosylphosphatidylinositol Analogues. ACS Chem. Biol. *3*, 625–634.

Urbaniak, M.D., Capes, A.S., Crossman, A., O'Neill, S., Thompson, S., Gilbert, I.H., and Ferguson, M.A.J. (2014). Fragment screening reveals salicylic hydroxamic acid as an inhibitor of *Trypanosoma brucei* GPI GlcNAc-PI de-*N*-acetylase. Carbohydr. Res. *387*, 54–58.

Uversky, V.N. (2002). Natively unfolded proteins: A point where biology waits for physics. Protein Sci. *11*, 739–756.

van der Vaart, J.M., Caro, L.H., Chapman, J.W., Klis, F.M., and Verrips, C.T. (1995). Identification of three mannoproteins in the cell wall of *Saccharomyces cerevisiae*. J. Bacteriol. *177*, 3104–3110.

Vainauskas, S. (2003). A Conserved Proline in the Last Transmembrane Segment of Gaa1 Is Required for Glycosylphosphatidylinositol (GPI) Recognition by GPI Transamidase. J. Biol. Chem. *279*, 6540–6545.

Vainauskas, S., and Menon, A.K. (2006). Ethanolamine Phosphate Linked to the First Mannose Residue of Glycosylphosphatidylinositol (GPI) Lipids Is a Major Feature of the GPI Structure That Is Recognized by Human GPI Transamidase. J. Biol. Chem. *281*, 38358–38364.

Vashist, S., Kim, W., Belden, W.J., Spear, E.D., Barlowe, C., and Ng, D.T.W. (2001). Distinct retrieval and retention mechanisms are required for the quality control of endoplasmic reticulum protein folding. J. Cell Biol. *155*, 355–368.

Verma, A.K., Prasad, K.N., Singh, M., Dixit, A.K., and Ayyagari, A. (2003). Candidaemia in patients of a tertiary health care hospital from north India. Indian J. Med. Res. *117*, 122–128.

Vermes, A. (2000). Flucytosine: an exploration of pharmacokinetics and toxicity (Amsterdam: Thela Thesis).

Victoria, G.S., Yadav, B., Hauhnar, L., Jain, P., Bhatnagar, S., and Komath, S.S. (2012). Mutual co-regulation between GPI-*N*-acetylglucosaminyltransferase and ergosterol biosynthesis in *Candida albicans*. Biochem. J. *443*, 619–625.

Vishwakarma, R.A., and Menon, A.K. (2005). Flip-flop of glycosylphosphatidylinositols (GPI's) across the ER. Chem. Commun. 453.

Wächtler, B., Citiulo, F., Jablonowski, N., Förster, S., Dalle, F., Schaller, M., Wilson, D., and Hube, B. (2012). *Candida albicans*-Epithelial Interactions: Dissecting the Roles of Active Penetration, Induced Endocytosis and Host Factors on the Infection Process. PLoS ONE *7*, e36952.

Wandinger-Ness, A., Bennett, M.K., Antony, C., and Simons, K. (1990). Distinct transport vesicles mediate the delivery of plasma membrane proteins to the apical and basolateral domains of MDCK cells. J. Cell Biol. *111*, 987–1000.

Watanabe, R. (2000). Initial enzyme for glycosylphosphatidylinositol biosynthesis requires PIG-P and is regulated by DPM2. EMBO J. *19*, 4402–4411.

Watanabe, N. -a., Miyazaki, M., Horii, T., Sagane, K., Tsukahara, K., and Hata, K. (2012). E1210, a New Broad-Spectrum Antifungal, Suppresses *Candida albicans* Hyphal Growth through Inhibition of Glycosylphosphatidylinositol Biosynthesis. Antimicrob. Agents Chemother. *56*, 960–971.

Watanabe, R., Inoue, N., Westfall, B., Taron, C.H., Orlean, P., Takeda, J., and Kinoshita, T. (1998). The first step of glycosylphosphatidylinositol biosynthesis is mediated by a complex of PIG-A, PIG-H, PIG-C and GPI1. EMBO J. *17*, 877–885.

Watanabe, R., Ohishi, K., Maeda, Y., Nakamura, N., and Kinoshita, T. (1999). Mammalian PIG-L and its yeast homologue Gpi12p are N-acetylglucosaminylphosphatidylinositol de-*N*-acetylases essential in glycosylphosphatidylinositol biosynthesis. Biochem. J. *339 (Pt 1)*, 185–192.

Weihofen, A., Lemberg, M.K., Ploegh, H.L., Bogyo, M., and Martoglio, B. (2000). Release of Signal Peptide Fragments into the Cytosol Requires Cleavage in the Transmembrane Region by a Protease Activity That Is Specifically Blocked by a Novel Cysteine Protease Inhibitor. J. Biol. Chem. *275*, 30951–30956.

Whiteway, M. (2000). Transcriptional control of cell type and morphogenesis in *Candida albicans*. Curr. Opin. Microbiol. *3*, 582–588.

Wiedman, J.M., Fabre, A.-L., Taron, B.W., Taron, C.H., and Orlean, P. (2007). *In vivo* characterization of the GPI assembly defect in yeast mcd4-174 mutants and bypass of the Mcd4p-dependent step in mcd4Delta cells. FEMS Yeast Res. *7*, 78–83.

Xue, B., Dunbrack, R.L., Williams, R.W., Dunker, A.K., and Uversky, V.N. (2010). PONDR-FIT: A meta-predictor of intrinsically disordered amino acids. Biochim. Biophys. Acta BBA - Proteins Proteomics *1804*, 996–1010.

Yadav, B., Bhatnagar, S., Ahmad, M.F., Jain, P., Pratyusha, V.A., Kumar, P., and Komath, S.S. (2014). First Step of Glycosylphosphatidylinositol (GPI) Biosynthesis Cross-talks with Ergosterol Biosynthesis and Ras Signaling in *Candida albicans*. J. Biol. Chem. *289*, 3365–3382.

Yi, L., Bozkurt, G., Li, Q., Lo, S., Menon, A.K., and Wu, H. (2017). Disulfide Bond Formation and N-Glycosylation Modulate Protein-Protein Interactions in GPI-Transamidase (GPIT). Sci. Rep. *8*, 45912.

Younes, S., Bahnan, W., Dimassi, H.I., and Khalaf, R.A. (2011). The *Candida albicans* Hwp2 is necessary for proper adhesion, biofilm formation and oxidative stress tolerance. Microbiol. Res. *166*, 430–436.

Yu, X.-C., and Strobel, H. (1996). Interactions of 8-anilino-1-napthalenesulfonic acid (ANS) and cytochrome P450 2B1: Role of ANS as an effector as well as a reporter group. Mol. Cell. Biochem. *162*.

Zhao, X. (2003). Allelic variation in the contiguous loci encoding *Candida albicans ALS5*, *ALS1* and *ALS9*. Microbiology *149*, 2947–2960.

Zhao, X. (2004). *ALS3* and *ALS8* represent a single locus that encodes a *Candida albicans* adhesin; functional comparisons between Als3p and Als1p. Microbiology *150*, 2415–2428.

Zhao, X. (2005). Analysis of the *Candida albicans* Als2p and Als4p adhesins suggests the potential for compensatory function within the Als family. Microbiology *151*, 1619–1630.

Zhao, X. (2006). *Candida albicans* Als3p is required for wild-type biofilm formation on silicone elastomer surfaces. Microbiology *152*, 2287–2299.

Zhao, X., Oh, S.-H., and Hoyer, L.L. (2007a). Deletion of *ALS5* , *ALS6* or *ALS7* increases adhesion of *Candida albicans* to human vascular endothelial and buccal epithelial cells. Med. Mycol. *45*, 429–434.

Zhao, X., Oh, S.-H., and Hoyer, L.L. (2007b). Unequal contribution of *ALS9* alleles to adhesion between *Candida albicans* and human vascular endothelial cells. Microbiology *153*, 2342–2350.

Zimmermann, H. (1992). 5'-Nucleotidase: molecular structure and functional aspects. Biochem. J. *285 (Pt 2)*, 345–365.

Chapter 10
A Signal with a Difference: The Role of GPI Anchor Signal Sequence in Dictating Conformation and Function of the Als5 Adhesin in *Candida albicans*

Mohammad Faiz Ahmad *, Pareeta Gajraj Mann *, and Sneha Sudha Komath

Introduction

GPI Anchors in C. albicans

GPI anchors are unique flexible glycolipid anchors that are used by eukaryotes to anchor proteins in the extracellular leaflet of the plasma membrane or to the cell wall. Elsewhere in this volume, Prof. Taroh Kinosita provides a succinct review of the GPI anchor biosynthetic pathway and its importance for mammalian cells. Although there are some species-specific differences, the core structure of the GPI anchor precursor is conserved in eukarya. As with higher eukaryotes, in lower eukaryotes too GPI anchored proteins perform a variety of extremely important functions (McConville and Ferguson 1993; Paulick and Bertozzi 2008). In lower eukaryotes, the pathway appears to be essential for growth and viability (McConville and Ferguson 1993). In *S. cerevisiae* and its closely related pathogenic fungal cousin, *C. albicans*, inhibiting GPI anchor biosynthesis is lethal (Leidich et al. 1994; Grimme et al. 2004; Pittet and Conzelmann 2007; Yadav et al. 2014). In *C. albicans*, a number of GPI anchored proteins are specifically required for adhesion and virulence and several of these are regulated with the morphogenetic switch that converts the yeast form of the organism into the invasive hyphal form (Martinez-Lopez et al. 2004). Prominent among these are the Als family of eight adhesins, the hyphal wall protein 1 (Hwp1) and Elf4E-associated protein 1 (Eap1) (Staab et al. 1999; Cormack et al. 1999; Hoyer and Hecht 2001; Sundstrom 2002).

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Not surprisingly, defects in GPI biosynthesis, therefore, also hamper the ability of the organism to successfully establish colonies on host tissues and inhibit its infection and virulence (Martinez-Lopez et al. 2004).

The Role of C-Terminal Signal Sequences in the Attachment of the Anchor

As interesting as the GPI anchors themselves are the GPI anchor attachment signal sequences (SSs) at the C-terminal ends of the proteins. These SSs dictate the attachment of pre-formed GPI anchors to the proteins within the lumen of the endoplasmic reticulum (ER).

Starting with their discovery in the late 1980s, a great deal of debate has centered upon the nature and role of the SSs in determining which proteins will receive the anchor and how they will function. We now know that the amino acid sequence of a SS varies with the protein. We also know that identities of the residues *per se* are not as important as conservation of the 'nature' of the residues in a sequence identified as a GPI-attachment SS. A general rule appears to be that the site of cleavage of SS, and its replacement with the GPI anchor precursor, is always an amino acid residue (ω-site) close to the C-terminal end of the protein with a very small side chain. This must be flanked on either side by residues with small side chains as well (ω − 1 to ω + 2). A polar linker segment of 8–12 residues beyond the ω + 2 site and a C-terminal hydrophobic stretch of 10–20 residues then completes the SS (Eisenhaber et al. 1998). Although present generally at the C-terminal end of proteins, the introduction of SS in the middle of a protein sequence can also apparently signal for cleavage and addition of the preformed glycolipid anchor at that position (Caras 1991). Subsequent to the transfer of the GPI anchor 'precursor', the anchor as well as the protein undergoes several remodeling steps/modifications within the ER as well as in the Golgi before it is finally targeted to the cell surface (as discussed in detail by Prof. Kinoshita in this book).

Do the SSs have any additional roles to play other than the obvious one of serving as flags for transfer of the GPI anchors? SSs also appears to work as markers for localization, dictating whether specific proteins should go to the cell wall or the plasma membrane in *S. cerevisiae* and other organisms that have a cell wall (Hamada et al. 1999; Frieman and Cormack 2004; Ouyang et al. 2013). There are also studies that indicate that the SS dictates sorting to the apical versus basolateral surfaces in mammalian cells (Ali et al. 1996; Paladino et al. 2008). Likewise, there are suggestions that the type of GPI anchor attached may itself be dependent on the SS (Nicholson and Stanners 2007). Since the GPI anchor undergoes several steps of remodeling within the endoplasmic reticulum (ER) as well as in the Golgi after it is attached to the protein, and before it is finally targeted to the cell surface, it is unclear whether the ER exit sites for the proteins are dictated by the SS or by the protein. Several studies suggest that the SS also dictates the fate of the protein under GPI deficiency conditions or when it enters the degradation pathway (Garg et al. 1997;

Ashok and Hegde 2008). Residues at and around the ω-site, appear to not only be important for GPI attachment, they also seem to influence the glycosylation pattern on the GPI-anchored proteins in the Golgi and thereby alter both oligomerization status as well as localization in mammalian cells (Miyagawa-Yamaguchi et al. 2014). There are also studies that suggest that the SS after its detachment from the protein could continue to dictate its fate (Guizzunti and Zurzolo 2014). However, it is not very clear how these functions are mediated by a peptide stretch that is removed from the protein at the time of the GPI anchor attachment.

Mutations in SS

What happens in the case of mutations in the SS? For proteins like the mammalian prion protein (PrP), for example, such a question also assumes clinical significance since mutations in the SS have been associated with neurodegenerative disorders (Poggiolini et al. 2013). One obvious outcome, for mutations that occur at or around the ω-site, would be that GPI anchors do not get attached to the proteins and these must then be routed either towards secretion as anchorless variants or towards the protein degradation pathways (Böhme and Cross 2002; Kiachopoulos et al. 2005; Hizume et al. 2010). The absence of the anchor could certainly affect the functionality of the protein in such cases, both because it alters its localization and because the anchor itself could have a say in the final conformation of some proteins (Frieman and Cormack 2003; Kiachopoulos et al. 2005; Nisbet et al. 2010). Other mutations in the SS that do not necessarily have any effect on anchor attachment can also nevertheless affect the functioning of mature proteins on the cell surface (Hoque et al. 1996; Windl et al. 1999). How and why this happens is not very clear. One hypothesis, based on the idea that the SS continues to have an independent existence even after its detachment from the protein, has been that these mutations alter the degradation patterns of the detached SS and thereby modulate the functionality of the mature GPI-anchored protein (Guizzunti and Zurzolo 2014). The mechanism for this however remains unclear. Another hypothesis, based on the hypothesis that the SS carries a functional specificity signal within itself, suggests that mutations could alter the kind of anchor attached to a protein and therefore alter its functionality/fate (Nicholson and Stanners 2007). Although differences are known to occur in processing of the GPI anchored proteins during their transport through the secretory pathway, including differences due to mutations in the SS (Miyagawa-Yamaguchi et al. 2014), no studies have so far indicated that the GPI precursor attached by the transamidase to nascently translocated proteins is different for different proteins.

Using the Als5 adhesin from *C. albicans* we propose a third possible hypothesis where SS has a role in controlling the conformation of the protein and thereby influencing GPI anchor attachment and functionality. The Als family of adhesins have three distinct features that mark them out for the unique tasks that they are required to perform on the surface of *C. albicans* (Sundstrom 2002). Besides binding to a wide variety of specific peptide ligands, they also possess the ability to adhere to

several basal lamina proteins and to undergo oligomerization via amyloid formation/β-aggregation that assist in the establishment of fungal colonies. We had shown previously that the GPI anchor attachment SS could significantly alter the conformation of Als5 (Ahmad et al. 2012). The presence of SS could not only keep the protein in a relatively well folded and predominantly α-helical conformation, it could also prevent it from adhering to collagen type IV or aggregating, both important functions of the adhesin. Deletion of SS from the protein resulted in a protein with predominantly pre-molten globule characteristics and complete functionality. We had hypothesized that this difference between the two proteins could be because the SS at the C-terminal end of Als5-SS folds back and interacts with the amyloid patch within the adhesin, forcing the protein into adopting an α-helical conformation and suppressing its functionality (Fig. 10.1). Als5 lacking the SS would of

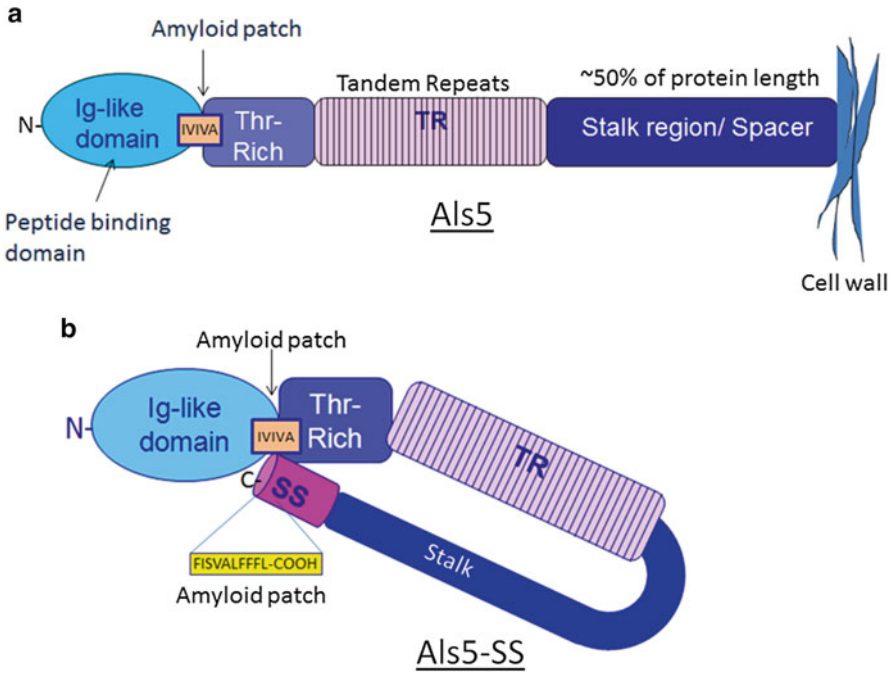

Fig. 10.1 Domain organization of Als5 andAls5-SS. Mature Als proteins on the cell surface are organized into several domains as shown in (**a**). Based on our previous studies on Als5, we had proposed that before the cleavage of the C-terminal SS and attachment of a preformed GPI anchor, SS folds back and interact with the N-terminal half of Als5 (Ahmad et al. 2012), as shown in (**b**). This folding back of SS influences the secondary structure of the protein leading to a more structured secondary conformation than predicted. We also proposed that the interaction takes place via the amyloidogenic regions within Als5-SS, one adjacent to the Ig-like peptide binding domain (shown in *orange*) and the other within its SS (shown in *yellow*), and thus inhibits the aggregation of Als5. Thus, presence of SS can influence the structure and function of Als5 protein. A mutation within the amyloidogenic regions could possibly disrupt or weaken this interaction, and thus affect the structure and function of the mutant protein

course be free from such a constraint and capable of adopting a more extended conformation as is expected for the functional protein on the cell surface. Likewise, mutations in Als5-SS that abrogate interaction between the two domains, as per our hypothesis, should result in a protein whose amyloid patch is exposed. In this manuscript we put this hypothesis to test. We show that mutant variants Als5-SS V309N (mutation in the amyloid patch) or Alss5-SS L1326R and Als5-SS F1327R (both mutations in the SS) are proteins which show better adhesion as well as aggregation, more like Als5 rather than Als5-SS.

Effect of Mutations on the Conformation and Function of Als5-SS

Identifying Mutations in Als5-SS That Would Abrogate β-Aggregation Potential of Putative Interacting Domains

In a previous manuscript, we showed by TANGO analysis that Als5-SS has two patches which have a significant amount of β-aggregation potential as predicted (Ahmad et al. 2012). One of these lies within the mature functional protein sequence, at the C-terminus of the Ig-like N-terminal domain of the protein that has been previously shown to bind specific peptide ligands (Klotz et al. 2004; Rauceo et al. 2006), while the other lies within the SS (Fig. 10.1). The internal site with the motif IVIVA was also shown by Peter Lipke and co-workers to be highly conserved in a variety of closely related adhesins, including the Als-family, and protein fragments of Als5 containing this motif were all capable of exhibiting aggregation (Ramsook et al. 2010). Using TANGO (http://tango.crg.es/), we also predicted what mutations within these sites would turn the amyloid patch into one with insignificant β-aggregation potential. We discovered that a V309N mutation within the protein reduced the β-aggregation potential of the first patch drastically (Fig. 10.2a). Likewise, L1326R or F1327R mutations reduced the β-aggregation potential of the SS to nearly zero (Fig. 10.2b, c). Thus, we hypothesized that if we introduced any of these mutations in Als5-SS, it should reduce the interactions between these two amyloidogenic regions of Als5-SS and result in better adhesion and aggregation.

In silico Predictions of GPI Anchor Attachment for Als5-SS and Its Mutant Variants

While introduction of the V309N mutation was not expected to affect GPI anchor attachment, it was pertinent to also ask whether mutations in the SS would affect GPI anchor addition. We used the GPI-SOM (http://gpi.unibe.ch/; Fankhauser and Mäser 2005) and Big-PI (http://mendel.imp.univie.ac.at/set/gpi/gpi_server.html;

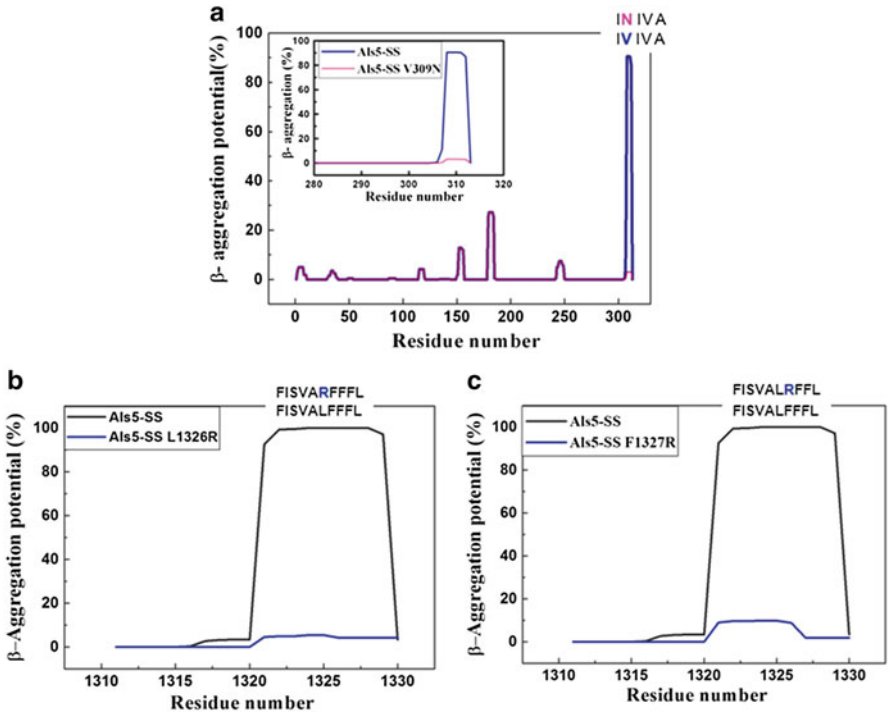

Fig. 10.2 TANGO prediction for **β**-aggregation potential. Introduction of V309N (**a**), or L1326R (**b**), or F1327R (**c**) mutations leads to reduced **β**-aggregation potential for Als5-SS. The sequences within which the mutations have been made are also shown. In each figure the sequence below represents the wild type and the one above shows the mutated sequence. The inset in (**a**) corresponds to the region containing the IVIVA domain

Table 10.1 Prediction of GPI anchor attachment by BIG-PI predictor and GPI-SOM

Proteins	BIG PI-predictor	GPI-SOM
Als5-SS	GPI anchored	GPI anchored
Als5-SS L1326R	Non GPI anchored	GPI anchored
Als5-SS F1327R	GPI anchored	GPI anchored
Als5-SS L1326R F1327R	Non GPI anchored	Non GPI anchored

Eisenhaber et al. 1999) software to predict whether the mutations affected GPI anchor attachment. We discovered that the L1326R mutation was drastic enough to convert the SS into a stretch that was no longer likely to be recognized as a putative SS (Table 10.1). On the other hand, mutating F1327R appeared to have no significant effect on its potential to act as a signal for GPI anchor attachment (Table 10.1). Needless to say, the double mutation L1326R F1327R resulted in a sequence with no potential to serve as a successful SS (Table 10.1).

Table 10.2 Prediction of ΔG_{app} for TM helix insertion of SS from Als5 by ΔG predictor software (http://dgpred.cbr.su.se)

	Predicted ΔG_{app} (kcal/mol)
SS	+2.955
SS L1326R	+3.675
SS F1327R	+3.585
SS L1326R F1327R	+4.313

An accompanying question we needed to address was about the nature of SS itself. For long it was believed that during translocation into the ER, a newly translated proprotein destined to be GPI anchored would have the hydrophobic segment of its SS inserted into the ER membrane where it would then be recognized by the GPI-transamidase (Berger et al. 1988; Caras and Weddell 1989). However, there is now growing experimental evidence to suggest that membrane-inserted SS domains are not absolutely essential (Wang et al. 1999; Dalley and Bulleid 2003). Several proproteins that get GPI anchored are perhaps soluble proteins. A thermodynamic assessment of the free energies (ΔG_{app}) for membrane insertion of a variety of SS suggested that SS in general have sequences with marginal hydrophobicities and $\Delta G_{app} \sim 0.0$ (Galian et al. 2012). Using ΔG predictor software (http://dgpred.cbr.su.se) developed by von Heijne's group (Hessa et al. 2007) the SS fragment of Als5-SS was predicted to have a helix insertion ΔG_{app} of +2.6 kcal/mol (Table 10.2). In other words, Als5-SS could perhaps be a soluble ER luminal protein. This also implies that the SS is likely to be free to interact with the rest of the protein, as hypothesized in our model, and may not be constrained to be inserted in the membrane. Mutation of V309N, as expected, does not in any way alter the value of ΔG_{app} for SS (+2.955 kcal/mol). Mutations within SS, both L1326R and F1327R, make the ΔG_{app} value more positive (Table 10.2), suggesting that these mutations too by themselves are unlikely to promote membrane insertion of the SS.

Generating ALS5-SS Variants Carrying the Different Mutations

Next we set about introducing these mutations, both L1326R and F1327R, within Als5-SS for wet lab experiments. Although the mutation L1326R was predicted to convert SS into a sequence that may not be recognized by the transamidase, as discussed above, we persisted in generating the mutant variant of Als5-SS possessing this mutation for two major reasons: 1) Theoretical predictions only give a general picture. They are neither fool-proof nor 100 % accurate and are based on the experimental data set available at this point in time. It is possible that predictions would change when more data or information become available. 2) The mutation, as per our hypothesis, should still be able to successfully disrupt the interaction between the internal amyloid patch and the SS. Thus, as a proof of concept, this is still a variant worth generating. The mutant, as a candidate for the transamidase, is currently being evaluated in wet lab experiments in the lab.

Table 10.3 List of primers used

	Primer sequence
Als5-SS FP	5′GCG*GGATCC*ATGATTCAACAATTTACATTGTTATTC3′
Als5-SS RP	5′GCG*CTCGAG*TCATAGAAAGAAGAATAATGCAACG3′
Als5-SS V309N FP	5′GATGCCGGATCTAACGGTATTAATATTGT 3′
Als5-SS V309N RP	5′TGTTCTAGTTGTAGCAACAATATTAATACC3′
Als5-SS SS L1326R FP	5′CTTAAATTTATTAGCGTTGCACGGTTCTTC3′
Als5-SS SS L1326R RP	5′CTCGAGTCATAGAAAGAAGAACCGTGCAAC3′
Als5-SS F1327R FP	5′AAATTTATTAGCGTTGCATTACGGTTCTTT 3′
Als5-SS F1327R RP	5′CCGCTCGAGTCATAGAAAGAACCGTAATGC3′

Since we required Als5-SS as a proprotein with its SS intact for these studies, we expressed the protein in bacterial cells that lack the GPI biosynthetic machinery. We previously generated the construct *Als5-SS-pGEX-6p-2* for expression of the protein in bacterial cells (Ahmad et al. 2012). We used the same plasmid to amplify these mutations in *GST-Als5-SS* using mutagenic primers (sequences of the primers are given in Table 10.3). The template plasmid, without any mutation, used for PCR was digested using Dpn1 enzyme and then transformed in DH5α strain of *E. coli*. The colonies obtained after transformation were screened by colony PCR using gene specific primers, FP and RP of *ALS5-SS*, and the colonies confirmed positive were further confirmed by complete sequencing of the gene of interest. We successfully generated three mutants (*GST-ALS5-SS V309N, GST-ALS5-SS L1326R* and *GST-ALS5-SS F1327R*) in the *GST-ALS5-SS* gene (data not shown).

The proteins were expressed and purified as already described previously (Ahmad et al. 2012). Briefly, plasmids without (for GST-Als5-SS) or with mutations (for GST-Als5-SS V309N, GST-Als5-SS L1326R and GST-Als5-SS F1327R) were transformed into BL21 strain of *E. coli*. Primary cultures were obtained by incubation of these transformed strains at 37 °C at 220 rpm overnight. Secondary cultures were generated using 1 % inoculum of the primary culture in each case after incubation at 37 °C at 220 rpm for 3 h. Protein expression was induced for 4 h using 0.1 mM IPTG at 16 °C when OD_{600nm} of the cultures reached ~0.6. The cells were pelleted down at 8,500 rpm at 4 °C for 10 min and the pellet resuspended in lysis buffer (50 mM sodium phosphate buffer, pH 8.0, 150 mM NaCl, 10 μM PMSF, 0.1 mg/mL lysozyme, and 5 % glycerol). After incubation at 4 °C on a rocker for 1 h, the cells were lysed using digital probe sonicator (30 s ON and 30 s OFF for 7 min). The cell lysate was centrifuged at 8,500 rpm for 45 min at 4 °C, the supernatant mixed with glutathione-agarose beads and incubated for 4 h on a rocker at 4 °C. The beads were washed using wash buffer (50 mM sodium phosphate buffer, pH 8.0, 3 M NaCl), and proteins eluted using 10 mM glutathione in elution buffer (50 mM sodium phosphate buffer, pH 8.0, 150 mM NaCl, 20 % glycerol). The homogeneity of the protein samples were >90 % as assessed from runs on 6 % SDS polyacrylamide gels stained with Coomassie brilliant blue R250 (data not shown).

The proteins were dialyzed to remove glutathione before they were characterized. As explained in our previous paper as well, the GST-tag is attached to the

protein via a PreScission™ protease site, however, this site did not appear to be accessible to the protease and the tag could not be removed (Ahmad et al. 2012). Thus, all the wet lab experiments included purified GST as a control. The tag did not appear to significantly affect our results.

The Als5-SS Mutant Variants Had a Conformation That Was Different from That of Als5-SS

The mutant proteins were assessed for secondary structure formation by CD spectroscopy by a Chirascan CD spectrometer (Applied Photophysics). Dialyzed proteins at ~0.09 mg/mL were used for obtaining the spectra between 200 and 260 nm in a 1 mm path length cuvette with 1 nm step length. In all cases the buffer background was subtracted. The final spectra were averages of five repeat scans. The spectrum of GST was then subtracted from that of each of the fusion proteins to get the spectrum of Als5-SS V309N, Als5-SS L1326R and Als5-SS F1327R using ProData software that came with the instrument.

We had previously shown that Als5 and Als5-SS had very different secondary structures as assessed by CD spectroscopy (Ahmad et al. 2012). While Als5-SS was a relatively well folded protein, Als5 itself was predominantly a pre-molten globular protein. This was one of the reasons why we had hypothesized that the SS at the C-terminal end probably folded back and interacted with the amyloid patch on the Als5 and thereby induced a significant amount of structure into a protein that would otherwise be largely a natively unfolded protein.

We found that each of the single mutations that we introduced during the course of this study perturbed the secondary structure of Als5-SS, although none of them had CD spectra that completely resembled that of Als5 (Fig. 10.3). In other words,

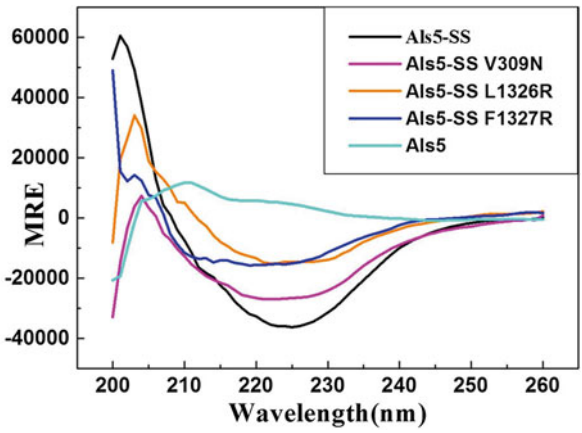

Fig. 10.3 Secondary structure prediction by CD. The secondary structures of Als5-SS V309N, Als5-SS L1326R and F1327R mutant proteins differed from Als5-SS, suggesting these mutations alter the conformation of the protein possibly by disruption of interaction between the N-terminus amyloidogenic patch and SS. The spectrum of Als5 lacking the SS is also shown for comparison

as per our model, the mutations appeared to alter the interactions between the domains without completely abrogating it.

We were now ready to examine adhesion by and aggregation of the mutant proteins.

The Als5-SS Mutant Variants Adhere Better to Collagen Type IV than Als5-SS Itself

We first examined the ability of the mutant proteins to adhere to collagen type IV. We had previously shown that GST-Als5 had approximately twofold better adherence to the basal lamina protein in comparison to GST-Als5-SS while GST by itself showed little or no adhesion to collagen type IV under similar conditions.

Using a similar protocol as before, we coated a 96 well plate with 0.5 mg/mL of collagen type-IV and incubated it at 4 °C overnight. Next day the wells were washed thrice with PBS and twice with elution buffer before adding 200 μL of ~0.07 mg/mL of each protein (GST, GST-Als5-SS, GST-Als5-SS V309N, GST-Als5-SS L1327R and GST-Als5-SS F1327R) to the respective wells. The plate was incubated at 37 °C for 1 h. Two controls, one using GST and the other without primary antibody were taken. 200 μL of 5 % skimmed milk in PBS were added to block the wells and incubated at 37 °C for another hour and washed 5 times with antibody buffer (1 % skimmed milk in PBS + 0.025 % Tween-20). Anti-GST primary antibody (1:1,000 diluted) was added to each well and incubated at 37 °C for 1 h. After incubation all the wells were washed 5 times with antibody buffer and 200 μL of HRP-conjugated secondary antibody (1:20,000 diluted) was added and incubated at 37 °C for 1 h. The wells were washed 5 times with antibody buffer and 100 μL of Tetramethylbenzidine (TMB) solution (1 mg/mL TMB in DMSO + 9 mL of 0.05 M phosphate citrate buffer, pH 5 and 2 μL H_2O_2) was added to each well and incubated at 37 °C for 1 h. OD_{650nm} was recorded using a plate reader (Thermo Scientific Multiscan GO).

We observed that the GST-Als5-SS mutants showed significantly better adhesion in comparison to GST-Als5-SS (Fig. 10.4).

The Als5-SS Mutant Variants Show Much Better Aggregation That Wild Type Als5-SS

We next explored the aggregation ability of the mutant variants of Als5 using transmission electron microscopy (TEM) with the help of a JEOL 2100F system. The proteins (0.09 mg/mL) were incubated at 37 °C for different time points (12, 24 h,

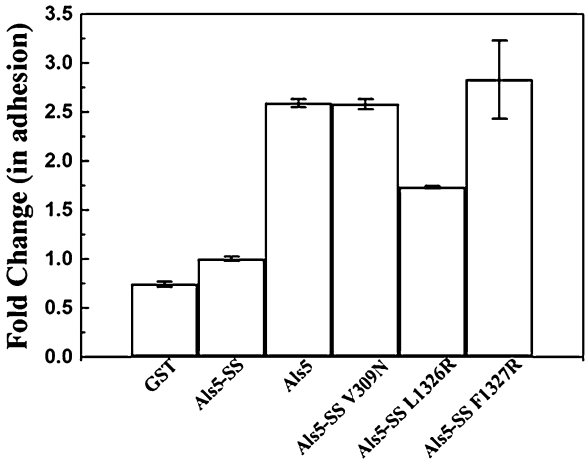

Fig. 10.4 GST-Als5-SS mutants show better adherence to collagen type IV than GST-Als5-SS. GST-Als5-SS V309N, GST-Als5-SS L1326R and GST-Als5-SS F1327R showed significantly higher adherence to collagen type IV (basal lamina protein) as compared to GST-Als5-SS. GST alone was used as control

1 week and 2 weeks) before analysis. Carbon coated copper grids were used for spotting the proteins, which were then stained with 2 % uranyl acetate and dried, before TEM images were recorded at 200 kV accelerating voltage.

We had previously shown that GST-Als5-SS could not form significant aggregates even if incubated for 2 weeks at 37 °C. On the other hand, GST-Als5 could form extensive aggregates within 24 h of incubation under similar conditions (Ahmad et al. 2012). As before, we observed that neither GST nor GST-Als5-SS formed aggregates even after 2 weeks of aggregation, while GST-Als5 formed significant aggregates within 12 h of incubation under similar conditions (Fig. 10.5). In contrast to GST-Als5-SS, GST-Als5-SS V309N, GST-Als5-SS L1326R and GST-Als5-SS F1327R could all show a significant amount of aggregation when incubated at 37 °C for 12 h (Fig. 10.5). The aggregation, as expected, was better when the mutant variants were incubated for longer periods of time.

Mutations in a Synthetic SS Peptide Prevent It from Inhibiting Self-Aggregation of Als5

In order to confirm the above results, we used a synthetic SS peptide (synthesized by Custom Peptide Synthesis) composed of just the last 20 residues at the C-terminus of Als5-SS as a competitor in our TEM studies. We previously showed that the synthetic SS peptide at 1:1 M concentrations could significantly inhibit the self-aggregation of GST-Als5 (Ahmad et al. 2012). If our hypothesis was correct, we

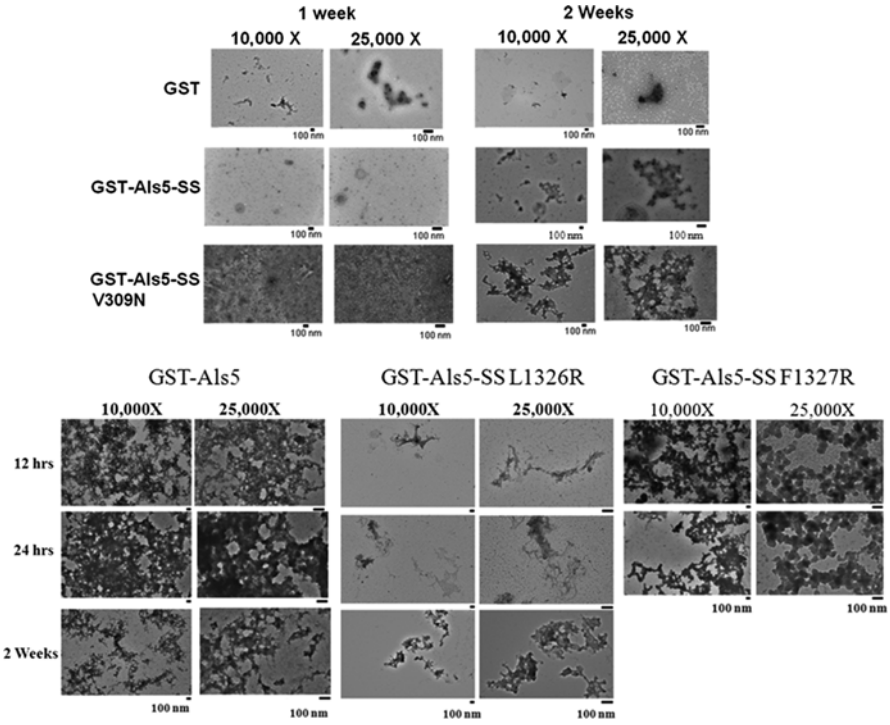

Fig. 10.5 Effects of mutations in amyloidogenic region and SS on the aggregation of GST-Als5-SS. GST-Als5-SS V309N**,** GST-Als5-SS L1326R and GST-Als5-SS F1327R showed much better aggregation than GST-Als5-SS. Thus, these mutations probably alter the interactions between SS and amyloidogenic domain near the Ig-like domain in GST-Als5-SS, thereby enhancing the aggregation of the mutant proteins. The aggregation of GST alone is also shown as control to demonstrate that the aggregation is not due to the N-terminal GST tag on the protein variants

expected to see reduced effect of the peptide on the self-aggregation of GST-Als5 if we introduced the L1326R or the F1327R mutation in it. Hence, we tested whether a synthetic peptide that included either the L1326R or the F1327R mutation would be able to inhibit GST-Als5 aggregation. Indeed, while SS continued to inhibit the aggregation of GST-Als5, neither SS L1326R nor SS F1327R could inhibit the self-aggregation of GST-Als5 to any significant degree (Fig. 10.6). As expected, the peptide with the double mutation also did not inhibit the aggregation of GST-Als5 (Fig. 10.6).

Thus, all in all, the data presented here confirms the hypothesis that we began with at the start of this work.

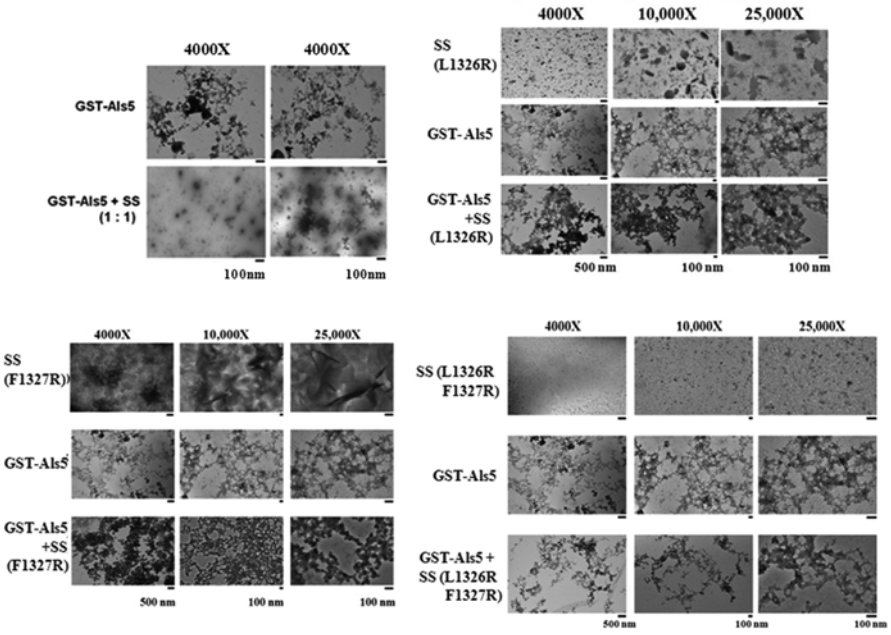

Fig. 10.6 Inhibition of aggregation of GST-Als5 by synthetic SS peptide variants. Incubation of GST-Als5 with equimolar concentration of synthetic SS peptide inhibits the aggregation property of GST-Als5, as also shown previously (Ahmad et al. 2012). However, SS peptide with L1326R and F1327R, or double mutations of L1326R F1327R failed to inhibit the aggregation of GST-Als5, suggesting the inability of these mutagenic SS peptides to interact with the amyloidogenic region of GST-Als5

Concluding Remarks

There is growing evidence to suggest that the SS plays multiple roles in GPI anchor attachment to proteins and these could vary depending both on the nature of the proteins and the SS themselves. But given the fact that the SS gets removed from the proprotein before GPI anchor attachment and the fact that the mature protein does not carry it, direct experimental proof on the exact mechanisms by which these additional roles may be performed by SS has remained sketchy.

The results described in this manuscript support a model wherein the SS domain of Als5-SS interacts with the rest of the protein and alters both its conformation and function. What could be the implications of these results? As we show from *in silico* analysis, Als5-SS is likely to be completely translocated into the lumen of the ER before the GPI-transamidase works on it to cleave off the SS and attach the pre-formed GPI anchor at its ω-site. As long as the protein remains in the ER and retains its SS, it is likely to be a reasonably well-folded protein with its amyloid domain protected from extraneous interactions by the SS. Thus, this proprotein has no

Table 10.4 Prediction of ΔG_{app} for TM helix insertion of SS from all Als adhesins by ΔG predictor software (http://dgpred.cbr.su.se)

SS of the following adhesins	Predicted ΔG_{app} (kcal/mol)
Als1	+2.347
Als2	+6.641
Als3	+1.689
Als4	+2.530
Als5	+2.955
Als6	+2.955
Als7	+2.045
Als9	+1.838

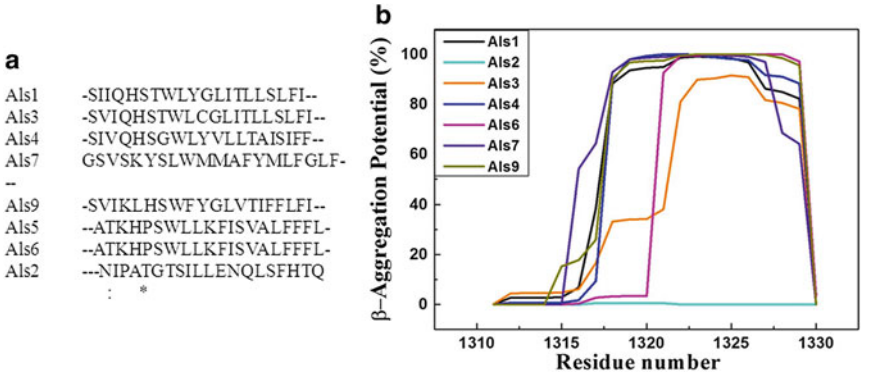

Fig. 10.7 Analysis of sequence similarity and **β**-aggregation potential for SS from all Als family adhesins: (**a**) multiple sequence alignment using Clustal Omega shows poor sequence conservation amongst the SS of the different Als adhesins from *C. albicans*. (**b**) TANGO analysis shows the presence of a **β**-aggregation patch with significant aggregation potential in all Als adhesins with the exception of Als2

tendency to self-aggregate, a property so critical for the functioning of the mature protein at the cell surface. Upon removal of the SS, however, the amyloid patch becomes available for interactions and makes the protein functional. The presence or absence of the SS not only alters the folding of the protein and its functionality, but it could possibly also determine its interactions with other proteins of the GPI biosynthetic machinery, the ER exit sites and those of the secretory pathway. Mutations in the SS, which do not affect attachment of the GPI anchor, could nevertheless alter the conformation of the proprotein sufficiently to modulate these interactions and thereby alter the final fates of the mutant variants.

Is there a bigger picture too? *In silico* analyses suggest that all other members of the Als family are also likely to be have signal sequences that are unlikely to be membrane-inserted by themselves (Table 10.4). In addition, despite poor conservation (Fig. 10.7a), the SSs of all the Als adhesins, with the exception of Als2, have C-terminal ends with >90 % β-aggregation potential (Fig. 10.7b) and a highly

conserved amyloid domain (Ramsook et al. 2010). Thus, it is possible these proteins too could possibly fit our model. In other words, for the Als family of adhesins from *C. albicans*, the SS may not only provide the appropriate signal for the transamidase to recognize the site for transfer of the GPI anchor it could also probably provide a mechanism for keeping the proprotein variants from prematurely aggregating in the ER. The absence of conservation in the sequences, therefore, may not only serve to provide differential kinetics of GPI transfer to the Als proteins by the GPI-transamidase it may also be necessitated by the conformational requirements of the proproteins.

Whether this model could be extended beyond the Als family of proteins, remains to be tested. While a general set of rules appear to govern the overall arrangement of residues within the anchors, the significant variations between the sequences and their thermodynamic characteristics would argue against a one-model-fits-all hypothesis. Nevertheless, neither the evidence presented by Nicholson and Stanners (2007) nor that by Guizzunti and Zurzolo (2014) (which provide alternative models to explain how mutations in SS affect localization or functionality of the mature protein), would preclude such an interpretation. The issue in both cases may just as well be one of altered conformation of the proprotein itself resulting in alterations in its downstream interacting partners and therefore alterations in transportation and functionality of the mature protein.

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References

Ahmad MF, Yadav B, Kumar P et al (2012) The GPI anchor signal sequence dictates the folding and functionality of the Als5 adhesin from Candida albicans. PLoS One 7:e35305. doi:10.1371/journal.pone.0035305

Ali S, Hall J, Hazlewood GP et al (1996) A protein targeting signal that functions in polarized epithelial cells in vivo. Biochem J 315(Pt 3):857–862

Ashok A, Hegde RS (2008) Retrotranslocation of prion proteins from the endoplasmic reticulum by preventing GPI signal transamidation. Mol Biol Cell 19:3463–3476. doi:10.1091/mbc.E08-01-0087

Berger J, Howard AD, Brink L et al (1988) COOH-terminal requirements for the correct processing of a phosphatidylinositol-glycan anchored membrane protein. J Biol Chem 263:10016–10021

Böhme U, Cross GAM (2002) Mutational analysis of the variant surface glycoprotein GPI-anchor signal sequence in Trypanosoma brucei. J Cell Sci 115:805–816

Caras IW (1991) An internally positioned signal can direct attachment of a glycophospholipid membrane anchor. J Cell Biol 113:77–85

Caras IW, Weddell GN (1989) Signal peptide for protein secretion directing glycophospholipid membrane anchor attachment. Science 243:1196–1198

Cormack BP, Ghori N, Falkow S (1999) An adhesin of the yeast pathogen Candida glabrata mediating adherence to human epithelial cells. Science 285:578–582

Dalley JA, Bulleid NJ (2003) How does the translocon differentiate between hydrophobic sequences that form part of either a GPI (glycosylphosphatidylinositol)-anchor signal or a stop transfer sequence? Biochem Soc Trans 31:1257–1259

Eisenhaber B, Bork P, Eisenhaber F (1998) Sequence properties of GPI-anchored proteins near the omega-site: constraints for the polypeptide binding site of the putative transamidase. Protein Eng 11:1155–1161

Eisenhaber B, Bork P, Eisenhaber F (1999) Prediction of potential GPI-modification sites in pro-protein sequences. J Mol Biol 292:741–758. doi:10.1006/jmbi.1999.3069

Fankhauser N, Mäser P (2005) Identification of GPI anchor attachment signals by a Kohonen self-organizing map. Bioinformatics 21:1846–1852. doi:10.1093/bioinformatics/bti299

Frieman MB, Cormack BP (2004) Multiple sequence signals determine the distribution of glycosylphosphatidylinositol proteins between the plasma membrane and cell wall in Saccharomyces cerevisiae. Microbiology 150:3105–3114. doi:10.1099/mic.0.27420-0

Frieman MB, Cormack BP (2003) The omega-site sequence of glycosylphosphatidylinositol-anchored proteins in Saccharomyces cerevisiae can determine distribution between the membrane and the cell wall. Mol Microbiol 50:883–896

Galian C, Björkholm P, Bulleid N, von Heijne G (2012) Efficient glycosylphosphatidylinositol (GPI) modification of membrane proteins requires a C-terminal anchoring signal of marginal hydrophobicity. J Biol Chem 287:16399–16409. doi:10.1074/jbc.M112.350009

Garg N, Tarleton RL, Mensa-Wilmot K (1997) Proteins with glycosylphosphatidylinositol (GPI) signal sequences have divergent fates during a GPI deficiency. GPIs are essential for nuclear division in Trypanosoma cruzi. J Biol Chem 272:12482–12491

Grimme SJ, Colussi PA, Taron CH, Orlean P (2004) Deficiencies in the essential Smp3 mannosyltransferase block glycosylphosphatidylinositol assembly and lead to defects in growth and cell wall biogenesis in Candida albicans. Microbiology 150:3115–3128. doi:10.1099/mic.0.27254-0

Guizzunti G, Zurzolo C (2014) The fate of PrP GPI-anchor signal peptide is modulated by P238S pathogenic mutation. Traffic 15:78–93. doi:10.1111/tra.12126

Hamada K, Terashima H, Arisawa M et al (1999) Amino acid residues in the omega-minus region participate in cellular localization of yeast glycosylphosphatidylinositol-attached proteins. J Bacteriol 181:3886–3889

Hessa T, Meindl-Beinker NM, Bernsel A et al (2007) Molecular code for transmembrane-helix recognition by the Sec61 translocon. Nature 450:1026–1030. doi:10.1038/nature06387

Hizume M, Kobayashi A, Mizusawa H, Kitamoto T (2010) Amino acid conditions near the GPI anchor attachment site of prion protein for the conversion and the GPI anchoring. Biochem Biophys Res Commun 391:1681–1686. doi:10.1016/j.bbrc.2009.12.128

Hoque MZ, Kitamoto T, Furukawa H et al (1996) Mutation in the prion protein gene at codon 232 in Japanese patients with Creutzfeldt-Jakob disease: a clinicopathological, immunohistochemical and transmission study. Acta Neuropathol 92:441–446

Hoyer LL, Hecht JE (2001) The ALS5 gene of Candida albicans and analysis of the Als5p N-terminal domain. Yeast 18:49–60

Kiachopoulos S, Bracher A, Winklhofer KF, Tatzelt J (2005) Pathogenic mutations located in the hydrophobic core of the prion protein interfere with folding and attachment of the glycosylphosphatidylinositol anchor. J Biol Chem 280:9320–9329. doi:10.1074/jbc.M412525200

Klotz SA, Gaur NK, Lake DF, Chan V, Rauceo J, et al. (2004) Degenerate peptide recognition by Candida albicans adhesins Als5p and Als1p. Infect. Immun. 72, 2029–2034.

Leidich SD, Drapp DA, Orlean P (1994) A conditionally lethal yeast mutant blocked at the first step in glycosyl phosphatidylinositol anchor synthesis. J Biol Chem 269:10193–10196

Martinez-Lopez R, Monteoliva L, Diez-Orejas R et al (2004) The GPI-anchored protein CaEcm33p is required for cell wall integrity, morphogenesis and virulence in Candida albicans. Microbiology 150:3341–3354. doi:10.1099/mic.0.27320-0

McConville MJ, Ferguson MA (1993) The structure, biosynthesis and function of glycosylated phosphatidylinositols in the parasitic protozoa and higher eukaryotes. Biochem J 294(Pt 2):305–324

Miyagawa-Yamaguchi A, Kotani N, Honke K (2014) Expressed glycosylphosphatidylinositol-anchored horseradish peroxidase identifies co-clustering molecules in individual lipid raft domains. PLoS One 9:e93054. doi:10.1371/journal.pone.0093054

Nicholson TB, Stanners CP (2007) Identification of a novel functional specificity signal within the GPI anchor signal sequence of carcinoembryonic antigen. J Cell Biol 177:211–218. doi:10.1083/jcb.200701158

Nisbet RM, Harrison CF, Lawson VA et al (2010) Residues surrounding the glycosylphosphatidylinositol anchor attachment site of PrP modulate prion infection: insight from the resistance of rabbits to prion disease. J Virol 84:6678–6686. doi:10.1128/JVI.02709-09

Ouyang H, Chen X, Lü Y et al (2013) One single basic amino acid at the ω-1 or ω-2 site is a signal that retains glycosylphosphatidylinositol-anchored protein in the plasma membrane of Aspergillus fumigatus. Eukaryot Cell 12:889–899. doi:10.1128/EC.00351-12

Paladino S, Lebreton S, Tivodar S et al (2008) Different GPI-attachment signals affect the oligomerisation of GPI-anchored proteins and their apical sorting. J Cell Sci 121:4001–4007. doi:10.1242/jcs.036038

Paulick MG, Bertozzi CR (2008) The glycosylphosphatidylinositol anchor: a complex membrane-anchoring structure for proteins. Biochemistry (Mosc) 47:6991–7000. doi:10.1021/bi8006324

Pittet M, Conzelmann A (2007) Biosynthesis and function of GPI proteins in the yeast Saccharomyces cerevisiae. Biochim Biophys Acta 1771:405–420. doi:10.1016/j.bbalip.2006.05.015

Poggiolini I, Saverioni D, Parchi P (2013) Prion protein misfolding, strains, and neurotoxicity: an update from studies on mammalian prions. Int J Cell Biol 2013:910314. doi:10.1155/2013/910314

Ramsook CB, Tan C, Garcia MC et al (2010) Yeast cell adhesion molecules have functional amyloid-forming sequences. Eukaryot Cell 9:393–404. doi:10.1128/EC.00068-09

Rauceo JM, De Armond R, Otoo H, Kahn PC, Klotz SA, et al. (2006) Threonine-rich repeats increase fibronectin binding in the Candida albicans adhesin Als5p. Eukaryotic Cell 5, 1664–1673

Staab JF, Bradway SD, Fidel PL, Sundstrom P (1999) Adhesive and mammalian transglutaminase substrate properties of Candida albicans Hwp1. Science 283:1535–1538

Sundstrom P (2002) Adhesion in Candida spp. Cell Microbiol 4:461–469

Wang J, Maziarz K, Ratnam M (1999) Recognition of the carboxyl-terminal signal for GPI modification requires translocation of its hydrophobic domain across the ER membrane. J Mol Biol 286:1303–1310. doi:10.1006/jmbi.1999.2584

Windl O, Giese A, Schulz-Schaeffer W et al (1999) Molecular genetics of human prion diseases in Germany. Hum Genet 105:244–252

Yadav B, Bhatnagar S, Ahmad MF et al (2014) First step of glycosylphosphatidylinositol (GPI) biosynthesis cross-talks with ergosterol biosynthesis and Ras signaling in Candida albicans. J Biol Chem 289:3365–3382. doi:10.1074/jbc.M113.528802