CHAPTER 1

General Introduction

1.1. *Candida albicans*

Drug-resistant fungal infections and mostly invasive fungal infections from complex surgical procedures are emerging as an important clinical problem (Arendrup et al., 2017). In immunocompromised patients, the use of corticosteroid and chemotherapeutic drugs cause decrease in the amount of saliva and its pH in the oral cavity. So these therapies mostly affect the whole microbiota of the gut and the mostly individuals are infected by yeast form of *Candida albicans*.[3] *Candida albicans* is present as a commensal organism in nearly 75% of the human population present in divergent niches such as skin, oral cavity, gastrointestinal and urogenital tracts of humans (Perlin et al., 2017). However, if the balance of the normal flora is disrupted or the immune defenses are compromised, *Candida albicans* becomes pathogenic causing candidiasis and candidemia. Bloodstream infection caused by *C. albicans* is the fourth most common life-threatening disease in immunocompromised patients (Dartevelle et al., 2018), claiming 4,50,000 to 7,00,000 lives per annum worldwide according to a survey conducted by the Centers for Disease Control and Prevention (CDC) and World Health Organization (WHO).

1.2. Candidiasis

Candidiasis is a fungal infection caused by the yeast *Candida spp*. Some species of *Candida* can cause infection in people; the most common is *Candida albicans*. *Candida* normally lives on the skin and inside the body, such as the mouth, throat, gut, and vagina, without causing problems. *Candida* can cause infections if it grows out of control or if it enters deep into the body. For example, it can cause infections in the bloodstream or internal organs like the kidney, heart, or brain.

Figure 1.1 Types of Candidiasis

1.2.1. Vaginal Candidiasis

Candida can cause an infection if conditions change inside the vagina to encourage its growth. Vaginal candidiasis is common. Women who are more likely to get vaginal candidiasis include those who: (i) are pregnant, (ii) use hormonal contraceptives, (iii) have diabetes, (iv) have a weakened immune system (for example, due to HIV infection or medicines such as steroids and chemotherapy), (v) are taking or have recently taken antibiotics. The symptoms of vaginal candidiasis include vaginal itching or soreness, pain during sexual intercourse, pain or discomfort when urinating and abnormal vaginal discharge.

1.2.2. Invasive Candidiasis

Invasive candidiasis is a serious infection. It is not a particularly localized infection as it can affect the blood, heart, brain, eyes, bones, or other parts of the body. The specific type and dose of antifungal medication used to treat invasive candidiasis usually depends on the patient's age, immune status, location and severity of the infection. For most adults, the initial recommended antifungal treatment is echinocandin (caspofungin, micafungin, or anidulafungin) given intravenous. Fluconazole, amphotericin B, and other antifungal medications may also be appropriate in certain situations (Pappas et al., 2016).

1.2.3. Oral Candidiasis

Candidiasis in the mouth and throat is also called thrush or oropharyngeal candidiasis. Candidiasis in the esophagus is called esophageal candidiasis or candida esophagitis. Esophageal candidiasis is one of the most common infections in people living with HIV/AIDS. symptoms, including white patches on the inner cheeks, and tongue, redness or soreness, cotton-like feeling in the mouth, Loss of taste, Cracking and redness at the corners of the mouth, Symptoms of candidiasis in the esophagus usually include pain when swallowing and difficulty swallowing (Buchacz et al., 2016). The treatment for candidiasis in the esophagus is usually fluconazole. Other antifungal medicine is applied to the inside of the mouth for 7 to 14 days. These medications include clotrimazole, miconazole, or nystatin. For severe infections, the most common treatment is fluconazole (an antifungal medication) taken by mouth or through a vein (Pappas et al., 2016).

1.3. Head and neck radiotherapy effect in immunocompromised human patients

Most of the patients with head and neck cancers receive a dose between 50 and 70 Gy with a therapeutic intent. The head and neck region, a complex area composed of variety of tissues that respond differently to radiotherapy. Ionizing radiation induces damage in normal tissues, salivary gland situated in the field of radiation. The most notable salivary changes are a decreased pH and buffering capacity, salivary electrolyte imbalance, and altered nonimmune and immune antibacterial systems. The decreased production of immunoprotein and lysozyme levels are relatively more than the reduction in salivary flow rate and pH which results in a significant immunoprotein deficit compromise in immunologic mechanisms and oral clearance will lead to poor host protection, causing change in the oral microbiota of irradiated patients. In recent decades many case studies came out to light that more severe condition of candidiasis occurs in immunocompromised patients such as cancer patients when they go for radiotherapy treatment. One report study of hospital patients showed that 26.16% of patients were mycologically positive for *Candida* before radiotherapy with CFUs 100. 14 ± 59.11 that increased to 60.74% of patients during radiotherapy with an increase in CFUs to 490.15 ± 207.97 (Suryawanshi et al., 2012). In 2008 another report showed that *C. albicans* becomes more resistant to γ-radiation during the process of filamentation (Cagnacci et al., 2008).

1.4. Factors that influence the virulence of C. albicans

Morphogenesis: *Candida albicans* exhibits remarkable morphological plasticity, transitioning between yeast, pseudohyphal, and hyphal forms. In the yeast form, C. albicans can colonize on host surfaces and evade immune detection. However, under certain conditions, such as nutrient limitation or exposure to host factors, C. albicans can undergo morphogenesis to form filamentous structures, including pseudohyphae and true hyphae. Hyphal forms are associated with tissue invasion, dissemination, and host damage. The transition to hyphal growth is regulated by various environmental cues, signaling pathways, and transcription factors, allowing C. albicans to adapt to different host niches and modulate its pathogenic behavior accordingly (Lo et al., 1997).

Adhesion: Adhesion is the initial step in Candida colonization and infection. C. albicans expresses a family of cell surface adhesins, including Agglutinin like sequence (Als) proteins, which mediate adherence to host epithelial and endothelial cells, extracellular matrix components (such as collagen and fibronectin), and medical devices. Als proteins contain adhesive domains

that interact with specific host ligands, facilitating fungal attachment to host surfaces. Adhesion promotes the establishment of Candida infections by facilitating colonization, biofilm formation, and evasion of host immune responses (Hoyer et al., 2008; Sundstrom, 2002).

Biofilm Formation: *Candida albicans* has the ability to form biofilms on both biotic and abiotic surfaces, such as mucosal tissues, indwelling medical devices (e.g., catheters, prosthetic implants), and environmental substrates. Biofilms are structured communities of microorganisms encased in an extracellular matrix composed of polysaccharides, proteins, and DNA. Within biofilms, C. albicans cells are protected from host immune defenses, antimicrobial agents, and environmental stresses. Biofilm-associated infections are often chronic, difficult to eradicate, and associated with increased morbidity and mortality in immunocompromised patients (Ramage et al., 2009; Finkel & Mitchell, 2011).

Secreted Enzymes: *Candida albicans* secretes various enzymes that contribute to its pathogenicity by facilitating tissue invasion, nutrient acquisition, and immune evasion. Secreted aspartyl proteinases (SAPs) are a family of proteolytic enzymes that degrade host proteins, including immunoglobulins, cytokines, and extracellular matrix components, promoting tissue damage and immune evasion. Phospholipases hydrolyze host cell membranes, facilitating fungal dissemination and evasion of host immune responses. Lipases, esterases, and hemolysins are additional enzymes produced by C. albicans that contribute to its virulence by disrupting host cell membranes and promoting tissue damage (Naglik et al., 2003).

Antifungal Resistance: *Candida albicans* can develop resistance to antifungal agents commonly used in clinical practice, such as azoles, echinocandins, and polyenes. Resistance mechanisms include alterations in drug targets (e.g., mutations in the lanosterol 14α-demethylase enzyme targeted by azoles), overexpression of efflux pumps (e.g., Cdr1p, Mdr1p), and biofilm formation. Antifungal resistance poses a significant challenge in the management of Candida infections, particularly in immunocompromised patients and those with underlying comorbidities (Lee et al., 2023).

Iron Acquisition: Iron is an essential nutrient for microbial growth, and *Candida albicans* has evolved mechanisms to acquire iron from the host environment. These mechanisms include the production of high-affinity iron uptake systems, such as reductive iron assimilation and siderophore-mediated iron acquisition. Candida cells express cell surface receptors and transporters that bind and internalize iron-bound siderophores or heme from host proteins. Iron

acquisition enhances the survival and virulence of C. albicans in iron-limited host niches, such as the mucosal surfaces (Heymann et al., 2002).

Escape from Host Immune Response: *Candida albicans* has developed multiple strategies to evade host immune defenses and establish infection. These strategies include masking of cell wall components (e.g., β-glucan) to evade recognition by host pattern recognition receptors (PRRs), inhibition of phagocytosis through the secretion of factors that interfere with host immune cell function (e.g., candidalysin, secreted aspartyl proteinases), and modulation of host immune signaling pathways (e.g., Toll-like receptor signaling, inflammasome activation). Candida cells can also switch between different morphological forms (yeast, pseudohyphae, hyphae) to evade immune detection and clearance by host immune cells (Netea et al., 2008).

Toxin Production: *Candida albicans* produces various toxins and virulence factors that contribute to host cell damage and immune activation. Candidalysin, a cytolytic peptide toxin, disrupts host cell membranes, leading to host cell lysis and the release of pro-inflammatory cytokines and chemokines. Candidalysin promotes tissue inflammation, neutrophil recruitment, and fungal dissemination, contributing to the pathogenesis of Candida infections. Additionally, C. albicans produces other toxins, such as phenol-soluble modulins (PSMs), which have cytotoxic and immunomodulatory effects on host cells, further exacerbating tissue damage and inflammation (Moyes et al., 2016)

1.5. Hyphal Stage Virulence Switch and its Blockers

Cues from outside the cell can prompt the transition of yeast to hyphae in *C. albicans*, including elevated temperature (around 37°C), increased CO_2 levels (approximately 5%), neutral pH, lack of nutrients, exposure to serum, peptidoglycan, and N-acetylglucosamine. Conversely, molecules involved in quorum sensing, whether originating from within the organism or from external sources, can inhibit this transition. In addition to environmental cues from the host, *C. albicans* morphogenesis is influenced by quorum sensing molecules (QSMs) of both internal and external origin. Notably, tyrosol and farnesol, produced by *C. albicans*, respectively promote and inhibit the yeast to hyphae transition. Farnesol inhibits by blocking the ubiquitin ligase mediated degradation of a transcriptional repressor CUP9 through ubiquitination and downregulation of cAMP/PKA signaling. The other QSMs, including aliphatic alcohols like ethyl and isoamyl

alcohol, interact with pathways such as Ras1-cAMP, where farnesol's effect can be counteracted by dibutyryl-cAMP. Farnesol also inhibits filamentous growth via negative regulators Tup1 and Nrg1 (Chow et al., 2021).

Moreover, interactions with other microorganisms, like Gram-negative bacteria found in cystic fibrosis patients' lungs, such as *P. aeruginosa*, *S. maltophilia*, and *B. cenocepacia*, impact the morphogenesis of *C. albicans*. Exogenous QSMs like 3-oxo-C12-homoserine lactone and phenazines from *P. aeruginosa* have been identified to inhibit *C. albicans* hyphal development. Diffusible signal factor (DSF) produced by *S. maltophilia*, and BDSF (cis-2-dodecenoic acid) produced by *B. cenocepacia* increase Ubi4 & Sfl1 suppressors of hyphal transition (Chow et al., 2021).

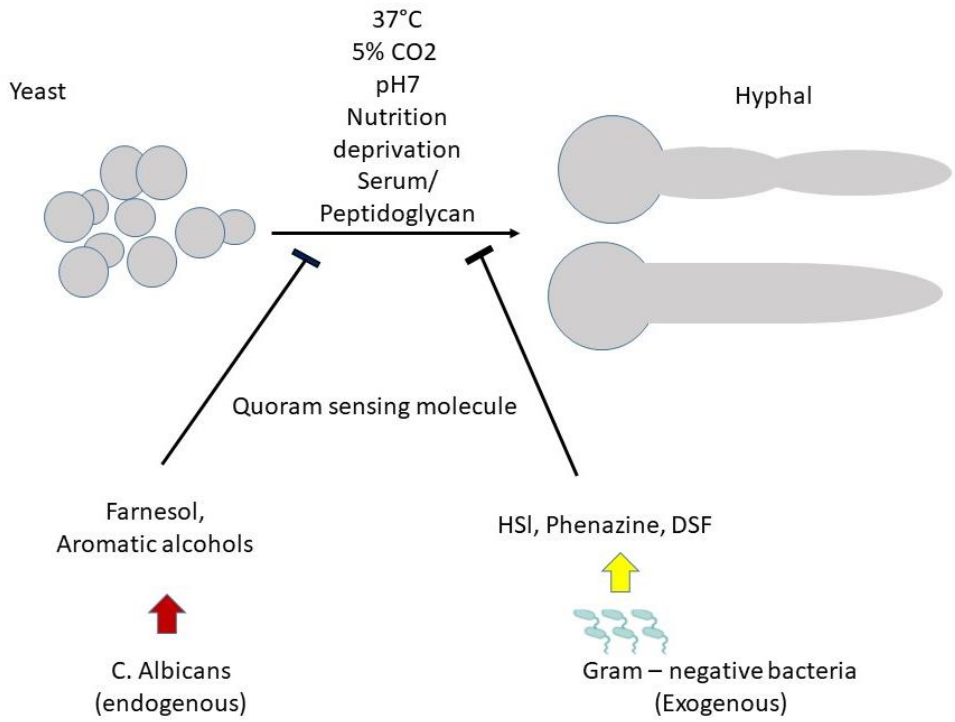


Figure 1.2 Morphology Switch relation with Exogenous and Endogenous quorum sensing molecule.

1.6. Cell Surface Composition

The cell wall structure of *C. albicans* includes an inner framework composed of chitin and β-glucans (the (1→3)-β-D-glucan and (1→6)-β-D-glucan), as well as an outer layer rich in glycosylated mannoproteins, with approximately 84% being water insoluble (1→3)-β-D-glucan. The adhesion of yeast cells to host surfaces is facilitated by adhesins. Upon contact with host cells, this adherence triggers the transition from yeast to hyphae and directed growth through thigmotropism (Hua et al., 2023). The expression of invasins then facilitates the uptake of the fungus by the host cell via induced endocytosis. Additionally, another invasion mechanism involves adhesion, physical forces, and secretion of fungal hydrolases to aid in the breakdown of barriers, enabling fungus-driven active penetration into host cells.

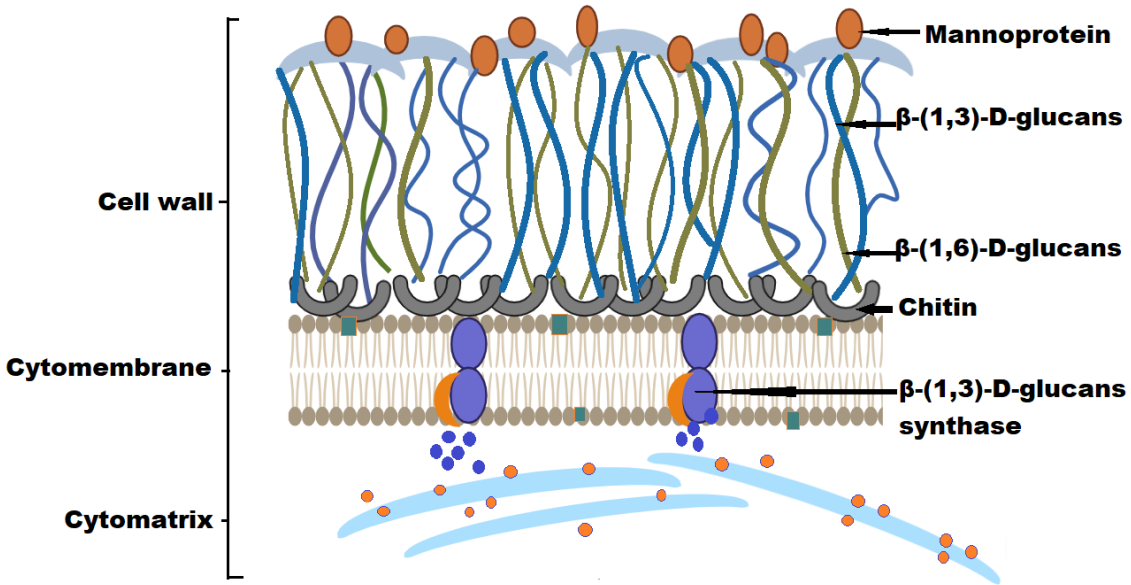


Figure 1.3 C. albicans Cell suface composition

1.7. *C. albicans* Cell cycle

Moreover, the morphogenetic transitions in *C. albicans* are intimately linked to the cell cycle progression. Eukaryotic cells cycle through distinct phases G1, S, G2, and M are coordinated by proteins such as cyclins, cyclin-dependent kinases (CDKs), and checkpoint proteins. In yeast

forms, cell cycle events are coordinated with bud emergence and nuclear division, leading to the formation of separated rounded cells. However, in filamentous forms, cell cycle progression differs; pseudohyphae remain attached and elongate due to a prolonged G2 phase, while hyphae exhibit a unique cell cycle pattern. Here, a germ tube forms before the G1 phase to S phase transition. Nuclear division occurs within the elongating hypha, with cytokinesis failing to separate the cells.

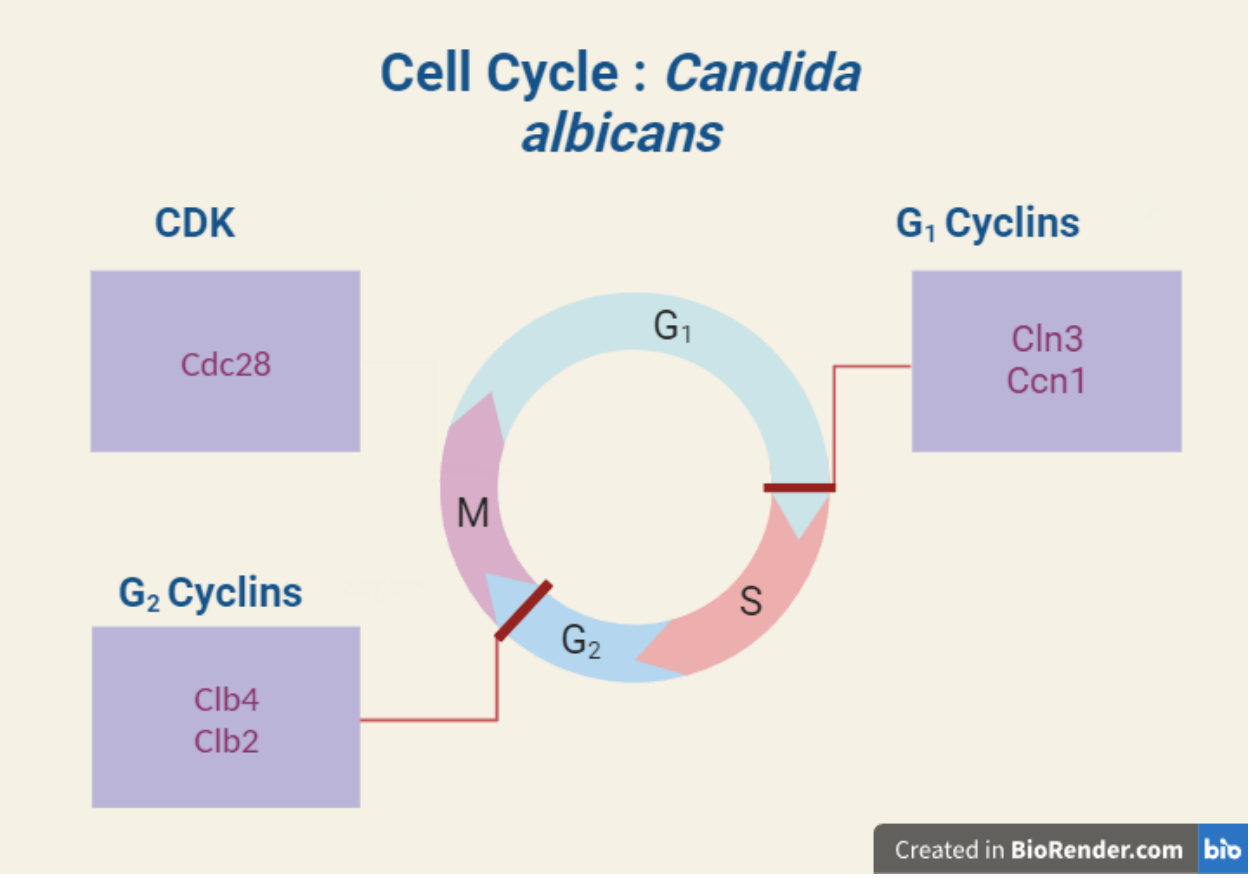


Figure 1.4 Cell cycle stage cyclins/CDK

1.8. Cyclins/ CDK levels in Yeast vs hyphal morphogenesis

The polarized growth of hyphae is associated small GTPases tightly regulated by cell cycle-associated cyclins and CDK most of *C. albicans*. *C. albicans* possesses three G1 cyclins (Ccn1, Cln3, Hgc1) and two essential B-type mitotic cyclins (Clb2, Clb4). Cdc28, the master regulator cyclin dependent kinase (CDK), governs cell cycle progression at G1/S and G2/M phases via specific cyclin interactions (Umeyama et al., 2006). Cyclin levels oscillate during the cell cycle, with stable Cdc28 levels. In yeast cells, the concentrations of Ccn1 and Cln3 are elevated during the G1 phase, aligning with the emergence of buds and apical growth, but decrease as cells progress into early G2. On the other hand, levels of Clb2 rise to their maximum during the early G2/M phase (Bensen et al., 2005), while Clb4 reaches its peak during the mid-G2/M phase. Both B-cyclin levels decline as cells enter the M phase and eventually vanish upon exiting mitosis (Clemente et al., 2006; Bensen et al., 2005). In hyphal cells, polarized growth continues at the apical site throughout the cell cycle, due to the initial accumulation of Ccn1 and Cln3 and their persisting presence for a long time during hyphal growth (Loeb et al., 1999; Beson et al., 2005), that leads to extension of G1 phase. Due to the extended time the mitotic cyclins Clb2 and Clb4, are delayed in hyphal cells. The elevated levels of Ccn1 maintain hyphal growth, along with Cln3. The forkhead transcription factor Fkh2 generally undergoes cell cycle-dependent phosphorylation to regulate the cell cycle progression by expressing cyclins. However, Fkh2 in hyphal condition is phosphorylated by Ccn1/Cln3-Cdc28 in a cell cycle independent manner (Beson et al., 2002; Greig et al., 2015), redirecting it to enhance the expression of hyphal-specific genes such as the hyphal-specific G1 cyclin HGC1. In addition to being suppressed by Tup1 and Nrg1, the activity of the transcription factor Ume6 positively promotes the expression of *HGC1* (Zheng et al., 2004) . Ume6 ensures continuous expression of Hgc1 throughout the cell cycle as long as the conditions inducing hyphal growth persist. Induction of hyphal growth triggers the phosphorylation of Fkh2, leading it to enhance the expression of genes specific to hyphae. Additionally, the hyphal-specific cyclin Hgc1, under the regulation of Ume6, governs hyphal morphogenesis regardless of cell cycle control. It interacts with Cdc28 to regulate diverse cellular processes

1.9. Ubiquitin

Ubiquitin is a small polypeptide consisting of 76 amino acid residues, well conserved during evolution (Finley et al., 1991). Protein sequence and structure of ubiquitin are the best examples of evolutionary conservation with only three or fewer replacements in amino acid residues from yeast to higher animals and plants (Lin et al., 2014). Inside the cells ubiquitin is mostly present in the free form but under physiological conditions, ubiquitin is covalently joined to a variety of protein species through the action of E1 Ub-activating enzyme, E2 Ub-conjugating enzyme, sequentially, to target their degradation or trafficking (Figure 1.7). The choice between trafficking and degradation processes depends upon the lysine linkage pattern between ubiquitin molecules in the polyubiquitin chain built on the substrate protein (Hershko et al., 1998; Ciechanover et al., 1998). In *C. albicans* only two ubiquitin genes are present, the UBI3 and *UBI4*. *UBI3* gene encoding a ubiquitin fusion protein composed of one ubiquitin sequence with a 3P tail of 225 bp is highly homologous to the S. cerevisiae UBI3 3P tail. The *UBI4* gene encoding polyubiquitin which contains three ubiquitin repeats in a head to tail spacerless arrangement (Sepulveda et al., 1996). *UBI3* Expression in *C. albicans* is repressed by stress conditions, such as upshift in temperature, starvation, as described for other genes of *C. albicans* (SSB1) involved in translation (Hershko et al., 1998; Ciechanover et al., 1998).

1.10. Ubiquitin Structure

The three-dimensional structure of ubiquitin was first solved by X-ray crystallography at 2.8 Å and then 1.8 Å resolution in the mid 1980s (Vijay kumar et al., 1985). Its structure is finely compact, characterized by the presence of two short α helices (α1 and α2) and five β strands (β1 to β5) that collectively form a β-sheet fold (Figure1.5).

These secondary structural elements are interlinked by flexible loops, facilitating dynamic conformational changes essential for its multifaceted functions. The α helix contributes to the stability of ubiquitin's structure and are instrumental for mediating protein-protein interactions, while the β strands create a scaffold that allows for the formation of a compact, globular fold. This structural arrangement not only confers stability but also enables ubiquitin to engage in intricate molecular interactions.

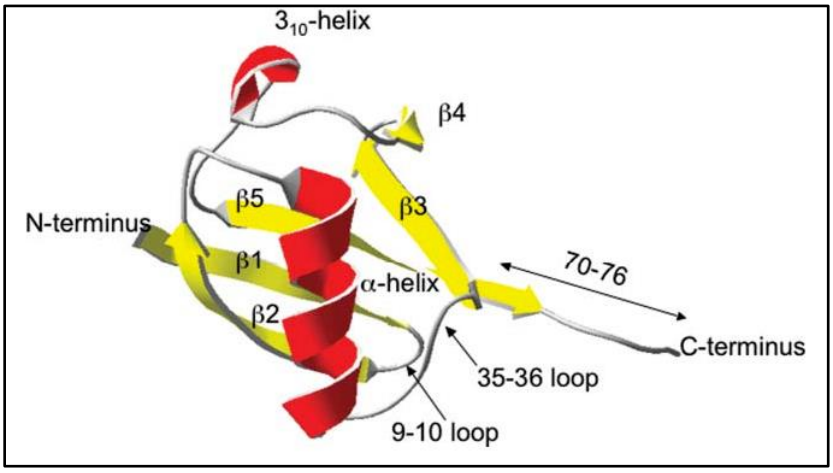
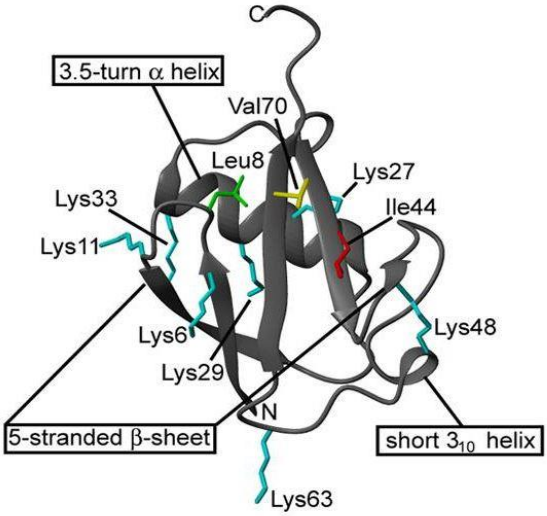


Figure 1.5 Tertiary structure of ubiquitin (Jackson & S. E. 2006)

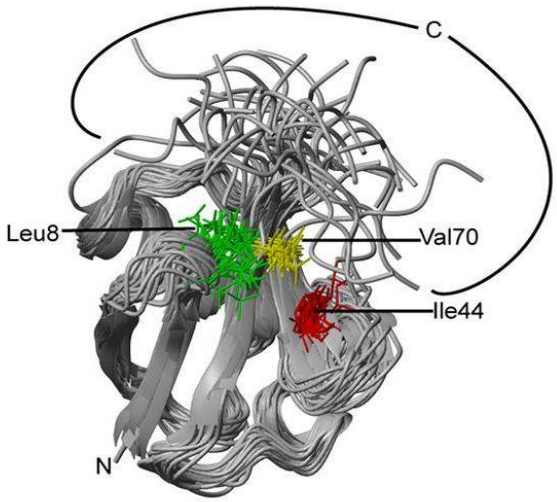
1.11. Proximal and Distal ubiquitin structural diversity

Ubiquitin, a crucial regulatory protein, exhibits a complex structure consisting of a 5-stranded β-sheet, a 3.5-turn α-helix, and a short 3_{10} helix. This structure includes solvent exposed Lys residues that facilitate the assembly of ubiquitin chains, important for cellular signaling. Additionally, hydrophobic residues like Leu8, Ile44, and Val70 provide a platform for interactions with ubiquitin-binding domains (UBDs) (Figure 1.6 a). In solution, ubiquitin displays conformational diversity, with distinct conformations influenced by specific UBD interactions. For instance, Lys48-linked diubiquitin forms compact structures due to inter-moiety interactions (Figure 1.6 c), whereas Lys63-linked and linear diubiquitin chains adopt extended conformations (Figure 1.6 d). These variations in linkage and conformation contribute to the robustness of ubiquitin-mediated signaling pathways.

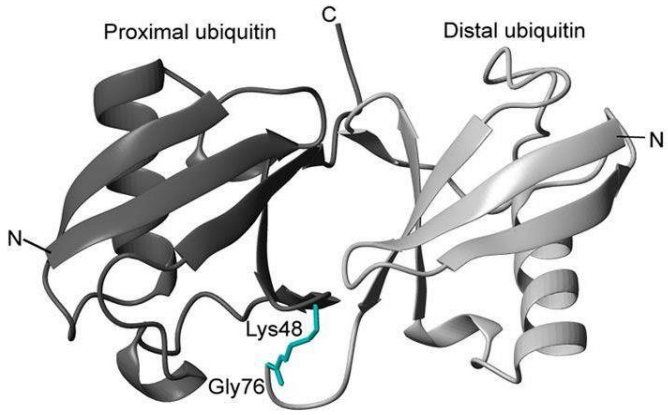
a Ubiquitin



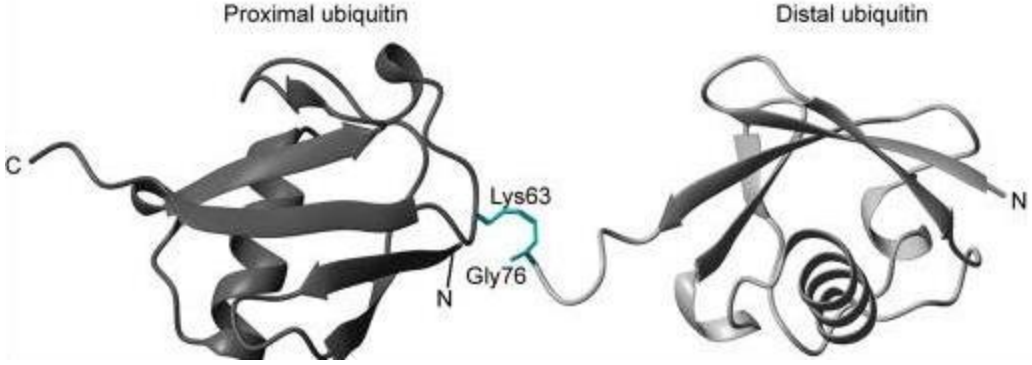
b Conformational diversity of ubiquitin



c Lys48-linked diubiquitin



d Lys63-linked diubiquitin



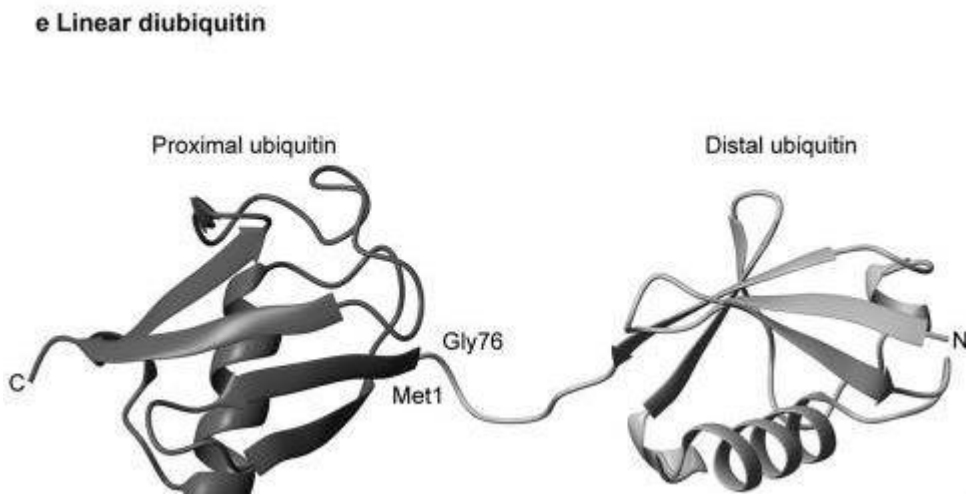


Figure 1.6 Proximal and Distal ubiquitin structural diversity under K48, K63, and linear ubiquitin (Dicic et al., 2009).

1.12. UPS key functions as a central player in the cell cyle and filamentation of *C. albicans*

The Ubiquitin-Proteasome System (UPS) is a crucial, evolutionarily conserved mechanism in eukaryotes that maintains cellular proteostasis, regulates the cell cycle, and influences filamentation (Finley et al., 2012). In *C. albicans*, disruption of the UPS components leads to severe consequences such as protein aggregation and cell-cycle disruption, profoundly affecting morphogenesis (Hossain et al., 2020; Kornitzer, 2019). Polyubiquitinated proteins are preferentially targeted to the proteasome for degradation, whereas monoubiquitinated substrates have diverse cellular roles (Finley et al., 2012; Weissman, 2001). In *C. albicans*, polyubiquitin is encoded by *UBI4*, and its depletion induces abnormal morphogenesis (Sepulveda et al., 1996).
The putative E2 Rad6 represses morphogenesis and its deletion inducing filamentation likely through cAMP-PKA signaling (Leng et al., 2000). A thorough characterization of all *C. albicans* ubiquitin-modifying enzymes remains to be achieved and is likely to reveal additional functional connections (Finley et al., 2012). Moreover, the UPS is essential for the degradation of cyclins and CDK inhibitors to ensure orderly progression through the cell cycle (Adams, 2004).

Two multiprotein E3 complexes, the Skp1-Cullin-F-box (SCF) complex and the anaphase-promoting complex/cyclosome (APC/C) complex, coordinate the degradation of key cell-cycle regulators (Finley et al., 2012). Disruption of SCF complex activity induces morphogenesis by stabilizing its substrates, which include proteins like G1 cyclins, CDK inhibitors, and

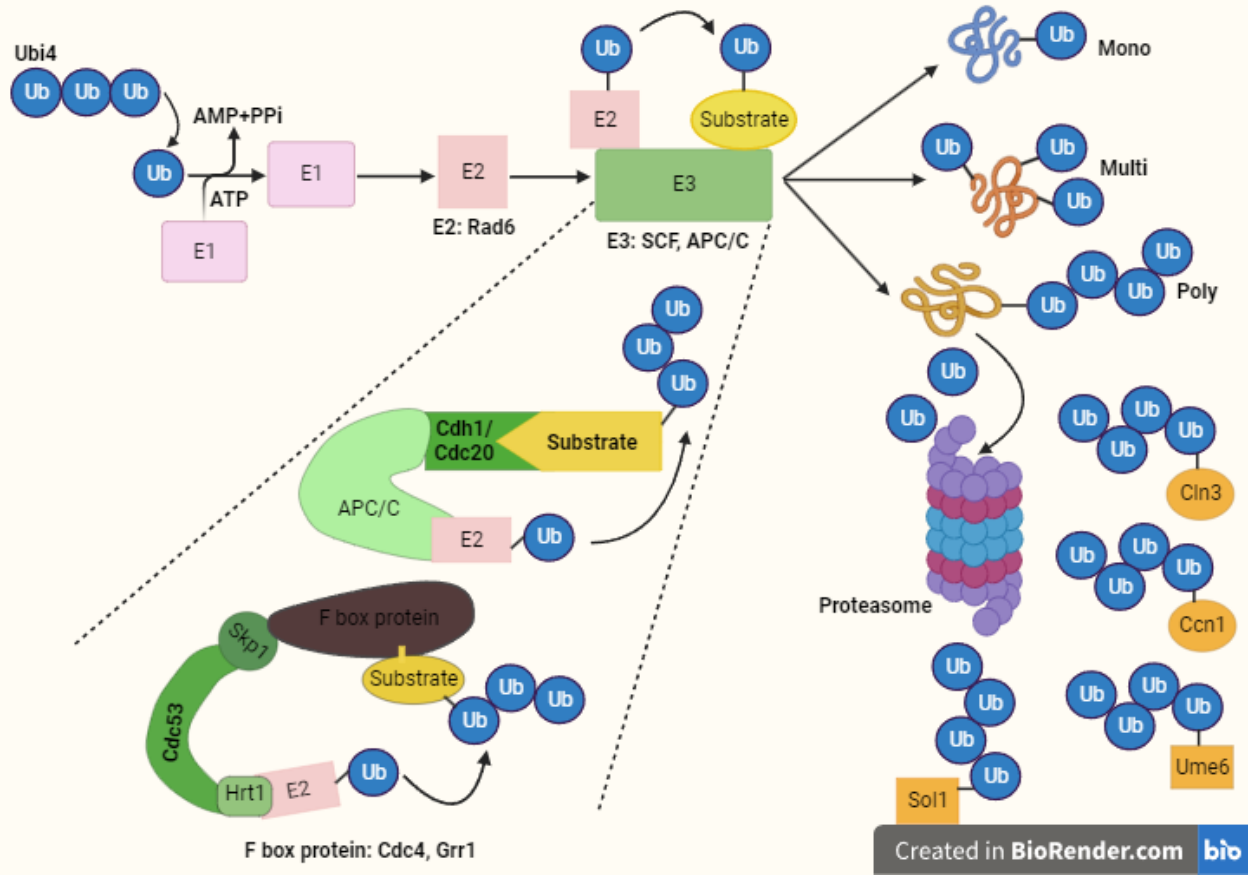


Figure. 1.7 Ubiquitin proteasome regulates cyclin and CDK inhibitors through ubiquitin ligase.

filamentation regulators such as Ume6 (Atir-Lande et al., 2005; Li et al., 2006; Mendelsohn et al., 2017). Similarly, the APC/C complex, although less characterized in *C. albicans*, has connections with morphogenesis. CDC20 and CDH1 are regulatory proteins known as activators of the anaphase-promoting complex/cyclosome (APC/C) in *Candida albicans*, similar to their counterparts in other eukaryotic organisms. Depletion of CDC20 or CDH1 leads to aberrant cell cycle and morphological phenotypes, highlighting the importance of these complexes in regulating morphogenesis through proteolysis (Chou et al., 2011).

Furthermore, inhibition of the proteasome interferes with cell cycle progression and induces filamentation in *C. albicans* by accumulating ubiquitinated proteins (Hossain et al., 2020). The UPS thus establishes another hub of connections for proteostasis, cell cycle, and morphogenesis. Additionally, the UPS influences filamentation by modulating the stability of morphogenetic regulators. For instance, Nrg1 degradation is mediated by the E3 Ubr1, enabling hyphal formation (Lu et al., 2014). Ume6 degradation is regulated by CDK phosphorylation and SCF-mediated ubiquitination, impacting filamentation (Lu et al., 2019).

1.13. Transcriptional factors (Activators/Inhibitors)

In *C. albicans*, transcriptional regulators play crucial roles in controlling gene expression and regulating various cellular processes. Among the transcriptional regulators.

1.13.1. Activators

Fkh2 (Forkhead box protein H2): Fkh2 is known to function as a transcriptional activator in C. albicans, regulating genes involved in cell cycle progression, morphogenesis, and virulence (Cote et al., 2009). Efg1 (Enhanced filamentous growth protein 1): Efg1 acts as a positive regulator of filamentation in C. albicans, promoting the transition from yeast to filamentous forms, which is important for virulence and biofilm formation (Basso et al., 2009). Stp1 (Ssy1-Ptr3-1): Stp1 is involved in the regulation of amino acid permease genes in response to extracellular amino acid concentrations. It functions as a positive regulator of amino acid uptake and metabolism. Tec1 (Transcriptional enhancer factor 1): Tec1 is a key transcription factor in *C. albicans* that regulates genes involved in hyphal development and biofilm formation. It acts as an activator of genes promoting filamentous growth.

1.13.2. Inhibitors

Rbf1 (Repressing factor binding protein 1) acts as a transcriptional repressor in *C. albicans*, regulating genes involved in cell cycle progression, morphogenesis, and virulence. It typically opposes the activity of Fkh2. Rim101 (Regulator of pH response 101) is involved in the alkaline pH response in *C. albicans* and acts as a negative regulator of filamentation under alkaline conditions. It represses genes involved in filamentous growth.

1.14. Rationale for selecting the ubiquitin mutations characterized in *S. cerevisiae* to investigate cell cycle and morphogenesis of *Candida albicans*

The rich repository of information available on *S. cerevisiae* positions it as an excellent initial model for hypothesis testing before exploring similar queries in a more complicated yeast such as *C. albicans*, an opportunistic human pathogen. Both *C. albicans* and *S. cerevisiae* trace back to a common ancestor, which had existed over 300 million years ago. Notable parallels in the molecular details of cell cycling, mating behavior, metabolism, cell wall formation, and signaling processes have been thoroughly documented for both organisms. However, the CUG codon of *S. cerevisiae* encodes Leucine (Leu), while the same codon encodes Serine (Ser) in *C. albicans*. To date very little has been reported about ubiquitin regulation and its relation to morphological switching in *C. albicans*. Ubiquitin mutations studied to gain insights into various cellular processes in *S. cerevisiae* (Prabha et al., 2010; Mishra et al., 2011; Doshi et al., 2017) have been employed here to understand cell cycle dynamics and morphogenesis of *C. albicans*, especially concerning the transition from yeast to hyphal form, an essential step in pathogenesis.

1.15. Partial disruption of ubiquitination by lethal mutants of ubiquitin reveals its importance over cell cycle and morphogenesis in the opportunistic human pathogen *Candida albicans* bringing into light the involvement of various molecular factors

Post-translational modifications play regulatory role by acting as on-off switches in many metabolic activities. Ubiquitination, one of the post-translational modifications, playing pivotal role in diverse activities of the eukaryotic cells by affecting DNA packing, transcription, protein degradation and signaling a gamut of other activities is not fully understood. The objective of this study is to understand the influence of ubiquitination over cell cycle and morphogenesis associated virulence of the opportunistic human pathogen *Candida albicans*, and provide information on the possible targets of designing novel drugs. Here mutant forms ubiquitin were employed to probe the process of ubiquitination and its possible recipient prtein substrates.

Chapter 1 provides a comprehensive overview of *Candida albicans*, emphasizing its role as a human commensal organism and opportunistic pathogen. It highlights the emergence of drug-resistant fungal infections and the clinical challenges associated with invasive candidiasis,

particularly in immunocompromised individuals. The different types of candidiasis, such as vaginal, invasive, and oral candidiasis, are discussed along with their clinical manifestations and treatment options.

Furthermore, the introduction delves into the impact of head and neck radiotherapy on immunocompromised patients, particularly in exacerbating candidiasis. It also explores the virulence factors of C. albicans, including morphogenesis, adhesion, biofilm formation, secretion of enzymes, antifungal resistance, iron acquisition, evasion of host immune response, toxin production, and cell surface composition. The role of ubiquitin-proteasome system (UPS) in regulating the cell cycle and morphogenesis of C. albicans is addressed, highlighting its importance in maintaining cellular proteostasis and influencing filamentation. Additionally, the introduction discusses transcriptional factors as activators and inhibitors of gene expression in C. albicans, shedding light on their roles in regulating various cellular processes. The rationale for selecting ubiquitin mutations characterized in S. cerevisiae to investigate cell cycle and morphogenesis of C. albicans is also provided, emphasizing the evolutionary conservation and similarities between the two yeast species. Overall, the introduction sets the stage for a detailed exploration of the molecular mechanisms underlying *Candida albicans* pathogenesis, highlighting the interconnectedness of various cellular processes and their implications for human health.

In **Chapter 2**, the study involved several key experiments to investigate the functional implications of mutant ubiquitin proteins in *Candida albicans*. Initially, site-directed mutagenesis was employed to generate CUG codon to UUG codon at 56^{th} position in previously generated mutant forms of the ubiquitin gene of *S. cerevisiae*, which were subsequently cloned into the pRC2312 vector. Confirmation of successful cloning was achieved through various molecular techniques, including gel electrophoresis and restriction digestion. Upon transformation into DH5α cells, the presence of mutant ubiquitin genes was tested, followed by further confirmation through PCR amplification and DNA sequencing.

Expression of the mutant ubiquitin genes in C. albicans BWP17 strain using the pTET25M-NC vector allowed for functional analysis. Dosage-dependent lethality and survival capacity were assessed under varying doxycycline concentrations, revealing distinct responses among mutants. Notably, mutants UbEP42, UbL50P, and UbI61T exhibited compromised growth, reduced

survival, and impaired thermotolerance under heat stress conditions. Similarly, these mutants displayed decreased viability under antibiotic stress, highlighting their susceptibility to external stressors.

Furthermore, the study explored the impact of mutant ubiquitins on critical cellular processes. Mutants UbEP42, UbL50P, and UbI61T disrupted the yeast-to-hyphal transition, impairing the infective capability of C. albicans. Additionally, these mutants caused cell cycle arrest in the G0/G1 phase, delaying entry into the S phase. This effect was associated with a decrease in Cdc28 protein kinase levels, suggesting a disruption in cell cycle regulation. Moreover, impaired polyubiquitination with both K63 and K48 linkages was observed in mutants UbEP42, UbL50P, and UbI61T, indicating alterations in protein degradation pathways.

In summary, the study elucidated the multifaceted impacts of mutant ubiquitin proteins on various aspects of C. albicans biology, including growth, stress response, cell cycle regulation, and protein ubiquitination. Scanning Electron Microscopy (SEM) highlighted irregularities in the cell surface of mutants UbEP42, UbL50P, and UbI61T, indicating disruptions in the fungal cell wall structure. Further analysis revealed uneven chitin deposition and increased β-glucan exposure on the cell wall of these mutants, impacting cell aggregation and immune recognition.

Moreover, the study investigated the regulation of aspartyl protease secretion under different pH conditions, elucidating the role of ubiquitin mutations. Mutants UbEP42, UbL50P, and UbI61T displayed significantly decreased protease secretion, particularly at acidic pH levels, affecting virulence factors and morphological transitions in C. albicans. Gelatine zymography further corroborated the reduced protease activity in these mutants.

Overall, the study provides insights into the molecular mechanisms underlying the impact of ubiquitin mutations on cell morphology, cell cycle progression, protease secretion, and cell wall composition in C. albicans.

In **Chapter 3**, the impact of ubiquitin mutations on cyclin levels throughout the cell cycle in *Candida albicans* was investigated. Prior studies in Saccharomyces cerevisiae revealed intriguing effects of ubiquitin mutations on cellular functions, prompting an investigation into C. albicans. Quantitative real-time PCR was employed to analyze cyclin expression levels in BWP17 transformants expressing wild-type and mutant ubiquitin forms. Notably, G1 cyclins Ccn1 and Cln3 exhibited upregulation and sustained expression in mutants UbEP42, UbL50P,

and UbI61T, disrupting the typical transition to G2 cyclins Clb2 and Clb4. This imbalance likely contributed to cell cycle arrest and impaired morphogenesis, evidenced by the failure of yeast-to-hyphal transition observed with these mutants.

The study provides molecular insights into the decreased viability, morphogenesis failure, and cell cycle arrest induced by ubiquitin mutations in C. albicans. The mutants' impact on cyclin levels, particularly the sustained expression of G1 cyclins and inhibition of G2 cyclin expression, underscores the disruption of normal cell cycle progression. Further research, including proteomics analysis, is warranted to unravel the underlying metabolic pathways and potential therapeutic targets for Candida infections.

In **Chapter 4**, the impact of ubiquitin mutations on the molecular mechanisms underlying the pathogenesis of *Candida albicans* were investigated by focusing on three specific ubiquitin mutants: UbEP42, UbL50P, and UbI61T. Previously it was shown that these mutants cause dosage dependent by affecting various cellular processes in C. albicans. The main aim of this study was to understand the regulation of transcription factors involved in cell cycle and morphogenesis in C. albicans when these ubiquitin mutations are expressed. We employed real time PCR and proteomics to understand their influence on cellular physiology.

Through quantitative real-time PCR analysis, we observed dysregulation of key transcription factors governing morphogenesis, including Fkh2, Nrg1, Efg1, Rbf1, Rim101, and Tec1, in C. albicans expressing ubiquitin mutations. These mutations led to altered mRNA levels of the transcription factors, potentially impacting the ability of C. albicans to transition into hyphal structures and respond to environmental cues.

Proteomic analysis revealed differential expression of proteins associated with various cellular processes. The results identified common downregulated proteins involved in metabolism, stress response, cell wall synthesis, protein folding, and ubiquitin-mediated proteolysis. These findings suggest potential disruptions in crucial cellular pathways, compromising the viability, stress tolerance, and virulence of C. albicans.

Furthermore, we investigated protein-protein interactions and metabolic pathways affected by the dysregulated proteins. Analysis of the results provided insights into the interconnectedness of these proteins in biological processes and metabolic pathways, highlighting the complex regulatory networks affected by ubiquitin mutations.

Notably, the study identified common upregulated proteins associated with cell cycle regulation, stress tolerance, and ubiquitin signaling in C. albicans expressing ubiquitin mutants. These proteins may represent compensatory mechanisms to counteract the deleterious effects of ubiquitin dysregulation on fungal viability and survival.

Overall, the study contributes to a better understanding of the molecular mechanisms underlying the pathogenicity of C. albicans and provides valuable insights into the consequences of ubiquitin dysregulation.

In **Chapter 5**, the role of the C-terminal di-glycine motif in ubiquitin mutants and its significance in polyubiquitination chain formation in *Candida albicans* were investigated. The research was focused on understanding the mechanism of polyubiquitin chain assembly and its implications for the pathogenicity of C. albicans. It began by highlighting the importance of the di-glycine motif in ubiquitin for initiating polyubiquitin chain formation, crucial for various cellular processes including protein degradation and signal transduction. The result were discussed in relation to the significance of K48 and K63-linked polyubiquitination chains, emphasizing their distinct regulatory functions.

Using a di-glycine deletion strategy, we generated ubiquitin mutants and investigated their impact on polyubiquitin chain formation and K48/K63 chain linkages in C. albicans. Our results showed that mutants lacking the di-glycine motif exhibited impaired polyubiquitin chain assembly, indicating the essential role of this motif in facilitating ubiquitin polymerization.

Furthermore, Western blot analysis revealed differential regulation of K48 and K63 chain linkages in ubiquitin mutants with and without the di-glycine motif. Cell expressing the mutants lacking the motif showed decreased chain linkages, suggesting altered substrate recognition and degradation pathways. Structural bioinformatics analysis provided insights into the molecular basis of di-glycine deletion in ubiquitin mutants, highlighting distinct inter-residue geometries potentially affecting protein-protein interactions and substrate binding.

Overall, the study underscores the critical role of the C-terminal di-glycine motif in ubiquitin-mediated processes in C. albicans. Understanding the molecular mechanisms underlying di-glycine-mediated ubiquitin signaling pathways, holding a promise for the development of targeted therapeutic strategies against fungal infections and related diseases.