Chapter 5

Generating di-glycine deletions of ubiquitin mutants to understand the mechanism of polyubiquitin chain formation in C. albicans expressing ubiquitin mutations.

5.1 Introduction:

The ubiquitin molecule unique C-terminal tail composed of two glycine residues (referred to as C-terminal di-glycine). This di-glycine motif is crucial for the attachment of ubiquitin to substrate proteins and serves as the initiation point for the formation of polyubiquitin chains. Polyubiquitination (Burns et al., 2010), the covalent attachment of ubiquitin molecules to target proteins, regulates various cellular processes, including protein degradation, signal transduction, and DNA repair. The assembly of polyubiquitin chains involves the sequential attachment of ubiquitin moieties through their C-terminal glycine residues, forming isopeptide bonds with lysine residues on target proteins or other ubiquitin molecules (Komander et al., 2009).

5.1.1. Di-glycine motif significance in K48, K63 polyubiquitination chain formation

The di-glycine motif at the C-terminal of ubiquitin plays a pivotal role in this process, serving as a recognition site for ubiquitin-conjugating enzymes (E2) and facilitating the transfer of ubiquitin to target substrates. Among the diverse types of ubiquitin modifications, K48- and K63-linked polyubiquitination are two prominent forms with distinct regulatory functions. K48-linked polyubiquitin chains typically target proteins for proteasomal degradation, whereas K63-linked polyubiquitination is involved in non-proteolytic signaling events such as DNA repair, endocytosis, and cellular differentiation Specifically, K48 and K63 polyubiquitin chains have emerged as key regulators in these processes. (Xie et al., 2017). The importance of the C-terminal di-glycine motif in ubiquitin becomes particularly evident in the context of polyubiquitin chain formation. During ubiquitination, the C-terminal glycine residues of ubiquitin are covalently attached to the ε-amino group of a lysine residue on the target protein via an isopeptide bond, mediated by ubiquitin ligases (Cohen et al., 2014, Burns et al., 2010). This process can be repeated to form polyubiquitin chains, where subsequent ubiquitin molecules are linked through their C-terminal glycine residues to the lysine residues of the preceding ubiquitin in the chain. In K48-linked polyubiquitination, the C-terminal di-glycine motif is essential for the recognition and subsequent degradation of target proteins by the 26S proteasome. The formation of K48-linked polyubiquitin chains serves as a molecular tag that signals the target protein for degradation, thereby regulating protein turnover and maintaining cellular homeostasis. Similarly, in K63-linked polyubiquitination, while the roles of the C-terminal di-glycine motif in chain initiation are conserved, the regulatory outcomes differ

(Neutzner et al.,2012). K63-linked polyubiquitin chains participate in various cellular processes such as DNA repair, immune signaling, and protein trafficking. The versatility of K63-linked polyubiquitination lies in its ability to serve as a scaffold for the recruitment of effector proteins, thereby orchestrating diverse cellular responses.

Furthermore, the deletion or mutation of the C-terminal di-glycine motif in ubiquitin severely impairs its ability to form polyubiquitin chains and subsequently affects its regulatory functions. Studies utilizing ubiquitin mutants lacking the C-terminal di-glycine residues have provided crucial insights into the specific roles of ubiquitin linkages in various cellular pathways. Additionally, understanding the importance of the C-terminal di-glycine motif in ubiquitin has implications for the development of therapeutic strategies targeting ubiquitin-mediated processes in diseases such as cancer, neurodegenerative disorders, and immune-related disorders (Zhang et al., 2023). *Candida albicans*, an opportunistic fungal pathogen, relies on ubiquitin-mediated processes for virulence and adaptation to various environmental stresses. Understanding the mechanism of polyubiquitin chain formation in C. albicans is crucial for elucidating its pathogenicity and identifying potential targets for antifungal therapy. **In this study, we investigated the significance of our ubiquitin mutant's interaction in polyubiquitination chain formation and K48 and K63 chain aggregation/degradation to understand the importance of lethality in C. albicans. So here we used the di-glycine motifs deletion strategy at the C-terminal of our ubiquitin mutants to study the polyubiquitin chain assembly.**

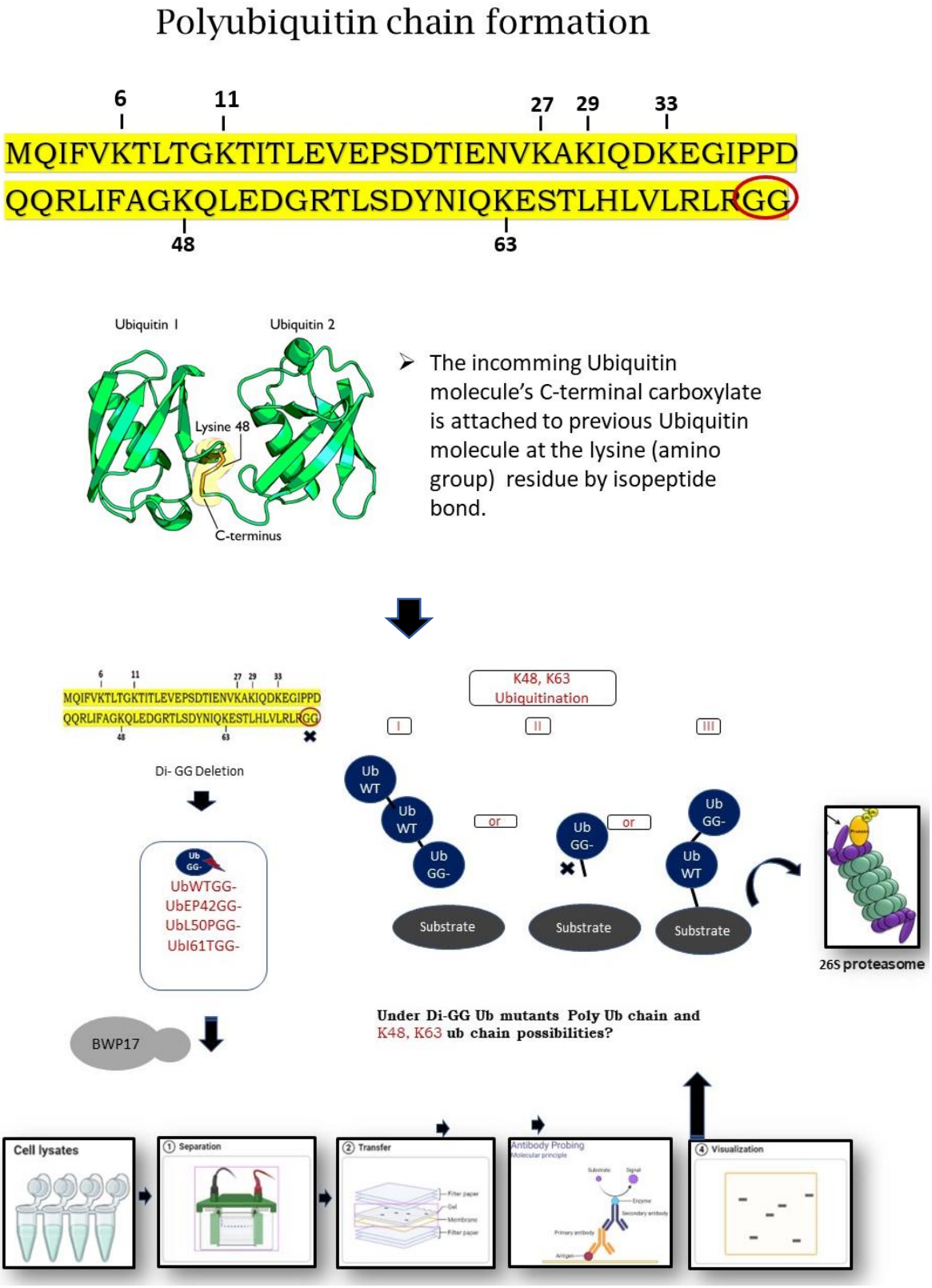


Figure 5.1. Hypothetical illustration for possibility of polyubiquitin chain formation

5.2 Materials and Methods:

5.2.1. Strains and growth conditions:

A strain of BW17(ura3::imm434/ura3:imm434 his1::hisG/his1::hisGarg4::hisG/arg4::hisG), and pTET25M-CH plasmid (Lai et al., 2011) gifted by Prof. Jia-Ching Shieh, Department of Biomedical Sciences, Chung Shan. A Di-glycine deletion ubiquitin mutants' strain, designated as UbWT. UbEP42, Ub L50P and UbI61T. Strain BWP17 was grown routinely in the yeast phase in YPD medium YPD(Chapa et al., 2005), (1% w/v, yeast extract; 2 % w/v, peptone; 2 % w/v, dextrose) and synthetic dextrose (SD) medium containing 0.67% Hi-media yeast nitrogen base and 2% glucose as carbon source, arginine and histidine, with or without uracil (20 mg L-1) as supplements [19]. Escherichia coli strain E. coli DH5α was used for plasmid propagation and cloning purposes. DH5α transformants were grown at 37°C in LB medium supplemented with ampicillin (100 mg ml).

5.2.2. Cloning and Transformation:

(a) Generation of Di- glycine deleted ubiquitin mutants:

Previously cloned ubiquitin mutant UbEP42 and its segregated four individual mutants UbWT. UbEP42, Ub L50P and UbI61T cloned from Yeast Episomal plasmid (YEP) (Finley et. al., 1994) to pTET25 M-CH histidine tag vector between the *Bgl*II and *Xma*I restriction sites. Plasmid pTET25M-CH having Tet- inducible promoter. Polyubiquitination chain formation examined

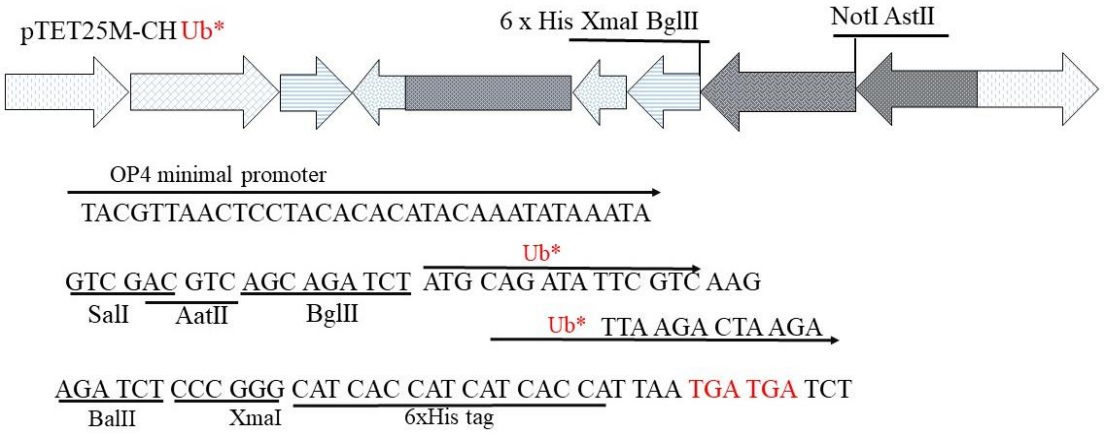


Figure 5.2. Vector map of P-TET25MC-H for ubiquitin Di- glycine mutants cloning and screening.

under Di- glycine deletion ubiquitin mutant UbEP42 overexpression in *C. albicans*. The *C. albicans* UBEP42 ubiquitin mutants and its derivative UbL50P, and UbI61T in the pTET25M--CH vector can be replaced by coding sequences of other genes with several restriction sites of choice

Table 5.1. Primers used for this study

Primer ID	Sequence (5' —> 3')	Length	GC(%)	T_m (°C)	ΔG (kcal/mol)
P1	GCTAAGCTTATGCAGATCTTCGTCAAGACG(fw HindIII)	30	46.67	66.38	-32.06
P2	GATGGATCCTCAACCACCACCTCTTAAGA(Rev.BamH1)	29	48.28	66.18	-30.35
P3GG-	GAGGACGGTAGAACGTTGTCTGATTACAAC(Fw. Mutagenic)	30	46.67	65.85	-31.55
P4GG-	GTTGTAATCAGACAACGTTCTACCGTCCTC(Rev. Mutagenic)	30	46.67	65.85	-31.55
IntF	CATGTCAAAGGATTCAAC	18	39	55	-30.45
IntR	GTATGGTGCCTATCTAAC	18	44	56	-32.11

Table 5.2. C.albicans strains used in this study

Strain	Parental Strain	Genotype	Plasmid	Protein expressed	Reference
BWP17		*ura3::imm434/ura3:imm434 his1::hisG/his1::hisG*	None	None	(Wilson et al., 1999)
UbWTGG-	BWP17	*ADH1/adh1::Ptet-UbWT:URA3-dpl200*	pTET25M-CH UbWT	C-terminal 6×His tag UbWTGG-	This Study
UbEP42GG-	BWP17	*ADH1/adh1::Ptet-UbEP42:URA3-dpl200*	pTET25M-CHUbEP42	C-terminal 6×His tag UbEP42GG-	This Study
UbL50PGG-	BWP17	*ADH1/adh1::Ptet-UbL50P:URA3-dpl200*	pTET25M-CH UbL50P	C-terminal 6×His tag UbL50PGG-	This Study
Ubl61TGG-	BWP17	*ADH1/adh1::Ptet-Ubl61T:URA3-dpl200*	pTET25M-CH Ubl61T	C-terminal 6×His tag Ubl61TGG-	This Study

(b) Transformation in C. albicans:

The pTET25M-CH vector containing wild-type and mutant ubiquitin sequences was transformed into the C. albicans BWP17 strain using standard protocols. Briefly, the DNA fragment carrying the Tet-on promoter along with the gene of interest was removed from the plasmid pTET25 M-CH by digestion with *Sac*II and *Kpn*I, purified using the Thermo Gel extraction kit, and transformed into *C. albicans* cells by the LiAc-PEG-ssDNA method [25]. Selection of the transformants of *C. albicans* was performed as previously described (Shieh *et al*., 2005). The DNA was specifically integrated into the *ADH1* locus of the *C. albicans* genome as a stable integrant and the integration was confirmed by selecting for the Ura^+ prototrophs on minimal medium plates lacking uridine [26]. The integrants confirmed by colony PCR, using the diagnostic primers intF and intR **(Table 5.1)**.

5.2.3. Sequence analysis:

The plasmid with deleted Di- glycine at C- terminal in the ubiquitin mutant gene was sequenced through Sanger's dideoxy nucleotide sequencing method for the detection of deletion at the C-terminal of the ubiquitin mutants. Agri genome sequence the plasmid sample with allied partner IDT, pTET25MC-H plasmid as a backbone through a single forward reaction using primer 5'AGATCTTTATTTGTATAGTTCATCC 3'

5.2.4. Western analysis:

Expression of wild-type and mutant ubiquitin proteins in C. albicans was confirmed by Western blot analysis using specific antibodies against ubiquitin. To examine the polyubiquitination profile in C.albicans under di glycine deleted mutants, Cell lysates from C. albicans expressing wild-type and mutant ubiquitin proteins were subjected to SDS-PAGE followed by Western blotting to detect polyubiquitin chains. Briefly, To detect expression of the tagged UbGG-mutants fusion proteins, *C. albicans* strains were grown in YEPD medium at 30 ◦C overnight, and diluted (1 : 10) into fresh YEPD, for log phase growth and after log phase addition of 40 μg/ml doxycycline (Hi-media) and then grown for 3 h. Lysates were prepared in ice-cold lysis buffer (150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM PMSF, protease inhibitor cocktail) using acid-washed glass beads. The cell lysate was centrifuged at 15000 rpm for 10-15 min at 4 ºC and the supernatant was used for further analysis. The proteins concentration of lysates was estimated using BCA protein quantification method. Proteins samples were separated by 12% SDS-PAGE. Proteins was transferred to the PVDF membrane at 50V overnight followed by blocking with 5% skimmed milk in PBS with 0.05% Tween-20 or 5% BSA in PBS with 0.05% Tween-20 for 1 h. The blot was then probed with primary antibody [1:3000 for either anti-Ub (Santa Cruz), anti-K48 (Cell Signaling), or anti-K63 (Cell Signaling) antibody. Appropriate secondary antibodies (Goat anti-rabbit IgG-HRP; sc-2004; Santa Cruz Biotechnology/ Goat anti-mouse IgG-HRP; 114068001A; GeNei) was then added at a dilution of 1:5000 for 2 h followed by three washes with PBST. The blot was developed on an X-ray film using ECL reagent (BioRAD). All commercially available antibodies were used according to the manufacturer's instructions.

5.2.5. TrRosseta Structural Bioinformatics:

To predict the comparative structural analysis of ubiquitin mutants with Di-GG motif or without Di-GG motif named as Ubiquitin mutant UbWTGG-, UbEP42GG-, UbL50PGG- and UbI61TGG-, the sequence for residues 1-228/222 were submitted to the TrRosetta de novo structure prediction server.

5.3 Results:

5.3.1. Plasmid PTET25M-CH isolation and Pcr for amplification of mutants:

Primarily isolation of plasmid PTET25M-CH incorporated with UbWT, UbEP42, UbL50P, and UbI61T ubiquitin mutants from DH5α cells through alkaline lysis method. PTET25MCH plasmid 7.5 kb long. Further amplification of ubiquitin mutants from PTET25M-CH (Figure 5.3)

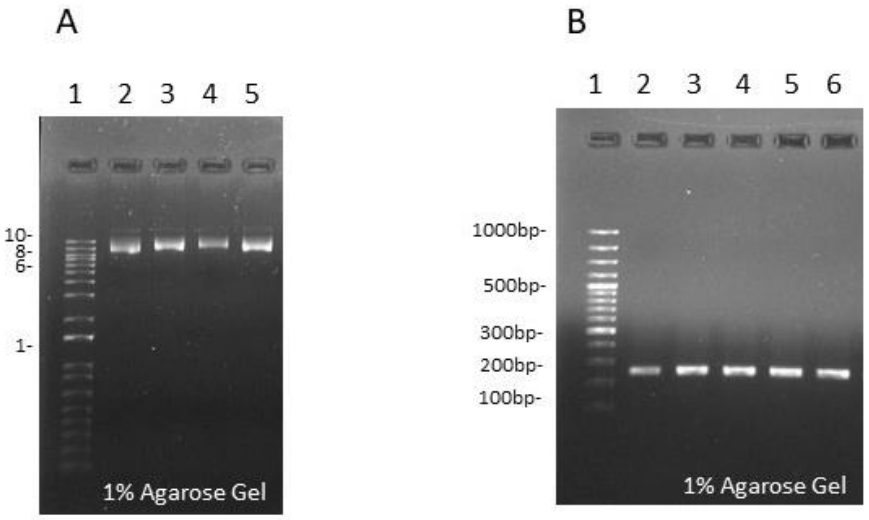
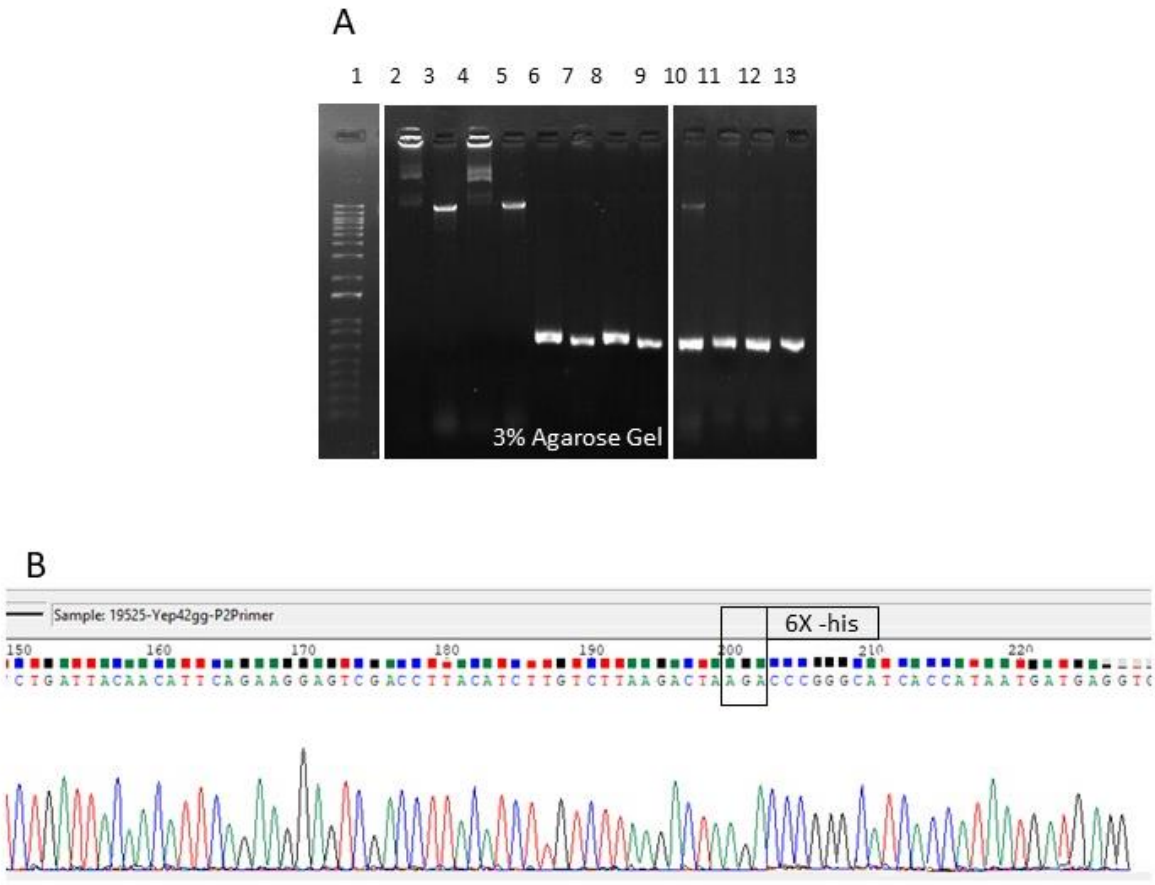


Figure 5.3. Plasmid PTET25M-CH isolation and PCR for amplification of mutants. (A) 1% agarose gel showing (A) PTET25M-CH 7.5 kb plasmid isolation. lane 1 shows lambda HindIII DNA marker, Lane 2-5 is the plasmid PTET25M-CH containing UbWT, UbEP42, UbL50P, UbI61T respectively (B 1% agarose gel showing shows PCR amplicon 228 bp of ubiquitin mutants, lane 1 contains lambda HindIII 1 kb DNA marker, Lane 2-5 showing PCR amplified ubiquitin mutants UbWT, UbEP42, UbL50P, UbI61T using forward and reverse primers listed in Table5.1.

5.3.2. Plasmid PTET25M-CH Restriction Digestion and Di glycine Deletion Sequencing:

The PTET25M-CH vector was subjected to restriction digestion using appropriate enzymes *Bgl*II and *Xma*I to facilitate the insertion of a di-glycine deletion ubiquitin mutants' cassette. The di-glycine deletion cassette was cloned to obtain c- terminal tag with histidine into the digested PTET25M-CH vector using standard molecular cloning techniques. Presence of the di-glycine deletion was confirmed through PCR amplification and sequencing (Figure. 5.4). Restriction

Figure 5.4 PTET25M-CH Di glycine ubiquitin mutants cloning. 3% agarose gel showing



Plasmid PTET25M-CH double digested (Lane 3,5) with *Bgl*II and *Xma*I restriction enzyme. Lane 6 shows UbWT amplified without Di glycine deletion at the C- terminal and lane 7 shows di glycine deletion from UbWT. The difference in their migration pattern is visualized. Similarly, lanes 8, and 9 for UbEP42, lanes 10 and 11 for UbL50P, and lanes 12

and 13 for UbI61T show migration difference. (B) The electropherogram obtained through sequence analysis showed the deletion of Di- glycine in all ubiquitin mutants.

digestion of the PTET25M-CH vector followed by cloning of the di-glycine deletion cassette yielded positive transformants in BWP17 through Lithium acetate PEG ssDNA method. Sequence analysis confirmed the successful incorporation of the di-glycine deletion in the recombinant vectors. Comparison with the wild-type sequence revealed the precise deletion of the di-glycine motif in the modified vectors. This manipulation did not affect the overall integrity of the vector backbone. Primers used for Di- glycine deletion are listed in Table 5.1.

5.3.3. Polyubiquitination of substrate protein under Di- glycine deletion mutants condition in C. albicans:

Western blot analysis of cell lysates from C. albicans expressing ubiquitin mutants lacking the di-glycine motif UbEP42GG-, UbL50PGG- and UbI61TGG-) showed a notable absence of polyubiquitin chains, suggesting that our generated ubiquitin mutant having di- glycine motif at the C- terminal play a pivotal role in impairment in polyubiquitin chain formation in c. albicans that caused to lethality, further it also suggested that individual ubiquitin mutants as a monomer not aggregated in proteome pool and may be degraded through proteasomal machinery. However interestingly we observe that our ubiquitin mutants UbEP42, UbL50P and UbI61T with di-glycine motif conjugated with substrates not degraded and show aggregation of the ubiquitination pool (Figure 5.5C,D). K48 and K63 linkage in UbEP42GG- showing a decline in polyubiquitination chain aggregation.

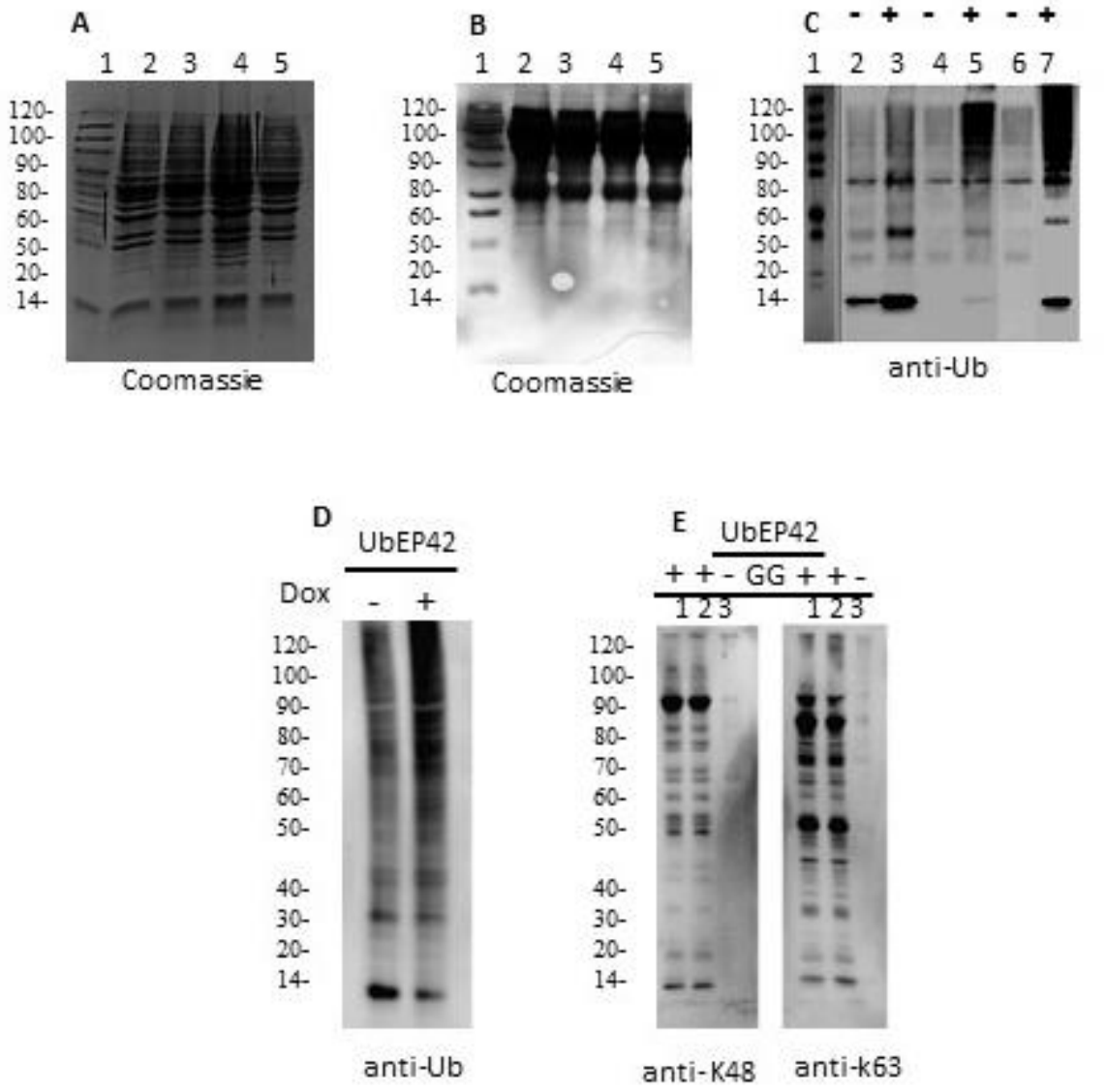


Figure 5.5. Ubiquitination and K48 and K63 linkage under Diglycine ubiquitin mutants. (A) Coomassie-stained SDS-PAGE gel (12%) of yeast stage lysate. Lane 1, SDS-PAGE marker; lane 2-5 total yeast extract proteome profile after induction of UbWTGG-, UbEP42GG-, UbL50PGG- and UbI61TGG- respectively. (B) Coomassie-stained 12% SDS-PAGE gel of hyphal stage lysate. Lane 1, SDS-PAGE marker; lane 2-5 total C. albicans hyphal stage extract proteome profile after induction of UbWT, UbEP42, UbL50P, and UbI61T respectively. (C) Western blot showing ubiquitination profile. *Candida albicans* strains harbouring the ubiquitin mutants with Di-glycine (+) and without Di-glycine (-) constructions were grown in YPD media doxycycline inducer for 3 h. Whole cell extract was separated by SDS-PAGE and probed with an

anti ub antibody, allowing the detection of the ubiquitin protein. Lane 2,3 UbWT, Lane 4,5 UbL50P, and Lane 6,7 UbI61T representing ubiquitination profile with or without Di-glycine (-/+) (D) UbEP42 comparative ubiquitination profile under di-glycine deletion condition. Indicating nondegradative substrate with polyubiquitin chain accumulation decline. (E) K48 and K63 linkage in UbEP42GG- showing a decline in polyubiquitination chain aggregation.

5.3.4. 3D & 2D structure prediction over -/+ Di-glycine ubiquitn mutants:

The online trRosetta server utilizes a method of 3D structure prediction that involves energy minimization, incorporating constraints from predicted inter-residue distances and orientations within the submitted sequence. This process consists of two main steps. Initially, 30 coarse-grained models are produced, focusing on backbone atoms and side-chain centers, using constrained energy minimization guided by predicted 2D geometries between residues. Subsequently, these initial models undergo relaxation to generate the final full-atom models, refining them further for improved accuracy. One of the key features in trRosetta is de novo modeling. Nevertheless, to enhance the performance for targets with detectable homologous structures, the server automatically includes homologous templates which built a deep multiple sequence alignment of 15000 homologous sequences from the uniclust30_2018_08 database) used a machine learning model to estimate the distances and relative angles of each pair of residues. These contact maps were then used to sample coarse-grain and full-atom protein folding conformation spaces to optimize a molecular mechanic's forcefield to find low-energy conformations. The top resulting conformations were highly consistent with pairwise full atom RMSD 0.15 Å, further, we align the Di-glycine deleted ubiquitin mutants structure model generated from trRosetta. Structural alignment was done through the RPSB PDB Mol* 3D viewer (Figure 5.6). The a -helix encompassing residues 24–33 region showing difference in inter residue interaction in Di- glycine deletion ubiquitn mutants. further we investigate to check whether there is any difference in Helix, strand and Coil no. in 2D structure through trRosetta. We don’t find any such difference. all the 2D structures having same no. of Helix, Strand, and Coil (Figure 5.7).

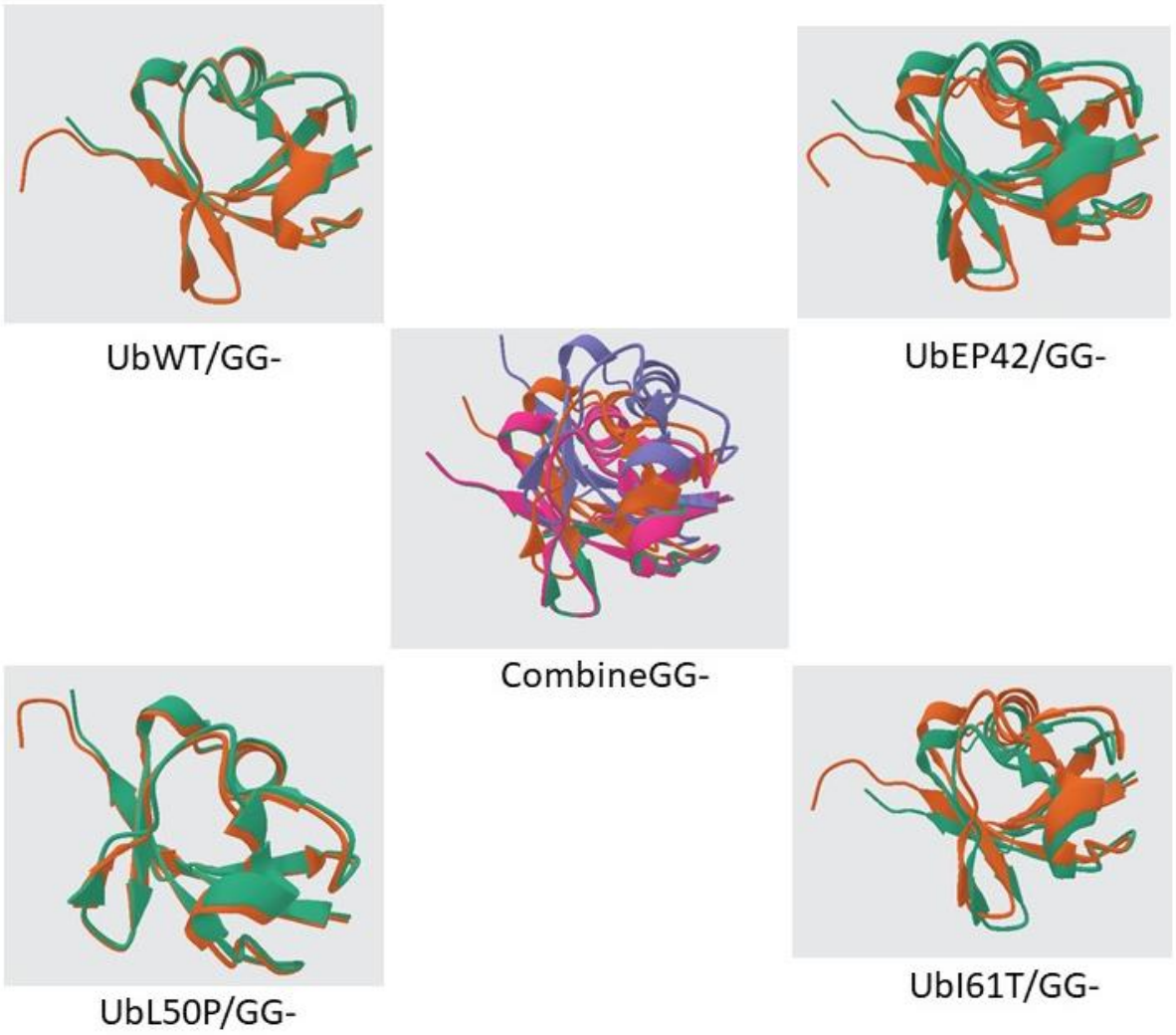


Figure 5.6. Prediction of Di- glycine Ubiquitin mutants inter-residue 3D geometries by trRosetta and align the structure with ubiquitin mutants having Di-glycine motifs at C-terminal using Mol* 3D viewer from protein data bank (RCSB PDB). The predicted structure models With Di-glycine shown in color cartoons (Orange) and without Di-glycine (Green). TM- scores 0.1 for all the constraints model. (B) Representing combine all Di-glycine deleted ubiquitin mutants structures align model using RPSB PDB Mol* 3D viewer.

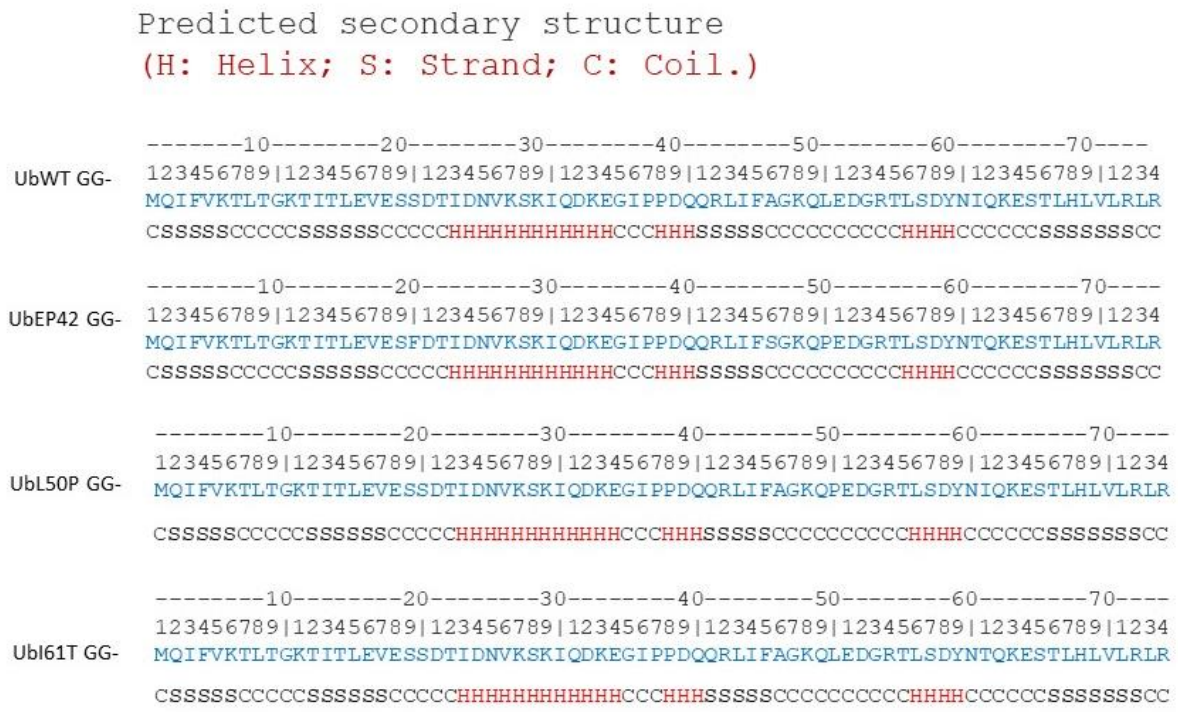


Figure 5.7. Prediction of Di- glycine Ubiquitin mutants inter-residue 2D geometries by trRosetta The predicted secondary structure showing that all ubiquitin mutants have the same no. Helix, Strand, and Coil.

5.4 Discussion:

In this study, we conducted a comprehensive analysis of polyubiquitination profiles and K48/K63 chain linkages in *Candida albicans*, focusing on the significance of previously studied UbWT, and dosage-dependent lethal ubiquitin mutants UbEP42, UbL50P, and UbI61T (Dantuslia et al., 2023). Deletion of di-glycine at c-terminal ubiquitin mutants named as UbWTGG-, UbEP42GG-, UbL50PGG-, and UbI61TGG-. In this study, our findings shed light on, how our ubiquitin mutants are involved in functional polyubiquitination. Our results revealed a notable absence of polyubiquitin chains in ubiquitin mutants lacking the di-glycine motif, indicating impaired ubiquitin polymerization. This impairment suggests that the di-glycine motif in our ubiquitin mutants plays a crucial role in facilitating polyubiquitin chain assembly for dosage dependent lethality in C. albicans, consistent with previous studies highlighting its importance in substrate recognition and ubiquitin conjugation (Peng et al., 2003).

Furthermore, Western blot analysis of UbEP42 and UbEP42GG- provided insights into the differential regulation of K48 and K63 chain linkages in ubiquitin mutants with and without the di-glycine motif. Mutants lacking the di-glycine motif exhibited decreased K48 and K63 chain linkages upon overexpression of UbEP42GG-, suggesting in the absence of substrate recognition motifs its monomer or polymer forms degradation through UPS pathway. This observation is consistent with the established roles of K48-linked chains in proteasomal degradation and K63-linked chains in non-proteolytic signaling events (Swatek & Komander, 2016).

Structural bioinformatics analysis using the trRosetta server further elucidated the molecular basis of di-glycine deletion in ubiquitin mutants. The predicted 3D structures revealed distinct inter-residue geometries, potentially affecting protein-protein interactions and substrate binding. These structural alterations may underlie the observed functional differences in polyubiquitination profiles and chain linkages, highlighting the importance of the di-glycine motif in maintaining ubiquitin's regulatory functions (Yang et al., 2020).

Moreover, the absence of significant differences in secondary structure elements between ubiquitin mutants with and without the di-glycine motif suggests that the functional disparities arise from alterations in inter-residue interactions rather than overall structural changes. This emphasizes the specific role of the di-glycine motif in mediating ubiquitin-protein interactions and substrate recognition (Hurley et al., 2006). Overall, our study provides comprehensive insights into the functional role of the C-terminal di-glycine motif in ubiquitin-mediated processes in C. albicans. The impairment of polyubiquitin chain formation and altered K48/K63 chain linkages underscore the critical nature of this motif in regulating cellular homeostasis and pathogenicity. Understanding the molecular mechanisms underlying di-glycine-mediated ubiquitin signaling pathways holds promise for the development of targeted therapeutic strategies against fungal infections and related diseases.