# *Summary*

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Proteins are modified post-translationally for different ends. Ubiquitination is one such posttranslational modification in which a small protein ubiquitin is added to target proteins to regulate their function, location or degradation.

Ubiquitination has been shown to play role in histone packing, transport of p53 out of nucleus, sorting of proteins like carboxypeptidase S, degradation of membrane proteins in lysosomes and most importantly targeted degradation of proteins by proteasomes. The levels of misfolded proteins are elevated as a consequence of intrinsic or extrinsic factors during the proteotoxic stress, hence, the removal is of greater significance (Bucciantini et al., 2002). The ubiquitin proteasome system (UPS) is responsible for removing abnormal, misfolded proteins or the proteins which have served their function.

Ubiquitination is catalyzed by a set of three enzymes E1, E2 and E3 which act in a relay system. E1 or ubiquitin activating enzyme binds free ubiquitin in an ATP dependent reaction and transfers it to E2 or ubiquitin conjugating enzyme. Ubiquitin Conjugating Enzymes (E2/ UBC) acts as an intermediary between E1 and E3 or ubiquitin ligase, transferring ubiquitin to a substrate protein bound to E3 either directly or through E3. In cells even though there is only a single kind of E1, several E2s and nearly thousand E3s are present, making the process of ubiquitination highly substrate specific.

The variety of functions carried out by E2s contradicts their image as simple carriers of activated ubiquitin. E2s are structurally and functionally diverse. *S.cerevisiae*, the simplest eukaryote has 13 genes for encoding E2 enzymes; while ~40 E2s are present in human (Stewart et al., 2016; Ye and Rape, 2009). All E2s possess a conserved ubiquitin conjugating (UBC) domain (Stewart et al., 2016). The other structural features of the enzymes are variable. E2 enzymes arranged into four different classes: Class I contain only UBC domain, Class have a tail C-terminal to UBC domain, Class III have an

extension N-terminal to UBC domain, while Class IV have extensions at both N and C terminals of UBC domain. To an extent, this structural diversity of E2s is responsible for the degree of specificity and complexity shown by them during ubiquitination. E2s bind to specific E3s. Since, there are more E3s than E2s, the combination of E2 and E3 enzymes can generate a high degree of specificity.

In *S. cerevisiae*, UBC1, UBC4 and UBC5 constitute a sub-family of E2s even though they belong to different classes of E2s (Seufert et al., 1990). UBC1 belongs to class II having a C-terminal extension (Merkley and Shaw, 2004); while UBC4 and UBC5 belong to class I possessing only the core domain (Cook et al., 1997). UBC1 plays important role in degradation of short-lived and abnormal proteins. It is also essential for cell growth and viability (Seufert et al., 1990). UBC4 and UBC5 are heat shock proteins with redundant functions which are essential for viability under stress conditions (Seufert and Jentsch, 1990). Single gene deletions of any one or two members of the three member group UBC1, UBC4 and UBC5 did not affect the viability of cells but strikingly the deletion of all three genes resulted in lethality (Seufert et al., 1990). Over expression of UBC1 in the Δubc4Δubc5 deletion mutant cells resulted in improved cell growth, conferred resistance to high temperatures and amino acid analogues (Seufert et al., 1990). Thus, these three genes have over lapping functions and hence belong to the group of genes important for cell growth and viability. Although these enzymes have partially complementing functions, they clearly have specific roles and hence they were chosen for the present study.

UBC1 is a class II E2 possessing the core catalytic domain and a C-terminal (van Leeuwen et al.) domain. The highly conserved core domain has α/β structure and UBA domain is a 3 helix bundle. The core domain and the UBA domain are connected by a 22 residue flexible linker (Merkley and Shaw, 2004). E2-25k is a human homolog of yeast UBC1. E2-25K plays a role in neurodegenerative disorders like Alzheimer's in association with $UBB^{+1}$, a frame-shift mutant of ubiquitin (Ko et al., 2010a). The only difference

between the structures of two proteins is the linker. E2-25k consists of 6 residue linker instead of 22 residues as of UBC1. A study revealed that, a chimeric protein containing E2 domain of UBC4 and linker with UBA domain of E2-25k showed no polyubiquitin chain synthesis activity (Haldeman et al., 1997). Pair-wise alignment of the two protein sequences revealed 43.81% conservation with major difference at the linker region between the two proteins. In order to understand the structural and functional importance of the linker the chimeric protein c-UBC1 was constructed by replacing the liner of UBC1 with that of E2-25K. c-UBC1 comprises of N-terminus and C-terminus of *S.cerevisiae* and linker of E2-25k. c-UBC1 was expressed in YWO5 cells carrying the ubc1 deletion mutant for in vivo functional characterization. Our observations suggested that c-UBC1 exhibits similar resistance as UBC1 to translational inhibitors (canavanine, hygromycin B and cycloheximide) and thermotolerance in vivo. Interestingly, c-UBC1 displayed increased resistance to heat stress. c-UBC1 was capable of forming polyubiquitin chain as UBC1. In vitro studies revealed that c-UBC1 has a compact structure, which unfolds in presence of a denaturant. Interestingly, c-UBC1 displayed minor alterations in its secondary structure. Therefore, c-UBC1 construct with shorter linker performed equally well in the yeast system as compared to longer linker suggesting that shorter linker is a product of evolution with very little functional significance. In conclusion, c-UBC1 was found to be functionally active and comparable with wild type.

The highly conserved class I enzymes of *S.cerevisiae* UBC4 and UBC5 show 92% sequence homology with difference of only eleven amino acid residues. It was known for a long time that at functional level, UBC4 can take care of the functions of UBC5 making the latter redundant (Seufert and Jentsch, 1990). Still they exist as two separate genes in nature. This suggests that there must be some specialized function served by either of them to meet selective conditions. Hence to understand the reason for evolving and maintaining UBC4 and UBC5 as two separate enzymes, 11 single mutants of UBC4 were generated with E15G, T20A, A42S, I68V, S69N, A81S, N82S, H126Q, R132K, P133A and R140K were constructed by substituting single

residues of UBC4 with the respective residues from UBC5. These variants of UBC4 were expressed in Δubc4Δubc5 double mutants for in vivo functional characterization. The observations suggested that cells expressing H126Q and P133A variants of UBC4 grow at a slower rate in comparison to those expressing wild type UBC4. Moreover, H126Q, R132K, P133A and R140K were the only variants which conferred sensitive phenotype towards three translational inhibitors tested namely, cycloheximide, L-canavanine and hygromycin B. It is known that proline because of its unique structure cannot be substituted with any other amino acid easily. This fact was further supported by our observation that cells expressing P133A variant of UBC4 could not withstand heat stress. S69N was the only substitution that showed survival similar to wild-type. The two mutations R132K and R140K of UBC4 made the cells behave like the double deletion mutant of UBC4 and UBC5. The variants indicated decrease in protein stability theoretically. Hence, the residues of UBC5 individually do not confer any structural advantage to UBC4. Interactive proteins of UBC4 are nearly three times more than those of UBC5. UBC5, therefore, is a functionally minimized version, evolved as another means of regulation to meet cell stage specific needs.

Summarising, the work presented in this thesis probes into structural features of ubiquitin conjugating enzymes UBC1 and UBC4 and their functional importance. UBC1 of *S. cerevisiae* shares 43.81% sequence homology with its human homolog E2-25K. UBC1 and E2-25K have two separate domains in their structure namely the N-terminal UBC domain and the C-terminal UBA domain. The domains are connected by a short linker. Structurally the two enzymes differ in the length of the linker. UBC1 has a linker made of 22 residues, while the linker is of 6 residues in E2-25K. To understand the importance of length of linker, the linker sequence of UBC1 was swapped with that of E2-25K. Functional characterization of the chimeric protein c-UBC1 showed that functionally it is more potent than UBC1 in conferring survival advantage to yeast cells under heat stress. Structural characterization showed that there are some changes in the content of secondary structure of c-UBC1. UBC4 and UBC5 are the products of a gene

duplication event, with 92% sequence homology and showing differences with respect to only 11 residues. They have several E3 binding partners in common. To understand the reason for maintenance of two proteins, single mutants of UBC4 were generated by replacing residues in UBC4 with those of UBC5. The resultant single mutants did not show any functional advantage. Theoretical analysis of the two proteins with respect to binding partners suggested that UBC5 with smaller number of binding partners is functionally limited, indicating one more mode of regulation. Hence, the two proteins which diverged in their sequence are conserved and sustained by the yeast cell to act selectively and exclusively on two different sets of proteins.