PUBLICATIONS

The Ubiquitin Proteasome System with Its Checks and Balances

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Abstract

Cells need to quickly change according to changing environment to survive, and for that, they must not just make new proteins but also degrade others equally promptly. For this purpose, cells have evolved the ubiquitin system, which consists of ubiquitin molecules which are used to tag proteins in a process called ubiquitination; E1, E2 and E3 enzymes which carry out the process of ubiquitination; and deubiquitinating enzymes (DUBs) that remove the ubiquitin from the substrate proteins in a process called deubiquitination. Ubiquitination involves various lysine residues on ubiquitin; among them K48 and K63 are the most significant and well understood. Ubiquitination with K48 linkage leads to degradation of substrate proteins by a multi-protein complex called proteasome. Proteasome-mediated degradation is involved in numerous different processes in cells, due to which defects in it are responsible for several diseases. But due to the high diversity of E3 enzymes and ubiquitin target proteins, there are many drug targets that can be utilized to treat diseases. This makes it vital to understand ubiquitin system for advancement of health care.

Keywords

Ubiquitin • Ubiquitin structure • Proteasomes • Ubiquitination • Deubiquitination

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S. Chakraborti, N.S. Dhalla (eds.), *Proteases in Physiology and Pathology*,
DOI 10.1007/978-981-10-2513-6_26

26.1 Introduction

Ubiquitin is used by eukaryotic cells as a tag, and covalent attachment of ubiquitin to proteins marks them for different activities. Ubiquitin was first discovered in the mid-1970s [1], and for some time, it was seen as a molecule exclusively involved in proteasome-mediated protein degradation, whose use by proteasome enabled fast and extensive changes in the cell protein composition by selective protein degradation, enabling cell to adapt to changing environment [2–5]. In due course of time, however, many functions other than proteasomal degradation have also been associated with ubiquitin. Among them are DNA repair [6], transcription regulation [7, 8], translation regulation [9–11], cell signalling [12], autophagy [13] and endocytosis [14]. Hence, the emerging picture of ubiquitin system is a general post-translational modification system that greatly increases the diversity and functional ambit of proteome, analogous to other post-translational modifications such as phosphorylation, methylation and acetylation. Besides, its role as a tag meant to direct protein degradation has immense importance in regulating many of the functions listed above. Other components of ubiquitin pathway are the E1, E2 and E3 enzymes, involved in ubiquitination of target proteins, deubiquitinating enzymes and ubiquitin-binding partners. An extremely diverse group of E3 enzymes has evolved to accomplish the difficult task of selectively ubiquitinating a particular protein while leaving others unaffected, with each type of E3 specific for a particular protein or group of proteins. In fact E3 genes in humans outnumber the genes for even protein kinases and G-protein-coupled receptors. These E3s in turn are activated or deactivated depending on whether or not their substrate proteins are to be ubiquitinated. Such diverse and specific E3s also offer opportunities to be used as drug targets in numerous diseases ranging from cancer to neurological disorders [15–17]. Here we summarize the current understanding of ubiquitin system based on developments in the last few decades, with particular emphasis on its proteolytic role. We first describe the essential components of ubiquitin system in the context of their functions and the role their structures play in performing them and then move on to different functions of ubiquitin system as tagging and de-tagging machinery. Lastly, we highlight the roles that a defective ubiquitin system plays in different diseases, as well as use of its components directly as drug targets or indirectly in other modes of treatment.

26.2 Players in Ubiquitin System

26.2.1 Ubiquitin

26.2.1.1 Molecular Structure of Ubiquitin

Ubiquitin is a small, monomeric, single-domain protein of 76 residues, which makes it a good model system to study protein structure, folding and stability. Its X-ray crystallographic structure was first determined in the mid-1980s at 2.8 Å [18] and later at 1.8 Å [19]. The globular structure of ubiquitin is formed by a mixed β-sheet and α-helix held together in a β-grasp fold. The protein sports nine reverse

turns and a 3_{10} helix. Additionally, ubiquitin has two β-bulges in its structure. β-bulges are regions in a β-sheet, where two residues in a β-strand are present opposite a single residue in the neighbouring strand. The space required by the extra residue causes this region to bulge out from the β-strand, hence the name. β-bulge affects β-sheet's structure firstly by accentuating the sheet's inherent twist and secondly by interfering with the alternating arrangement of side chains on the two sides of the backbone. The first β-bulge of ubiquitin is located in the N-terminal region between the two antiparallel β-strands forming a hairpin, while the second β-bulge is present in the β-sheet, adjacent to K63. The second β-bulge is a parallel G1β-bulge formed by residues E64 and S65 at positions 1 and 2 and Q2 at position X on the other strand.

Ubiquitin sequence shows a very high degree of conservation among all eukaryotes, with only three residues being replaced from yeast to humans.

In the 1990s, Makhatadze et al. studied the effects of different salts on ubiquitin's stability at pH 2.0, using differential scanning calorimetry, circular dichroism and fluorescence spectroscopy [20, 21]. They found that all salts tested increased the thermostability of ubiquitin through anion binding. They then studied the effect of surface charge on ubiquitin's stability using site-directed mutagenesis and specific chemical modifications. Robertson et al. too studied significance of charges and ion pairs on ubiquitin's surface using site-directed mutagenesis of specific surface residues and determining pKa of neighbouring charged residues by 2D NMR [22, 23]. Makhatadze et al. have produced an ubiquitin mutant more stable than its wild-type counterpart [24]. They first converted all arginine residues on ubiquitin surface to lysines and then carbamoylated their amino groups. They observed that ubiquitin was most stable when all these carboxyl groups were protonated and hence all surface charges were neutralized. Surface charges therefore do not appear to contribute to ubiquitin's stability. The outcome of this study helped in framing guidelines for engineering of surface charges to increase protein stability [25]. The importance of hydrophobic residues in ubiquitin's core was studied using site-directed mutagenesis and measurement of consequent heat capacity changes by differential scanning calorimetry [26, 27]. Replacing nonpolar residues with polar ones decreased the stability of ubiquitin, while replacing naturally occurring polar residues in core with nonpolar ones increased its stability. Replacing nonpolar residues with other nonpolar residues had no significant effect. There have been other studies too on the importance of hydrophobic residues which are present in the core of ubiquitin [28–30]. Significance of the interaction between I30 and I36 at the C-terminus of the α-helix has also been studied. Out of 16 variants produced in the study, none were found as stable as the wild type [31].

Ubiquitin residues essential for vegetative growth of yeast are clustered in three regions on ubiquitin surface. They are hydrophobic patches formed by L8, I44 and V70 and their surrounding residues, F4 and its surrounding residues and the C-terminal tail [32]. I44 patch is essential for proteasomal degradation and endocytosis [33], F4 patch is essential for endocytosis and for proteasomal degradation as well [32–35], and the C-terminal tail is essential for most ubiquitin functions, owing to its fundamental role in ubiquitination. The L8, I44 and V70 patch interacts with

regulatory subunit(s) of proteasome, while this patch together with F4 patch may either form a common binding site for proteins involved in endocytosis, or both patches may bind separate sets of proteins is yet to be known.

26.2.1.2 Ubiquitination and Its Types

Ubiquitin, as the name suggests, is ubiquitously found across cell types and species, exhibiting sequence and structure conservation [36–41]. This indicates its vitality for cell survival. Cell uses ubiquitin as a post-translational modifier by forming an isopeptide bond between the ε amino group of a lysine on target protein and carboxyl group of C-terminal glycine of ubiquitin [42]. If only a single molecule of ubiquitin is attached to the target protein (which is often referred to as substrate protein), the process is called monoubiquitination. Alternatively, more than one ubiquitin can be attached to a substrate protein at different locations in multiubiquitination, or a linear chain of ubiquitins can be built on substrate-attached ubiquitin to produce chains of covalently linked ubiquitins, or polyubiquitins, attached to target protein in polyubiquitination [43]. Monoubiquitination is involved in numerous processes like DNA repair, transcriptional regulation, receptor transport, histone regulation, nuclear export, endosomal sorting and viral budding [44–48], while multiubiquitination is involved in endocytosis [14]. Polyubiquitination can be subclassified into different types based on the lysine residue ubiquitinated. Ubiquitin has seven lysine residues (K6, K11, K27, K29, K33, K48 and K63), all of which can be ubiquitinated [49, 50]. Hence, polyubiquitins may either have every ubiquitin in the chain ubiquitinated on the same lysine (homotypic chains), or different ubiquitin molecules in the same chain are ubiquitinated on different lysines (heterotypic chains). Moreover, the chain may sometimes contain not just ubiquitin but ubiquitin-like proteins such as SUMO [51] and Nedd8 [52] too (heterologous chains). The relative abundance of homotypic chains is in the order of K48 > K11 and K63 >> K6, K27, K29 and K33 [53]. Lys48 chains were the first to be discovered and are involved in proteasomal degradation [3–5]. Lys63 chains are involved in lysosomal degradation [13, 14], autophagy [13] and numerous nondegradative functions like DNA repair [6], regulation of ribosome activity [9, 10] and activation of protein kinases [12]. The functions of other polyubiquitins remain unclear. Mixed-linkage polyubiquitins are found in the lower proportion compared to homotypic polyubiquitins. Mixed-linkage polyubiquitins so far discovered are K6/11, K27/29, K29/48 and K29/33 [54]. Recently, 'linear ubiquitin chains' have also been documented, in which C-terminal glycine of one ubiquitin is covalently linked not to a lysine residue but to an N-terminal methionine residue of the preceding ubiquitin. These chains, assembled by linear ubiquitin chain assembly complex (LUBAC) [55, 56], are involved in NF-κB signalling [56–58].

We have focussed on the second G1 β-bulge of ubiquitin, as the residues present in the structure show low preference for these positions in G1 β-bulges in general. Moreover, G1 β-bulge itself is relatively rare. Yet the bulge and its residues are totally conserved in ubiquitin across all eukaryotes, highlighting their significance in ubiquitin biology. Due to its proximity to K63, residues of this G1 β-bulge may be necessary for the functions involving polyubiquitin chains with K63 linkage.

Functional studies with ubiquitin carrying E64G, S65D and Q2N substitutions and their combinations showed no effect on growth under normal conditions, survival under heat stress and adherence to N-end rule [59–62]. However, the mutations led to increased cycloheximide sensitivity. Absence of any significant structural changes in ubiquitin due to these substitutions establishes that increased cycloheximide sensitivity results from functional rather than structural defect. Ribosomal protein L28 is modified by K63-linked polyubiquitin chain, which is important for making the organism resistant to translational antibiotics. Here the mutations E64G, S65D and Q2N hamper the formation of K63 polyubiquitin chain, because of their spatial proximity to K63 [59–62]. PTEN-induced kinase 1 (PINK1) is a Ser/Thr kinase that phosphorylates S65 on ubiquitin. S65-phosphorylated ubiquitin in turn activates Parkin, which is an E3 implicated in Parkinson's disease, and ubiquitinates mitochondrial proteins. Phosphorylation of ubiquitin by PINK1 occurs in response to mitochondrial membrane depolarization. Parkin itself is also known to be phosphorylated by PINK1. Together, PINK1 and parkin are involved in mitochondrial membrane quality control [63]. Missense mutations in PINK1 gene are also implicated in autosomal recessive inherited Parkinson's disease.

Hence, the functional importance and in some cases the medical relevance of the conserved residues can be understood by generating mutations of ubiquitin and studying their differential effects. Ubiquitin gene was evolved in vitro, and a dosage-dependent lethal mutation UbEP42 was isolated in our laboratory [64]. UbEP42 carried amino acid substitutions in four positions, namely S20F, A46S, L50P and I61T. In spite of being incorporated into polyubiquitin chains, the mutant caused G1 phase arrest of the cell cycle by changing the Cdc28 protein kinase levels. Further, it displayed increased sensitivity towards heat stress and exposure to cycloheximide [65]. Ubiquitin's core is significantly hydrophobic, with 16 of its 21 leucine, isoleucine, methionine and valine residues buried in the interior. This may explain ubiquitin's high thermostability, which is necessary since the molecule's role in stress management often requires it to operate at high temperature.

26.2.2 Ubiquitin-Activating Enzyme or E1

E1 catalyses the first step in ubiquitination of a protein [35]. It binds an Mg ATP. ATP is hydrolysed into PP_i and AMP, and a high-energy mixed anhydride bond is formed between AMP and C-terminal glycine of ubiquitin [66, 67]. This step primes ubiquitin for subsequent attachment to substrate, as the energy released by ATP hydrolysis is used to form the high-energy mixed anhydride bond. Next, a high-energy thioester bond is formed between C-terminal glycine of the adenylated ubiquitin and a cysteine in E1, releasing AMP in the process [68–70]. This is a spontaneous step, as the thioester bond is at lower-energy level compared to mixed anhydride bond. From here, ubiquitin is transferred to an E2. The structure of E1 enzyme has been studied in great detail, and insights have been gained into its mechanism of action [71–73]. E1 can simultaneously bind to two ubiquitins, one adenylated and the other attached to cysteine. The C-termini of both ubiquitins are spatially close

[74], which probably allows easy transfer of ubiquitin from the first site to the second. Only a single E1 transfers primed ubiquitin to all the different E2 enzymes in most organisms. Catalysing the first step of the cascade, therefore, E1 is responsible for ensuring unlimited supply of primed ubiquitin to all the downstream conjugation reactions, and so it would be expected to be present in high concentrations. However, E1 concentration is less than total E2 concentration [4]. This requires E1 to be a highly efficient enzyme. Indeed, catalytic rate values for substrate ubiquitination are 10–100-fold slower than that of all E1-catalysed steps from ATP binding to thioester formation [75]. However, E1 affinity for ubiquitin increases tremendously after ATP binding [4, 66]. ATP binding causes a conformational change in E1, which makes the binding site more accessible to ubiquitin. The interactions between AMP-ubiquitin and E1 are extensive [76]. The only known residue in E1 active site is cysteine, but as arginine residues in ubiquitin are essential for its binding with E1, certain acidic residues may also be involved [77, 78]. E1s for ubiquitin and ubiquitin-like proteins (UBLs) are related. E1 for ubiquitin is a monomer of 110 K Da. Initially, the role of ubiquitin proteasome system in the degradation of a diverse array of short-lived proteins was demonstrated by using temperature-sensitive E1 mutants expressed in mammalian cell lines [79]. These mutants also help determine if a process is ubiquitin dependent [80, 81]. Hypomorphic allele of E1 was isolated, which produces mutant form of E1 that is less efficient than wild-type E1 in performing its function. It was demonstrated that the hypomorphic allele of E1 of yeast can successfully replace the temperature-sensitive variant in the experiments where ubiquitin dependence of degradation is investigated [35, 82].

26.2.3 Ubiquitin-Conjugating Enzymes or E2s

E2 accepts ubiquitin from E1. A thioester bond is formed between C-terminal glycine of the ubiquitin and active site cysteine in E2, as the Ub-E1 thioester bond breaks [4]. This step does not involve any change in energy level since energy stored in the thioester bond between ubiquitin and E1 cysteine is conserved in the thioester bond between ubiquitin and E2 cysteine. E2s are much more diverse than E1s. *S. cerevisiae* has 14 E2s, and higher organisms have even more. Some of these may be different isoforms of same E2 [83, 84], while others may have evolved independently [85–87]. This diversity confers first level of substrate specificity to ubiquitin system, the other being at the level of E3. Structural studies on E2 enzymes show that a core domain of 150 amino acids is conserved across all E2s and may account for their chief function of transferring ubiquitin to E3 enzymes. The core domain contains a four-stranded antiparallel β-sheet, four α-helices and a 3_{10} helix. Helix 2 and the β-sheet make up a central region bound by helices 3 and 4 on one side and helix 1 on the other. The loop connecting β-strand S4 to helix 2 contains the active site cysteine. It is located in a shallow groove formed by upstream residues of the same loop on one side and those of loop connecting helix 2–3 on the other. The region surrounding active site cysteine contains many of the most conserved residues [88–94], some of which interact with ubiquitin and others probably with E1.

The side opposite to the active site, on the other hand, contains most of the poorly conserved residues. Presently, it is not clear if this variation in sequence in different E2s is due to low selective pressure or because this region may be responsible for selecting specific E3s [95]. Some E2s also have N- or C-terminal extensions, which may be responsible for conferring either substrate or E3 specificity in these cases [96–98]. Recent structural studies on complexes of E3 with Ub-E2 conjugates are beginning to unveil the mechanism of ubiquitin transfer from E2 to E3 [99–104].

26.2.4 Ubiquitin Ligases or E3s

E3s catalyse the final step of ubiquitination, i.e. forming the isopeptide bond between the C-terminal glycine of ubiquitin accepted from an E2 and the lysine on the substrate protein. In some cases, substrate protein is ubiquitinated on the α-amino group of its N-terminal residue, which may not be lysine [105]. E3s are extremely diverse in their substrate specificity. This diversity enables selective ubiquitination of proteins and hence makes ubiquitination a powerful tool for regulating cellular activities. E3s are of two types, namely, RING E3s and HECT E3s. HECT E3s simply mimic the step of ubiquitin conjugation to E2, by breaking the thioester bond of Ub-E2 and forging another thioester bond between the C-terminus of ubiquitin and -SH group of the cysteine present in the active site of E3, leading to formation of Ub-E3. In this process, energy from E2-Ub thioester bond is conserved in E3-Ub thioester bond. Subsequently, Ub is transferred from E3 to a substrate protein, by forming an isopeptide bond between the carboxyl group of C-terminal glycine of ubiquitin and ε-amino group of lysine on the substrate protein. RING E3s, unlike HECT E3s, do not form a covalent bond with ubiquitin; instead, they act as adapters by binding to the substrate protein and E2 loaded with ubiquitin simultaneously. They facilitate transfer of ubiquitin from E2 to substrate protein directly.

Majority of E3s belong to RING domain type. RING domain was discovered and characterized in the early 1990s. The canonical sequence found in RING E3s is Cys-X2-Cys-X(9–39)-Cys-X(1–3)-His-X(2–3)-Cys-X2-Cys-X(4–48)-Cys-X2-Cys (where X is any amino acid). Cysteines 1, 2, 4 and 5 coordinate one Zn^{+2} ion, and cysteines 3, 6 and 7 along with the histidine coordinate a second Zn^{+2} ion. RING domain is thus structurally related to zinc finger domain. Unlike zinc finger, however, RING domain assumes a rigid and compact shape owing to the presence of the two zinc ions in the coordination sites. Sequence conservation in RING domain is not absolute. There are variants in which cysteines and histidines are swapped, as well as those in which another residue capable of coordinating zinc replaces a cysteine, e.g. Asp in Rbx1/Roc1. There is a third group of E3s known as U-box E3s, which also recruits E2s. They are often clubbed along with RING group of E3s, as the two are closely related. In U-box domain, which is structurally similar to RING domain, zinc ions are replaced by a network of hydrogen bonds [106].

HECT E3s have a modular architecture. HECT domain has a bilobal shape, with E2-binding site in the N-terminal lobe and the active site cysteine in the C-terminal lobe [86]. As the distance between the two lobes is large, both lobes must come

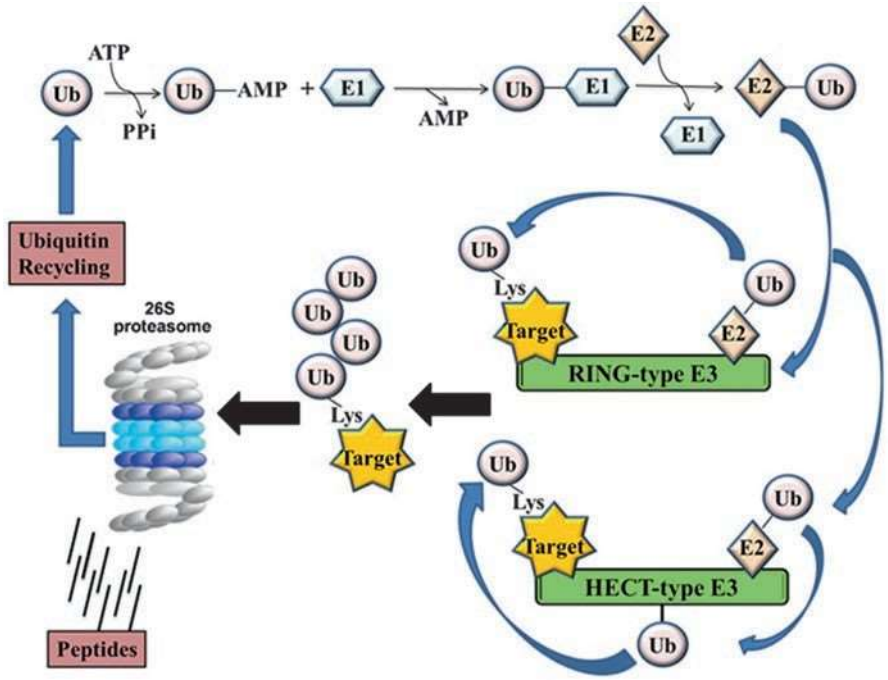

Fig. 26.1 The E1-E2-E3 pathway of ubiquitination of substrate proteins

close together for catalysis, perhaps covering a distance of around 50 Å [107]. Further, significant decrease in catalytic activity observed due to the mutations of the hinge between the lobes as a consequence of restricted movement supports the above model [108]. N-terminal region extending from N-terminal lobe of HECT domain differs from enzyme to enzyme and confers substrate specificity adding one more functional domain. Interestingly, substrate binding to E3 is not affected by the deletion of HECT domain [109–111]. Besides, isolated HECT domains retain binding to E2 enzymes. HECT domains do not show functional redundancy as they cannot substitute for each other [112], because they contribute to substrate specificity of their respective E3s [113] (Fig. 26.1).

26.2.5 Deubiquitinating Enzymes or DUBs

Deubiquitinating enzymes are proteases that remove substrate-conjugated ubiquitin from the target proteins. DUBs have several functions. Firstly, to maintain balance of any cellular process, negative regulation is as important as positive regulation. DUBs, by cleaving ubiquitin from target proteins, reverse the process of ubiquitination and hence act as negative regulators [114, 115]. Secondly, after the removal of polyubiquitin chains from target proteins either by other DUBs or by the proteasomes, DUBs replenish free ubiquitin pool by disassembling polyubiquitins into

individual ubiquitins [116, 117]. They also cleave free polyubiquitin chains produced by ubiquitin conjugation machinery in the absence of any target protein. Thirdly, three of the four ubiquitin genes (*UBI1*, *UBI2* and *UBI3* in yeast) produce ubiquitin fused to ribosomal proteins, and the fourth (*UBI4*) produces a fusion of 4 or 5 ubiquitins in yeast [118–120]. Hence, DUBs are needed for producing free and functional ubiquitin from the fusions. Lastly, unwanted reactions involving ubiquitin in cell create species, which do not seem to serve any function. Such species include thiol esters between ubiquitin C-terminus and cellular thiols like glutathione, amide derivatives of ubiquitin formed with lysine and spermidine and free ubiquitin adenylate. DUBs release free ubiquitin from these species [121, 122].

There are five different families of DUBs in humans, which together constitute about 100 known DUBs. Four of these families are cysteine-like proteases. These are Josephin domain or MJD proteases, ubiquitin-specific proteases (USP/UBP), ubiquitin C-terminal hydrolases (UCH) and ovarian tumour (OTU) families. The fifth one is of JAB1/MPN/Mov34 metalloenzyme (JAMM) domain zinc-dependent metalloprotease family. The active site in DUBs of Cys protease families consists of the catalytic cysteine and a histidine. Histidine increases polarity of cysteine -SH group, making S more negative, enabling it to make a nucleophilic attack on the isopeptide bond linking ubiquitin to substrate protein. Cleavage of isopeptide bond is followed by formation of a transient acyl intermediate-linking carboxyl group of ubiquitin to catalytic Cys of DUB. This acyl intermediate is then cleaved by a water molecule, releasing ubiquitin. In many, though not all cases, a third residue, which usually is Asn or Asp, polarizes and aligns histidine. The catalytic Cys, His and Asn/Asp are called the catalytic triad. All the four Cys protease families have the same basic catalytic site structure and mechanism. What distinguishes them from one another is their tertiary structure.

Besides the DUBs, which cleave SUMO and Nedd8, ubiquitin-like proteases (ULPs) include Adenain family of cysteine proteases [123–127]. The members of Adenain family resemble adenovirus protease. The diversity seen with DUBs is mainly responsible for their immense substrate specificity, which enables selective deubiquitination of proteins. Similar to DUBs, the feature of 'structural diversity resulting in functional selectivity' is observed with E3 enzymes as well, while catalysing ubiquitination. This makes deubiquitination as useful as ubiquitination in the regulation of cellular activities such as regulation of DNA repair [128], gene expression [129], cell cycle regulation [130], kinase activation [131, 132], proteasome-mediated degradation [133, 134], lysosome-mediated degradation [134], microbial pathogenesis [135, 136] and myriad other activities. Many pathogenic bacteria [136, 137] and viruses [135, 138–145] have acquired DUB genes through parallel transfer from eukaryotic genomes. These microbes may use DUBs to shield their proteins against host's ubiquitination machinery. For example, DUBs in Adenain family have been acquired by bacteria and viruses to cleave ubiquitin and interferon-stimulated gene 15 (ISG15) conjugates [123–127]. Mutant DUBs have also been implicated in numerous diseases [128, 146–148]. To date, very few DUBs have been characterized in terms of their substrates and physiological roles, and much of this area remains to be explored.

In UCH domain family UCH catalytic core is formed by a domain of 230 amino acid residues. The salient feature of this family is that the active site is covered by a loop. The loop restricts access to ubiquitinated proteins and polyubiquitin chains as they are too big and ensures that ubiquitin attached through its C-terminal to small peptides, and small chemical groups only can access the active site of the DUB for processing. The space afforded by this arrangement is too small for large, folded ubiquitin conjugates or even polyubiquitin chains to fit in. In a study conducted on UCHL3, the loop was systematically extended until UCHL3 gained ability to hydrolyse polyubiquitin. The amount of extension needed was significant. Hence, UCHs act not on ubiquitin linkage with whole proteins but with small peptides produced as by-products of proteasomal or lysosomal degradation, molecules like aldehydes and C-terminal extensions of polymeric proubiquitin. However, unfolded whole proteins may also be targeted, if they can manage to thread through confined space provided by the loop [149]. In some UCHs, active site exists in an unproductive conformation in the absence of activation signals [150].

Members of USP domain family consist of three subdomains in their structure, namely, finger, palm and thumb [151]. CYLD is the only USP that lacks finger subdomain [152]. Though sequence similarity of USP domain across the family is low, it is structurally well conserved [151–155]. The active site lies in a cleft between palm and thumb, and the C-terminus of ubiquitin binds the active site, while the globular portion of ubiquitin binds the fingers [151, 153, 155]. Crystal structures of both ubiquitin-bound and ubiquitin-unbound forms of many USPs have been solved, and their comparison shows that some USPs exist in inactive conformation in the absence of ubiquitin, while others maintain active conformation irrespective of ubiquitin's presence. In the former case, inactive conformation of the USP may be a result of either improper positioning of catalytic triad or blocking of correctly positioned triad by ubiquitin-binding surface loops. In many cases, USP domain has insertions or terminal extensions capable of folding into independent domains with some functional relevance. In USP5, these domains have additional ubiquitin-binding sites, whereas in CYLD, they determine subcellular localization [152].

In the OTU domain family, the structure of Otu1 covalently bound to ubiquitin shows that most of its interactions with ubiquitin are mediated by a large surface loop. In the absence of ubiquitin, this surface loop has been found to be disordered in OTUB1 and the other members of the family OTUB2 and A20. Superposition of ubiquitin-bound Otu1 structure on the structure of A20 shows that a helical domain blocks binding site for ubiquitin moiety, suggesting architectural variation of this site in A20. The structure of OTUB1 apoprotein shows nonproductive alignment of His residue with catalytic Cys in the catalytic triad, suggesting that OTUs, like USPs and UCHs, may exist in catalytically inactive conformation in the absence of ubiquitin. In OTU core domain, five β-strands are sandwiched between helical domains. However, the lengths of the β-strands are variable within the OTU family [152].

MJD family has four members in humans, of which the best studied is Ataxin 3 [156–160]. Ataxin 3 probably acts specifically on K63 polyubiquitin. It has an extended helical arm, which may regulate access of polyubiquitin chain to the active

site [157–159]. A second ubiquitin-binding site, which is distinct from the active site, lies at the back of this arm, suggesting that ataxin 3 may interact with two distal ubiquitins simultaneously in a polymer. Ataxin 3 also contains a polyglutamine stretch whose extension causes Machado-Joseph disease [161].

JAMM domain DUBs are commonly associated with large complexes [162–169]. Solving the crystal structure of one of the JAMM domain DUB, AMSH-like protease bound to a K63 diubiquitin has helped unravel the catalytic mechanism of JAMM domain DUBs [169]. JAMM domain coordinates two Zn ions, one of which primes an H_2O for hydrolysis of isopeptide bond, while the other is included in an AMSH-specific insert that forms a motif recognizing proximal ubiquitin of K63 diubiquitin. Another AMSH-specific insert recognizes distal ubiquitin. Hence, JAMM domain recognizes distal ubiquitin and sequence Gln62-Lys63-Glu64 in proximal ubiquitin, which makes AMSH-like protease K63 linkage specific. AMSH-specific inserts are absent in JAMM domain proteases that are not specific for polyubiquitin.

26.3 Ubiquitin-Binding Domains

The binding partners of ubiquitin recognize and bind to substrate-conjugated ubiquitin using domains called ubiquitin-binding domains or UBDs through noncovalent interactions. Ubiquitin exerts its effects not by structural modification of ubiquitinated protein but by itself serving as an additional interacting surface, which makes ubiquitin-binding domains necessary. Structure and sequence information about these domains may help identify new ubiquitin-binding partners. More than 20 different UBDs are known at present, and more are expected to be discovered. But since a comprehensive discussion of this topic is beyond the scope of this chapter, only the earliest discovered UBDs are described and compared here, namely, ubiquitin-interacting motif (UIM), ubiquitin-associated domain (UBA); coupling ubiquitin to endoplasmic reticulum degradation (CUE); polyubiquitin-associated zinc finger (PAZ (ZnF-UBP)); Gga and Tom1 domain (GAT); Npl4 zinc finger motif (NZF); Vps27, HRS, STAM (VHS); GRAM-like ubiquitin binding in Eap45 (GLUE); and ubiquitin-conjugating enzyme variant (UEV). Structurally, these domains are quite unrelated. UIMs consist of a single helix [170]. NZFs have three residues on loops of four strands, which in turn are stabilized by a Zn ion [171]. UBA and CUE domains are so closely related that the structures of their complexes with ubiquitin are superimposable. Both CUE and UBA domains consist of three helix bundles, of which two helices recognize ubiquitin [172–174]. Even though GAT consists of three helix bundles with two helices recognizing ubiquitin, it is unrelated to UBA and CUE, as the ubiquitin-interacting helices in CUE and UBA are antiparallel; however, they are parallel in GAT [175]. UEV domain is made up of α-helices and a β-sheet, and ubiquitin is recognized by the loop between two helices and a part of β-sheet [176, 177]. This structural diversity suggests that different UBDs recognize different parts of ubiquitin, which in turn may explain the reason for high sequence and structure conservation observed with ubiquitin. Most

UBDs interact with the L8-I44-V70 patch of ubiquitin, making their footprints on ubiquitin overlapping, although some recognize other surfaces such as Asp58 and Gln62 [178] or the C-terminal residues [179]. Moreover, the footprints on I44 patch show marked variation in spite of some overlap. Although most UBDs characterized so far contact the I44 face of ubiquitin, high degree of conservation seen with other surfaces of ubiquitin suggests that many more UBDs remain to be discovered. Besides, many proteins involved in ubiquitin-dependent processes have been shown to have unidentified ubiquitin-binding sites.

Mechanisms of action of UBDs are unclear, but their properties give key insights into their functioning in vivo. Affinity of UBDs for ubiquitin is typically low with their K_d values in the range of 10–500 μM. This may make complexes based on UBD-Ub interactions capable of rapid assembly and disassembly, making them more dynamic [180]. Low affinity may also make regulation of these complexes easier, as disruption of even a single interaction may destabilize the complex. Besides, as ubiquitin concentration in cell is very high [181], low affinity may be a strategy to ensure availability of free UBDs. Proteins needed to bind strongly to ubiquitin may do so by having multiple UBDs. The overlapping footprints of different UBDs on ubiquitin may prevent simultaneous binding of more than one ubiquitin receptor to ubiquitinated protein. This may be desirable in pathways that need sequential handing over of ubiquitinated protein from one ubiquitin receptor to another. Different footprints may also help UBDs in distinguishing between monoubiquitination and polyubiquitination and also between different linkages of polyubiquitination [172, 182, 183]. Structural studies show that ubiquitin structure slightly changes on binding to different UBDs, increasing their affinity for ubiquitin [184]. Some UBDs appear to be linkage specific, while others are not [185, 186]. Linkage specificity can be conferred either by interaction of UBD with linker region of polyubiquitin [187] or by fixed length of the linker sequence between tandem UBDs that favourably positions them to recognize a particular polyubiquitin [158, 188, 189].

26.4 Proteasome-Mediated Degradation

Proteasomal degradation is the most well-studied consequence of ubiquitination. One of the most valuable attributes of a cell is its ability to quickly adapt to changes in its internal and external environments, which often requires fast and extensive changes in its proteome. The set of existing proteins, which are not useful in the changed environment, are quickly degraded by proteasome, and the amino acids are used to produce a new set of proteins to meet the challenge. Besides, proteasome also degrades misfolded, truncated and denatured proteins formed during normal function or under stress conditions like high temperature, UV exposure or starvation. Proteasome's function can therefore be described as constructive destruction. Most proteases nonspecifically and nonprocessively degrade proteins by recognizing intrinsic cleavage sites that cannot be modified and releasing the products after every cleavage. Unlike most proteases which are nonspecific, proteasome has been

designed to specifically degrade certain proteins while leaving others unharmed. It is a hollow protein cylinder. The active sites are situated inside the cylinder, which ensures high processivity by preventing substrate proteins from diffusing away. The regulatory proteins associated with the two ends of the hollow cylinder, with few exceptions, recognize only ubiquitinated proteins. This confers specificity by ensuring that only ubiquitinated proteins are degraded.

The cylindrical protease also known as 20S proteasome is 15 nm in length and 11 nm in diameter and is made of four rings, two α-rings and two β-rings, stacked over one another in the order αββα. The α- and β-rings are made up of seven α-type and seven β-type subunits, respectively [190–196]. Amino acid sequences of α- and β-subunits share some homology. Proteasomes belong to a group of proteins called chambered proteases, all of which have same basic structure. In archaebacterial proteasome, all seven β-subunits are identical and have an active site for proteolysis on each of them [195, 197, 198]. In eukaryotic proteasome, however, not all β-subunits are identical, and only three of the seven β-subunits in each β-ring have proteolytic sites, presenting the proteasome with six of them. The three sites in each ring are located on β1, β2 and β5. The two β-rings therefore make a catalytic chamber lined by proteolytic sites, where proteins are degraded. β1, β2 and β5 sites have caspase-like, trypsin-like and chymotrypsin-like activities, respectively [196, 199, 200]. This diversity makes eukaryotic proteasome a highly efficient protease by increasing the probability of finding a cleavage site on substrate protein. Genetic studies have shown that the importance of these sites for cell growth decreases in the order β5 > β2 > β1. For example, β5β2 double mutants in yeast are lethal, while β5β1 and β2β1 are viable [199, 201, 202]. The α-rings form two additional chambers, called outer chambers or antechambers, which are present one on either side of the catalytic chamber. These outer chambers serve as antechambers for unfolded proteins before they can be inserted into catalytic chamber for degradation. The volume of outer chambers is 59 nm^3, while that of catalytic chamber is 84 nm^3 [197, 298]. Access to these chambers is controlled by pores, which may be either open or closed. When open, they are only 2 nm in diameter, which ensures that only unfolded proteins gain entry into chambers while leaving randomly colliding intact proteins unaffected. Besides, the N-terminal regions of α-subunits undergo conformational change to allow entry of the substrate protein into the proteolytic chamber [203, 204]. This conformational change is induced by the association of 19S complex with the 20S proteasome forming 26S proteasome. The 20S complex remains closed in the absence of 19S particle. PA28/11S, a complex involved in antigen processing by proteasomes, can also induce a similar conformational change. 28/11S helps proteasome generate immunopeptides from antigenic proteins that can be presented by MHC complexes for initiating an immune response [203, 205].

Attached on either end of the 20S complex is 19S complex. Composed of 19 subunits, it has two parts, the base and the lid [206, 207]. The base, composed of eight subunits, unfolds substrate proteins and inserts them into 20S complex. Since this process requires energy, six of the eight subunits in the base, namely, Rpt1–Rpt6, are ATPases [208]. Their C-terminal residues are also responsible for binding of 19S complex to 20S complex [209, 210]. These six subunits may assemble into a

heterohexameric ring [211], though the order in which they are arranged in the ring is debated [212–214]. The two non-ATPase subunits are Rpn1 and Rpn2. The lid also has eight subunits, Rpn3 and from Rpn5 to Rpn11. Out of these, Rpn10 attaches lid to base. The 19S complex imparts substrate specificity to proteasome by favouring K48 polyubiquitinated proteins over others [215]. The K48 polyubiquitin must contain at least four to five ubiquitins for recognition by proteasome [215], which suggests that both ubiquitin structure and polyubiquitin topology determine recognition. Only a few proteins are known to be recognized by 19S in nonubiquitinated state [216–220]. Rpn10 [221] and Rpn13 [222] are the subunits, which are known to bind polyubiquitinated proteins. Rpn10 binds ubiquitin through its ubiquitin-interacting motif (UIM) consisting of three helices connected by flexible linkers [223]. The UIM does not display any definite tertiary structure due to the flexible linkers. Rpn13 binds ubiquitin through a pleckstrin-like receptor of ubiquitin (PRU) domain [224]. Ubiquitin binds to loops in PRU domain, unlike other UBDs in which it binds to secondary structures. Studies suggest that Rpn1, Rpn2 and Rpt5 are ubiquitin receptors as well [225–227]. Rpt5 is also known to bind ornithine decarboxylase, the nonubiquitinated substrate of proteasome [218]. Before the unfolded proteins are inserted into 20S complex, the polyubiquitin tail is cleaved off by Rpn11 using its zinc finger containing JAMM motif [162, 228]. Rpn11 functions only in association with the rest of the 19S, as its deubiquitinating activity is ATP dependent. The 19S particle also opens the gates formed by N-terminal regions of α-subunits at the mouth of 20S complex. The 20S and 19S complexes together make the 26S proteasome. Proteasomes can exist in the nucleus and cytoplasm and attached to the outer surface of the endoplasmic reticulum.

The tertiary structures of proteasomal subunits exhibit properties typical of and necessary for subunits of large protein assemblies. Proteasomal-activating nucleotidase (PAN), an orthologue of proteasomal AAA ATPases, has coiled coils protruding from an oligonucleotide binding (OB) fold called PAN-N and an AAA fold [213, 229]. The long and slender topology enables coiled coils of PAN to establish extensive contact with their binding proteins. Rpn1 and Rpn2 have proteasome cyclosome (PC) repeats [230], which may form two helix ARM/HEAT units, which in turn form α-solenoids [231]. The α-solenoids are superhelical quaternary structures having extensive surface area for establishing contact with binding partners. Rpn3, Rpn5, Rpn6, Rpn7, Rpn9, and Rpn12 have PCI domain [232], which consists of an N-terminal helical bundle fold and a C-terminal-winged helix fold. Repetitive bihelical blocks preceding PCI domain in these subunits may form α-solenoids together with helical bundle fold in PCI domain [232].

26.5 Role of Proteasomal Degradation in Cell Physiology, Disease and Therapy

26.5.1 Role in Cell Physiology

There are various aspects of cell physiology that are regulated by proteasomal degradation, including DNA repair, transcription, protein synthesis, cell signalling, cell cycle and autophagy. During the progression of cell cycle, ubiquitin system ensures timely degradation of cell cyclins, inhibitors of cyclin-dependent kinase and other important proteins. Often defective regulation of UPS in relation to degradation of these key proteins is a major cause of tumorigenesis. There are two main families of E3s involved in cell cycle: the SCF (Skp1/Cul1/F-box protein) complex and the anaphase promoting complex or cyclosome (APC/C). APC/C is responsible for exit from mitosis and establishing a stable G0/G1 phase, while SCF is involved in all stages of cell cycle. In transcription-coupled DNA repair, proteasomal degradation of the largest subunit of RNA polymerase II occurs after its ubiquitination by Rsp5, which is a HECT domain E3 [110]. Besides, transcriptional activators are also degraded by proteasomes [233, 234]. Transcriptional activation domains (TADs) are responsible for signalling proteasomal degradation. Transcriptional factors containing TADs may be both activated and degraded through the same pathway. Proteasomal ATPases unfold the yeast Gal4 activator irreversibly, leading to its proteasomal degradation [7]. Interestingly, monoubiquitination is sufficient to serve as a marker for degradation in the case of Gal4, instead of polyubiquitination with K48 linkage. Genome-wide ChIP-chip studies show that proteasomal ATPases are resident on hundreds of yeast genes, as several trans- and co-activators of transcription undergo monoubiquitination. Hence, ubiquitination is used by cells as a mechanism in transcriptional regulation. Proteasomal degradation is also involved in protein synthesis. In order to prevent formation of defective ribosomes, cell must produce all ribosomal proteins in equimolar amounts. This requires an unattainably high level of coordination between the large numbers of ribosomal protein genes. Cells solve this problem by producing excess of all ribosomal proteins and subjecting those subunits which fail to get incorporated into ribosomes to proteasome-mediated degradation [11]. Proteasomal degradation therefore sculpts the ribosomal protein set into equimolarity. Interestingly, ribosomal proteins might be protected from proteasomal degradation before being incorporated into ribosomes. For example, *Ubi1* and *Ubi2* genes are expressed as ubiquitin fusions with small ribosomal subunit protein S27a, while *Ubi3* gene is expressed as ubiquitin fused to large ribosomal subunit protein L40 [120, 121]. Ubiquitin fused to these proteins may protect them not only from proteasomal degradation but also from N-terminal-specific proteases, as the ubiquitin is fused to their N-terminal. Proteasomal degradation is also involved in regulating the action of two prosurvival switches in TNFR1-mediated cell signalling. NF-κB pathway is driven towards cell survival by proteasome-mediated degradation of Ik-Bα, the inhibitor of NF-κB. Degradation of Ik-Bα enables NF-κB to migrate to nucleus and upregulate its target genes [235]. The second switch in the same pathway is receptor-interacting serine/ threonine protein

kinase 1 or RIP1. RIP1 serves as a dual switch. When RIP1 is conjugated with a K63-linked polyubiquitin chain, it functions as an inhibitor to proapoptotic pathway by NF-κB-independent mechanism initially and later by NF-κB-dependent mechanism [236]. Alternatively, a deubiquitinated RIP1 may also interact with FADD and caspase 8, causing apoptosis [237]. The enzyme A20 has both DUB and E3 activities and replaces the K63 chain on RIP1 with a K48 chain, causing its proteasomal degradation [238]. It is interesting to study the consequences when A20 fails to polyubiquitinate RIP1 with K48 chain, after removal of its K63 chain. Hence, the ubiquitination status of RIP1 acts as a checkpoint, as polyubiquitin chains with K48 linkage and K63 linkage serve as negative and positive regulators of NF-κB signalling, respectively, and in turn act as switches for apoptosis and cell survival. Ubiquitin system also plays a vital role in stress response. During stress, heat-shock proteins (HSPs) act as chaperones, folding unfolded proteins back to their native state. However, when the proteins are truncated or damaged beyond repair, these same HSPs associate with E3s like CHIP and Parkin and facilitate their degradation by proteasomes. If the rate of denaturation of proteins is so high that neither HSPs nor proteasomes can clear them quickly, the unfolded proteins form intracellular aggregates. Such potentially toxic aggregates may be responsible for neurodegenerative diseases like Parkinson's and Alzheimer's. Indeed, several protein aggregates have been shown to be ubiquitin positive. Interestingly, ubiquitin system plays a role not just in proteasomal degradation but also in lysosomal degradation [239–241]. Ubiquitin has been shown to be responsible for marking membrane proteins for selective degradation by lysosomes.

26.5.2 Role in Disease and Therapy

Owing to its complexity and involvement in multiple processes, ubiquitin system is associated with numerous diseases and also offers prime targets to cure them [15–17]. Mutation in E3s for specific target proteins can cause disease due to loss or gain of function associated with the target protein. Alzheimer's disease is characterized by extracellular amyloid plaques, containing misfolded β-peptides generated from the cleavage of amyloid precursor protein (APP) and intracellular neurofibrillary tangles containing Tau, a microtubule-associated protein. Defects in ubiquitin system may be involved in Alzheimer's disease in multiple ways. UBB^{+1} is a ubiquitin variant with a C-terminal extension of 19 amino acids. It cannot be conjugated to substrate lysines, although it may be incorporated into polyubiquitin chains. It was in the neurons of Alzheimer's disease that UBB^{+1} was first discovered, followed by other neurological disorders. Decreased proteasomal activity is observed in the severely affected regions of the brain of Alzheimer's patients. Besides, overexpression of UCH-L1, a DUB of ubiquitin hydrolase family, helped in reducing memory loss in mouse models of Alzheimer's disease, suggesting its role in the disease [15].

Parkinson's disease is a consequence of many unrelated causes, and studies implicate ubiquitin system as one of them. About 50% of juvenile patients of autosomal recessive Parkinson's disease have mutations in parkin gene, which encodes

a RING finger E3. Parkin protein contains an N-terminal Ubl domain and two C-terminal RING finger domains flanking an in-between RING (IBR) domain. Parkin binds to proteasome through its S5a subunit, and this binding is reduced by mutations at R42 residue. Such mutations may compromise substrate degradation through parkin, and resultant parkin substrate accumulation may result in Parkinson's disease pathogenesis. Possible parkin substrates include Parkin-associated endothelin receptor-like receptor (PAEL-R), α-synuclein, synaptotagmin XI, cyclin E, tubulin, misfolded dopamine transporters and polyglutamine-repeat proteins. None of them, however, showed increased abundance in neurons of parkin-lacking mice [15].

Huntington's disease is a CAG repeat disorder. The protein Huntingtin is crucial to the function of neuronal cells. Polyglutamine (polyQ) tracks are expanded in Huntingtin protein giving rise to a mutant form. Role of ubiquitin system is suspected in Huntington's disease as the inclusion bodies formed contain ubiquitin, E2/E3s and proteasomal subunits. Moreover, Huntingtin undergoes ubiquitination, and overexpression of its mutant form inhibits proteasomal activity, leading to cell cycle arrest [15].

Defects in ubiquitin system have also been implicated in several types of cancers. Products of numerous oncogenes and suppressor genes undergo ubiquitination [81, 242, 243].

The protein p53 is a tumour suppressor. It is involved in numerous cell proliferation and apoptosis pathways, which makes it a good drug target. About 50% of all human tumours contain p53 mutations and many of those that do not have defects in other components of p53 network. MDM2 is a RING finger E3 that ubiquitinates p53 causing its proteasomal degradation. It also inhibits p53 activity by physically blocking its N-terminal transactivation domain and preventing its nuclear export. p53 transcriptionally induces MDM2, creating a negative feedback loop. Hence, p53 activity could be boosted in cancer cells by inhibiting MDM2, helping tumour suppression. Moreover, MDM2 may also be responsible for degradation of other antioncogenic proteins.

SCF E3s are multisubunit E3s composed of four components. The subunit of SCF, CUL1 serves as the scaffold for assembly of the rest of ubiquitin-conjugating machinery. RBX1 is a RING finger protein interacting with C-terminus of CUL1, while N-terminus of CUL1 binds to SKP1, and in turn SKP1 interacts with F-box proteins. At least 68 F-box proteins are found in human genome, each of which recognizes multiple substrates. SCF E3s interact with different F-box proteins to recognize different substrates. F-box proteins therefore modulate specificity of SCF E3s. Mutations in many F-box protein-substrate pairs are involved in cancers. SCF-FBW7 is an E3 promoting degradation of proteins involved in cell proliferation, like cyclin E, c-Myc, c-Jun, Notch and sterol regulatory element-binding proteins (SREBPs). Arginine residues in WD40 repeats of FBW7 interact with phosphodegrons in these substrate proteins. Mutations targeting these arginines cause many types of cancers. Mutations in substrate phosphodegron, preventing their recognition by FBW7, may also cause malignant transformations. SCF-SKP2 is involved in the degradation of several negative cell cycle regulators like p130 (a protein

belonging to retinoblastoma family), FoxO (cell-cycle inhibitory transcription factor forkhead box protein O) and the CDK inhibitors p27, p21 and p57. Small CDK-interacting protein 1 (CKS1) is also an SCF-SKP2 subunit and is required for p27 degradation. It is probably needed for interaction of SKP2 with p27. SKP2 overexpression is involved in several human cancers and RNA interference. Intracellular injection of antiSKP2 antibodies can reduce cancer cell proliferation. IκB kinase phosphorylates IκB at S32 and S36, generating a phosphodegron, which is recognized by βTRCP, the E3 responsible for proteasomal degradation of IκB. βTRCP has numerous substrates, such as β-catenin (an oncogenic transcription factor), the cell-cycle regulatory proteins early mitotic inhibitor 1 (EMI1) and cell division cycle protein 25A (CDC25A) and progesterone receptor. Mutations in β-catenin phosphodegron are involved in the pathogenesis of several human cancers. VHL is an E3 that inhibits angiogenesis under normoxic conditions and is known for its antioncogenic role. Mutations in VHL are responsible for familial von Hippel-Lindau syndrome, a type of renal cancer, and somatic mutations of VHL genes are responsible for cancers like sporadic clear-cell renal carcinomas. E6-AP is a HECT domain E3 that degrades p53, proto-oncogene c-Myc and several other substrates. It is associated with E6 oncoprotein of human papillomavirus (HPV). E6-AP mutations are associated with Angelman's syndrome, and in certain sexually transmitted HPV serotypes like HPV-16 and HPV-18, P53 degradation by E6-AP causes transformation in uterine cervical epithelial cells, leading to cervical cancer. Mutations in components of ubiquitin system involved in DNA repair like E3 BRCA1 (discussed above) can also cause cancer [16].

Mutations and changes in the expression levels of various components of UPS are responsible for causing many diseases. Besides, ubiquitin system is also exploited by several pathogens for their benefit. Ubiquitin-mediated endocytosis of anthrax toxin-receptor complex leads to toxin activation [244]. *Yersinia pestis* produces a deubiquitinating enzyme YopJ, which prevents activation of NF-kB [245, 246]. Modulator of immune recognition 1 of Kaposi's sarcoma-associated herpes virus (KSHV) is an E3 enzyme, which ubiquitinates cysteines in MHC class I molecules, causing their endocytosis and degradation [247]. Certain tumour-causing viruses transform host cells by activating NF-κB pathway, using ubiquitin-dependent mechanisms. Tax protein of human T-cell leukaemia virus 1 (KSHV), an activator of IKK and NF-κB, is K63 polyubiquitinated by Ubc13 [248, 249]. However, no impairment is seen in Ubc13 knockdown cells [250]. STP-C of herpes virus *Saimiri* and LMP1 of Epstein-Barr virus also show ubiquitin-dependent activation of IKK [251, 252] by binding to TRAF6.

26.6 Conclusion

Ubiquitin-proteasome system is a very versatile protein-degrading machinery that selectively degrades polyubiquitinated proteins with a few exceptions. Unlike other proteases, this system gives cell the ability to make very specific changes in its proteome to adapt to changing environment. How much ubiquitin system increases

cell's survivability is evident from lethality of ubiquitin mutations like EP42. And the high degree of ubiquitin's sequence and structure conservation across species underlines its functional relevance. Numerous proteins have evolved to interact with different surfaces on ubiquitin through various ubiquitin-binding domains. E1, E2 and E3 are the enzymes involved in ubiquitination, and high diversity of E3s is responsible for the specificity of ubiquitination. Deubiquitinating enzymes do not just recycle of ubiquitin but confer additional control and specificity to the system. The simple structure of ubiquitin makes it an ideal system to study protein folding, and many structural studies have been carried out on it. Proteasomal degradation is involved in numerous cellular processes including DNA repair, transcription, protein synthesis and cell signalling. Besides, ubiquitination is involved not just in proteasomal degradation but lysosomal degradation as well. Owing to its diverse functions, defects in ubiquitin system have been implicated in several diseases like Alzheimer's disease, Parkinson's disease, Huntington's disease, various cancers and microbial infections. Nevertheless ubiquitin proteasome system along with deubiquitinating enzymes offers several prime drug targets which could be exploited in future to treat these diseases. This has triggered intense research on ubiquitin system and makes it an important field of study in our quest to improve human health.

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International Journal of Biological Macromolecules 179 (2021) 299–308

Contents lists available at ScienceDirect

International Journal of Biological Macromolecules

journal homepage: http://www.elsevier.com/locate/ijbiomac

Probing the effects of double mutations on the versatile protein ubiquitin in *Saccharomyces cerevisiae*

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article info

Article history:
Received 4 January 2021
Received in revised form 21 February 2021
Accepted 27 February 2021
Available online 01 March 2021

Keywords:
Yeast
Saccharomyces cerevisiae
Yeast ubiquitin
ubiquitin double mutations

abstract

Ubiquitin is an indispensable protein of eukaryotic origin with an extraordinarily high degree of sequence conservation. It is used to tag proteins post-translationally and the process of ubiquitination regulates the activity of the modified proteins or drives them for degradation. Double mutations produce varied effects in proteins, depending on the structural relationship of the mutated residues, their role in the overall structure and functions of a protein. Six double mutants derived from the ubiquitin mutant UbEP42, namely S20F-A46S, S20F-L50P, S20F-I61T, A46S-L50P, A46S-I61T, and L50P-I61T, have been studied here to understand how they influence the ubiquitination related functions, by analysing their growth and viability, Cdc28 levels, K-48 linked polyubiquitination, UFD pathway, lysosomal degradation, endosomal sorting, survival under heat, and antibiotic stresses. The double mutation L50P-I61T is the most detrimental, followed by S20F-I61T and A46S-I61T. The double mutations studied here, in general, make cells more sensitive than the wild type to one or the other stress. However, the excessive negative effects of L50P and I61T are compensated under certain conditions by S20F and A46S mutations. The competitive inhibition produced by these substitutions could be used to manage certain ubiquitination associated diseases.

1. Introduction

Ubiquitin is a small, monomeric, single-domain protein. It is used by the eukaryotic cell to tag other proteins in the process of ubiquitination [1]. Ubiquitination is an important post-translational modification that regulates the activity of the modified proteins or alters their concentration by driving them to degradation. Ubiquitination serves as one of the principal regulatory mechanisms of a eukaryotic cell controlling a variety of cellular processes including protein degradation [1], cell cycle [2–6], stress responses [7], protein sorting and trafficking [8,9], endocytosis [10–13], transcription [14] and translation [15]. Substrate proteins are ubiquitinated by the formation of a covalent bond between a lysine residue on them and the C-terminal glycine residue of ubiquitin [16]. Ubiquitination is catalysed by a set of three enzymes, namely ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2) and ubiquitin ligase enzyme (E3). Polyubiquitin chains are formed when a substrate ligated ubiquitin itself is ubiquitinated. Monoubiquitination and polyubiquitination are responsible for regulating varied cellular functions [17]. The sequence of ubiquitin is highly conserved across species [18–22]. In fact, the sequence and structure of ubiquitin have changed very little since it first appeared around 2×10^3–2.5×10^3 million years ago, in the unicellular organism, the Last Eukaryotic Common Ancestor [23]. Ubiquitin has seven lysine residues, all of which can be employed in ubiquitination.

The three-dimensional structure of ubiquitin was first solved at 2.8 Å resolution using X-ray crystallography [24], and was further refined at 1.8 Å [25]. Structurally, ubiquitin possesses an α helix made up of three and a half turns, a mixed β sheet made up of four strands and nine reverse turns. The core of the ubiquitin is significantly hydrophobic, and the molecule has extensive hydrogen bonding, both of which contribute to the high thermostability observed with ubiquitin. Further, the salt bridges between charged residues on the ubiquitin surface also add to its thermostability [26,27]. All the lysines involved in ubiquitination are located on the surface.

In the yeast *Saccharomyces cerevisiae*, ubiquitin is encoded by 4 different genes, namely *UBI1*, *UBI2*, *UBI3*, and *UBI4* [28]. *UBI1* and *UBI2* genes express ubiquitin molecule C-terminally fused to L40 ribosomal protein. *UBI1* and *UBI2* also have an intron. *UBI3* produces ubiquitin C-terminally fused to S31 ribosomal protein and also has an intron. *UBI4* produces a polymeric head to the tail fusion of four to five ubiquitins. This fusion is post-translationally hydrolysed into ubiquitin monomers by ubiquitin C-terminal hydrolase. *UBI1*, *UBI2* and *UBI3* are housekeeping genes, while *UBI4* is expressed in stress conditions and helps the cell deal with stress. The stress condition is recognised by the heat shock box element present upstream of the coding region of *UBI4*.

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https://doi.org/10.1016/j.ijbiomac.2021.02.208
0141-8130/

The high degree of sequence conservation of ubiquitin does not leave scope for too many sequence homologs in nature to understand the contribution of individual residues to its function [28–30]. In this background, the most useful methods to study structure-function relationships in ubiquitin are site-directed mutagenesis [31–34] and *in vitro* evolution. Natural evolution happens over a course of millions of years. On the other hand *in vitro* evolution is much faster due to fast-growing microbial model systems with very low doubling times, like yeast, and techniques that introduce random mutations with high frequency, like error-prone PCR. Error prone PCR is a PCR process in which the fidelity of DNA polymerase is reduced by various means, like varying the concentration of Mg ions, introducing Mn ions, and creating an imbalance in the concentration of dNTPs. To probe into structure-function relationships ubiquitin was subjected to *in vitro* evolution. The ubiquitin gene was amplified using error-prone PCR in our laboratory to generate mutants forms of the protein. Among the many mutants obtained from successive rounds of error-prone PCR, one mutant, namely UbEP42, proved to be lethal when over-expressed in permissive conditions [35].

The sequence of UbEP42 with four different amino acid substitutions, namely S20F, A46S, L50P and I61T, was characterized to understand the reasons behind dosage dependent lethality [36]. When each of these four substitutions was studied in isolation, I61T and L50P were unable to complement heat stress phenotype and antibiotic sensitivity [37]. Since all four of these substitutions are included in UbEP42 mutation, here we assess their effects in pairs of all combinations, namely S20F-A46S, S20F-L50P, S20F-I61T, A46S-L50P, A46S-I61T and L50P-I61T, to understand their effects on each other.

2. Materials and methods

2.1. Yeast strains and media

The two strains of *Saccharomyces cerevisiae* used in this study are SUB62 (Mata, lys2-801leu2-3,2-112 ura3-52 his3-Δ200 trp1-1) and SUB60 (Mata, lys2-801, leu2-3,112, ura3-52, his3-Δ200, trp1-1, ubi4-Δ2::LEU2) [3,38]. The cultures were grown at 30 °C at 200 r.p.m. One of the media used for growing yeast is synthetic dextrose (SD) medium containing 0.67% yeast nitrogen base (without amino acids) from Himedia and 2% glucose for carbon source. Additional supplements included in SD medium for selection are Histidine (20 mg L^{-1}), lysine (30 mg L^{-1}), uracil (20 mg L^{-1}), leucine (100 mg L^{-1}) and tryptophan (20 mg L^{-1}), depending on requirements of experiments [3]. Another medium used for growing yeast was Yeast Peptone Dextrose (YPD), the composition of which was 2% peptone (*w*/*v*), 2% dextrose (w/v), 1% yeast extract (w/v) and 2% agar (w/v).

2.2. Plasmids

The high copy number plasmid YEp96 was used to clone and express the genes of wild type and mutant forms of ubiquitins [3]. YEp96 has *TRP1* as a selection marker and can replicate in both *Escherichia coli* and *S. cerevisiae*, which enables it to be used as a shuttle vector. The desired gene cloned in YEp96 is expressed under *CUP1* promoter, which can be induced by $CuSO_4$. YEp96 plasmids carrying wild type and UbEP42 mutant ubiquitin genes are referred to here as YEp96/UbWt and YEp96/UbEP42, respectively. And YEp96 plasmids carrying the genes for ubiquitin double mutants derived from *UbEP42* gene are Yep96/UbS20F-A46S, YEp96/UbS20F-L50P, YEp96/UbS20F-I61T, YEp96/UbA46S-L50P, YEp96/UbA46S-I61T and YEp96/UbL50P-I61T. Following the protocols standardized in our laboratory, in the experiments completed in shorter durations, the cells were overexpressed with 100 mM $CuSO_4$ and where the experiments involved longer incubations, the variants of ubiquitin gene were expressed with 25 mM $CuSO_4$ to avoid any cytotoxic effects. SUB60 cells transformed with YEp96/UbWt, YEp96/UbEP42, YEp96/UbS20F-A46S, YEp96/UbS20F-L50P, YEp96/UbS20F-I61T, YEp96/UbA46S-L50P, YEp96/UbA46S-I61T and YEp96/UbL50P-I61T were grown in SD medium at 30 °C, and growth was monitored by measuring OD at 600 nm.

2.3. Generation time

Freshly grown cultures of untransformed SUB60 and SUB60 transformed with YEp96/UbWt, YEp96/UbS20F-A46S, YEp96/UbS20F-L50P, YEp96/UbS20F-I61T, YEp96/UbA46S-L50P, YEp96/UbA46S-I61T and YEp96/UbL50P-I61T were inoculated in SD medium containing 0.67% yeast nitrogen base (without amino acids) from Hi-media, 2% glucose, Histidine (20 mg L^{-1}), lysine (30 mg L^{-1}) uracil (20 mg L^{-1}) and 100 μM copper sulphate. Leucine (100 mg L^{-1}) was added to SD medium for SUB62, as the strain is auxotrophic for leucine. Cultures were then grown at 30 °C at 200 rpm to stationary phase. During their growth, OD of cultures at 600 nm was measured at intervals of 2 h up till stationary phase. To calculate generation time, two consecutive OD_{600} values were taken from mid log phase.

2.4. Viability assay

Untransformed SUB62, SUB60 strains and SUB60 cells transformed with YEp96/UbWt, YEp96/UbS20F-A46S, YEp96/UbS20F-L50P, YEp96/UbS20F-I61T, YEp96/UbA46S-L50P, YEp96/UbA46S-I61T and YEp96/UbL50P-I61T were inoculated in SD medium containing 25 μM copper sulphate. Leucine (100 mg L^{-1}) was added to SD medium for SUB62 as the strain is auxotrophic for leucine. Cultures were then grown at 30 °C at 200 rpm up to OD of 0.5–0.6 measured at 600 nm. Then their four-fold serial dilutions were prepared which were plated on SD agar and incubated at 30 °C. The composition of the SD agar was same as that of SD broth.

2.5. Heat stress complementation

Untransformed SUB60 cells and SUB60 cells transformed with YEp96/UbWt, YEp96/UbS20F-A46S, YEp96/UbS20F-L50P, YEp96/UbS20F-I61T, YEp96/UbA46S-L50P, YEp96/UbA46S-I61T and YEp96/UbL50P-I61T were grown in SD broth at 30 °C at 200 rpm, up to the optical density of 0.5-0.6 measured at 600 nm. Four-fold serial dilutions were prepared and were plated on SD agar containing 25 μM $CuSO_4$. The plates were incubated at 40 °C for time periods of 0, 4, 8, 12 and 16 h and transferred to 30 °C. Colonies were counted to measure survival after five days of incubation. This experiment was repeated three times using independent sets, and mean values were calculated.

2.6. Antibiotic sensitivity test

Antibiotic sensitivity test was carried out to test the complementation potential of UbEP42 derived double mutants [15]. SUB60 cells transformed with YEp96/UbWt YEp96/UbS20F-A46S, YEp96/UbS20F-L50P, YEp96/UbS20F-I61T, YEp96/UbA46S-L50P, YEp96/UbA46S-I61T and YEp96/UbL50P-I61T were grown up to optical density of 0.2 as measured at 600 nm. At this stage, four-fold dilutions of cultures were prepared and spotted on SD agar plates. Concentration of cycloheximide used was 4 μg/ml. Two plates were prepared, one without $CuSO_4$, used as negative control, and one with 25 μM $CuSO_4$. After incubation at 30 °C for ten days, the growth of the mutants was compared to that of SUB62 and untransformed SUB60.

2.7. K-48 linked polyubiquitination assay

SUB60 cells transformed with YEp96/UbWT, YEp96/UbS20F-A46S, YEp96/UbS20F-L50P, YEp96/UbS20F-I61T, YEp96/UbA46S-L50P, YEp96/UbA46S-I61T and YEp96/UbL50P-I61T were grown to log phase in YPD at 30 °C, up till OD 0.6 at 600 nm. Cells were then pelleted down, washed with normal saline and then suspended in normal saline.

OD of cells was then equalized and protease inhibitor cocktail was added to this suspension to prevent protein degradation, followed by lysing of cells by sonication. The total protein in the lysate was quantified by Folin Lowry method [39] and accordingly, samples of each lysate containing 50 μg of protein were subjected to SDS-PAGE [40] on 15% sodium dodecyl sulphate polyacrylamide gels. The gel was then subjected to western blotting [41,42] using nitrocellulose membrane. Ponceau staining of the blot was done. The blot was washed with PBS and then incubated in blocking buffer containing 5% (*w*/*v*) non-fat dry milk in PBS-T for 1 h, to block non-specific antibody binding sites. The blot was then incubated for 16 h with PBS-MT (mixture of PBS, milk powder and Tween 20) containing antibody specific for K-48 linked polyubiquitin. After incubation, the blot was washed with PBST, incubated in PBSMT containing secondary antibody (raised in rabbit), followed by few washes with PBST and PBS. Lastly, ECL solution (Horseradish peroxidase and H_2O_2) (Bio-Rad Clarity) was used to observe the bands corresponding to K-48 linked polyubiquitin.

2.8. Lysosomal degradation of uracilpermease

Fresh SUB60 cells transformed with YEp96/UbWt, YEp96/UbS20F-A46S, YEp96/UbS20F-L50P, YEp96/UbS20F-I61T, YEp96/UbA46S-L50P, YEp96/UbA46S-I61T and YEp96/UbL50P-I61T were grown till log phase and then treated with 100 μg/ml of cycloheximide for 20 min to inhibit the synthesis of uracil permease. Two western blots were then carried out, one with cells lysed immediately after cycloheximide treatment and other with cells incubated for 90 min after cycloheximide treatment. The incubation time of 90 min was required to allow for the lysosomal degradation of uracil permease that was synthesised before cycloheximide treatment. The antibody used was specific for uracil permease, a gift received from Rosine Haguenauer-Tsapis from Institute Jacques Monod. The procedure followed for lysis of cells and western blotting is same as that described for K48 linked polyubiquitination assay.

2.9. Cdc28 assay

Cdc28 is the gene that encodes CDK1, the cyclin dependent kinase that is the master regulator of cell cycle in yeast. To analyse levels of Cdc28 in cells, western blot involving antibody specific for Cdc28 was used. SUB60 cells transformed with YEp96/UbWt, YEp96/UbS20F-A46S, YEp96/UbS20F-L50P, YEp96/UbS20F-I61T, YEp96/UbA46S-L50P, YEp96/UbA46S-I61T and YEp96/UbL50P-I61T. The protocol for growth of cells, lysis and western blot is same as that described for K48 linked polyubiquitination assay.

2.10. N-end rule and UFD pathways

To analyse the degradation of protein substrates by N-end rule and UFD pathways, untransformed SUB60 cells and SUB60 cells transformed with YEp96/UbWt, YEp96/UbS20F-A46S, YEp96/UbS20F-L50P, YEp96/UbS20F-I61T, YEp96/UbA46S-L50P, YEp96/UbA46S-I61T and YEp96/UbL50P-I61T, were cotransformed with pUb23. Plasmid pUb23 carries the gene for ubiquitin-β-galactosidase fusion protein (Ub-X-βgal), and uses URA3 as selection marker [29,43]. Two different sets of cells were prepared. In the first set the plasmid carried gene for Ub-M-βgal fusion protein, where M or methionine was the N-terminal residues of β-galactosidase. It was used to study N-end rule pathway [44,45]. Second set expresses the gene for Ub-P-βgal fusion protein, where P or proline was the N-terminal residue of-β-galactosidase. It was used to study UFD pathway [45,46]. The degradation of β-galactosidase was analysed by western blotting using anti-β-galactosidase antibody specific for β-galactosidase. The protocol for the western blotting is same as that described for K48 linked polyubiquitination assay. The ubiquitin-β-galactosidase gene in pUb23 is under *GAL10* promoter, which is galactose inducible. To constitutively express Ub-M/P-β galactosidase, the cells were grown to mid-log phase in synthetic galactose medium at 30 °C. To these mid-log phase cultures, 100 μM $CuSO_4$ was added to express UbWt and UbEP42 derived double mutants of ubiquitin.

2.11. Confocal microscopy for endosomal sorting of GFP-CPS fusion protein

SUB60 cells transformed with YEp96/UbWt, YEp96/UbS20F-A46S, YEp96/UbS20F-L50P, YEp96/UbS20F-I61T, YEp96/UbA46S-L50P, YEp96/UbA46S-I61T and YEp96/UbL50P-I61T were grown in SD broth at 30 °C up to OD 0.8 measured at 600 nm. Cells were harvested and resuspended in YPD broth. The stock solution of FM4–64 dye was prepared in 16 μM DMSO and added up to a final concentration of 20 μM. Cells were then incubated in a stack incubator for 15 min. at 30 °C. Cells were then harvested by centrifugation at 700*g* for 3 min at 4 °C and resuspended in YPD broth. The cells from this suspension were deposited on a slide for observation under confocal microscope (Zeiss LSM 700).

3. Results

3.1. Overexpression of the UbEP42 derived double mutants S20F-L50P, A46S-L50P and L50P-I61T caused lethality

Previous studies have shown that overexpression of UbEP42 caused lethality in SUB60 cells. Similarly, overexpression of the two single mutations UbL50P and UbI61T isolated from UbEP42 was found to be lethal to SUB60 cells. But untransformed SUB60 lacking *UBI4* polyubiquitin gene, and SUB62 cells with overexpression of UbEP42 protein did not suffer lethality. This is because more copies of UbEP42 mutant ubiquitin are produced in transformed SUB60, which compete with wild type ubiquitin in its functions. On the other hand, in SUB62 there are enough molecules of wild type ubiquitin to outcompete defective copies of UbEP42. But lack of *UBI4* gene in SUB60 makes it more vulnerable to detrimental effects of UbEP42. The six different UbEP42 derived double mutants were also tested to see if any of them will cause similar negative effects when overexpressed or the presence of two mutations in a single molecule will produce complimentary effect (Fig. 1). SUB60 cells transformed with YEp96 plasmids encoding wild type ubiquitin and the double mutants were treated with 0, 100 and 200 μM $CuSO_4$. Results show that the mutant proteins UbS20F-L50P, UbA46S-L50P and UbL50P-I61T were lethal when overexpressed, while the mutant S20F-I61T is mildly lethal and the two other mutants S20F-A46S, and A46S-I61T were nonlethal even in 200 μM $CuSO_4$. Cells expressing UbWt served as positive control.

3.2. Expression of UbEP42 derived double mutations prolonged the generation times

The effect of UbEP42 double mutations on generation time of *Saccharomyces cerevisiae* was analysed. Two consecutive OD measurements were made at 600 nm for calculation of generation time. Results show that when overexpressed using $CuSO_4$, all the double mutants except A46S-I61T had increased generation time compared to wild type cells (Fig. 2). Generation time of A46S-L50P was significantly higher than wild type cells even in the absence of overexpression. It had a generation time of 4.3 h during overexpression. Mutants S20F-A46S, S20F-L50P, S20F-I61T and L50P-I61T had generation times of 3.58, 3.56, 6.16 and 5.40 h respectively when overexpressed.

3.3. The co-occurrence of nonlethal single mutations S20F and A46S along with the lethal L50P and I61T mutations increased cell viability

For testing the viability of SUB60 cells expressing UbEP42 derived double mutants, SUB60 cells expressing wild type ubiquitin and UbEP42 derived double mutations were grown to log phase. From these cultures, serial four-fold dilutions were made and plated on plates

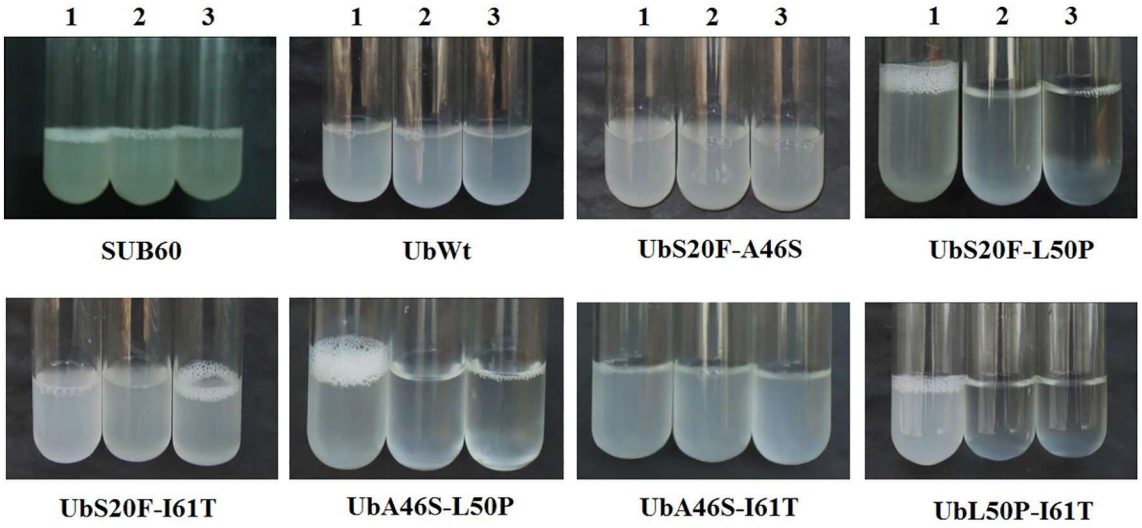

Fig. 1. Effect of the expression of UbEP42 derived double mutants of ubiquitin UbS20F-A46S, UbA46S-L50P, UbL50P-I61T, UbA46S-I61T, UbS20F-L50P, UbS20F-I61T and UbWt on SUB60 cells. The expression of ubiquitin wild type protein and its mutant forms from the plasmids was induced by adding 0, 100 and 200 μM $CuSO_4$, from left to right.

containing SD agar, both with and without induction by $CuSO_4$. Viability was measured by counting the number of colony forming units (Fig. 3). Comparison of viabilities of S20F-A46S, A46S-I61T and S20F-L50P showed no significant differences between induced and uninduced conditions. With the double mutants, S20F-I61T, A46S-L50P and L50P-I61T, viability under induced condition decreased considerably with respect to viability under uninduced condition.

3.4. The double mutants of ubiquitin except S20F-A46S render the cells more sensitive to heat stress

Analysis of heat stress sensitivity shows that the cells expressing double mutant forms of ubiquitin namely A46S-I61T, S20F-I61T, S20F-L50P, A46S-L50P and L50P-I61T are hypersensitive to heat stress with overexpression (Fig. 4). The double mutant forms of ubiquitin S20F-A46S and A46S-I61T to a lesser extent offer protection to cells by complementing the function of wild type ubiquitin. Remaining double mutations of

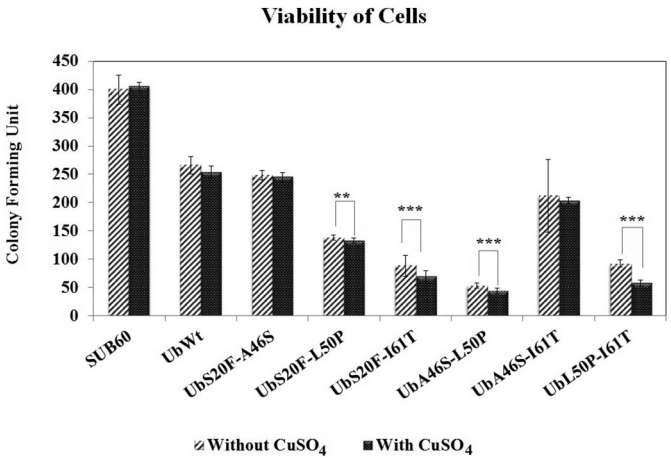

Fig. 3. Viability in terms of colony forming units of untransformed SUB60 and SUB60 transformed with YEp96/UbWt, YEp96/UbS20F-A46S, YEp96/UbS20F-L50P, YEp96/UbS20F-I61T, YEp96/UbA46S-L50P, YEp96/UbA46S-I61T and YEp96/UbL50P-I61T. Viabilities of the uninduced transformants were compared with those treated with 25 μM $CuSO_4$ inducer.

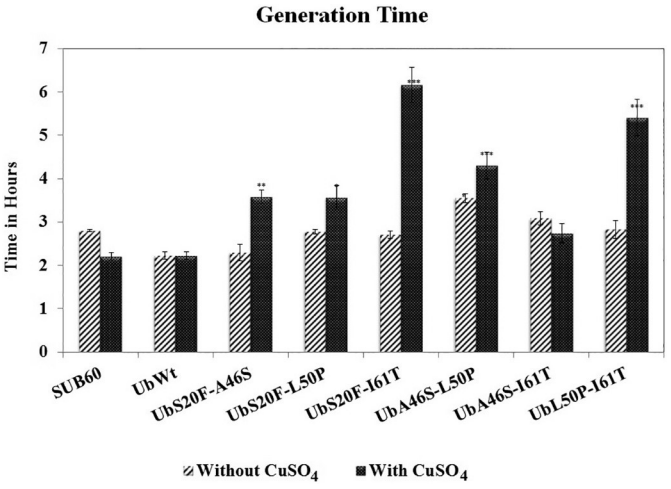

Fig. 2. Generation times of untransformed SUB60 and SUB60 transformed with YEp96/UbWt, YEp96/UbS20F-A46S, YEp96/UbS20F-L50P, YEp96/UbS20F-I61T, YEp96/UbA46S-L50P, YEp96/UbA46S-I61T and YEp96/UbL50P-I61T in SD medium without induction and under induction by 25 μM $CuSO_4$. The generation time values were represented as Mean ± SE. p*** <0.001 and p = NS represents non-significance and the experiment was repeated in three independent sets.

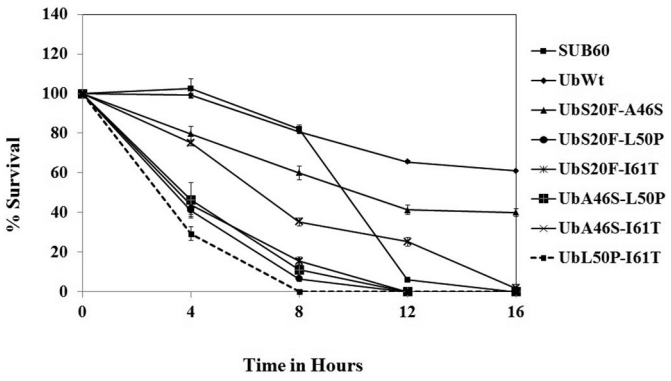

Fig. 4. Percent survival under heat stress of untransformed SUB60 and SUB60 transformed with YEp96/UbWt, YEp96/UbS20F-A46S, YEp96/UbS20F-L50P, YEp96/UbS20F-I61T, YEp96/UbA46S-L50P, YEp96/UbA46S-I61T and YEp96/UbL50P-I61T in SD medium under induction by 25 μM $CuSO_4$.

ubiquitin render the cells more sensitive than the untransformed SUB60. This suggests that they compete with the wild type ubiquitin and interfere with the degradation of proteins denatured under heat stress. The possible inhibitory mechanism of action of mutant forms of ubiquitin vis-a vis wild type ubiquitin presented in the Discussion.

3.5. Increased sensitivity observed with all the double mutants to the translational inhibitor cycloheximide

Ubiquitination is known to be involved in the regulation of protein synthesis. L28, a ribosomal protein, is known to undergo K-63 linked polyubiquitination, which makes yeast more resistant to certain translational inhibitors [47]. Many other ribosomal proteins are also known to undergo ubiquitination. The UbEP42 mutant cells have been found to be more sensitive than the wild type cells towards the translational inhibitor cycloheximide [48], from the previous studies in our laboratory. Hence, the sensitivity of UbEP42 derived double mutants towards cycloheximide was tested. The results indicate that double mutants S20F-L50P, S20F-I61T, A46S-L50P and L50P-I61T are more sensitive than wild type cells to cycloheximide (Fig. 5).

3.6. The double mutants of ubiquitin S20F-I61T, A46S-L50P and L50P-I61T interfere with UFD pathway of protein degradation

To study the effect of UbEP42 derived double mutants on protein degradation by N-end rule and UFD pathways, double transformants of SUB60 cells expressing the ubiquitin double mutations and Ub-M- β-galactosidase or Ub-P- β-galactosidase were employed. After growth and induction of the proteins as described in Materials and Methods section, these cells were subjected to western blot using antibody specific for β-galactosidase. In the results it was observed that with the mutants S20F-I61T, A46S-L50P and L50P-I61T, the intensity of bands of Ub-P-βgal was not significantly reduced than the intensity of bands of Ub-M-βgal (Fig. 6). This suggests that these three double mutants have a detrimental effect on the degradation of β-galactosidase through UFD pathway.

3.7. Double mutants of ubiquitin S20F-I61T, A46S-L50P and L50P-I61T have negative influence over the levels of Cdc28 protein kinase acting as roadblocks to cell cycle progression

Ubiquitin proteasome system is known to play a major role in cell cycle progression. The transition of a cell from one stage of the cell cycle to the other is accomplished by proteasomal degradation of specific regulatory proteins involved in the previous stage to allow proteins of the later stage to work. CDK1 (Cyclin Dependent Kinase 1) is a serine/threonine kinase that is involved in regulation of cell cycle. In *Saccharomyces cerevisiae*, it is produced by the gene *cdc28*. To study the effect of UbEP42 double mutations on Cdc28 levels, SUB60 cells carrying the double mutations were subjected to western blot using antibody specific towards Cdc28. The results show that bands of Cdc28 corresponding to the cells expressing three double mutants, namely S20F-I61T, A46S-L50P and L50P-I61T, of very low intensity when compared to that of wild type (Fig. 7).

3.8. Degradation of membrane proteins by lysosome is inhibited by the double mutants of ubiquitin S20F-I61T, A46S-L50P and L50P-I61T

Degradation of membrane proteins is carried out by the lysosome. Previous studies have shown that ubiquitin plays a role not only in proteasomal degradation but also in lysosomal degradation of proteins [49,50]. To test the effect of UbEP42 derived double mutations affect the lysosomal degradation of proteins, SUB60 cells transformed with YEp96 plasmids expressing wild type ubiquitin and UbEP42 derived double mutants were subjected to western blot using antibody specific for uracil permease, an integral membrane protein that transports uracil across the plasma membrane. Lysosomal degradation of uracil permease synthesised before and after cycloheximide treatment were analysed by western blotting using anti-uracil permease antibody. The results indicate that double mutations of ubiquitin S20F-I61T, A46S-L50P and L50P-I61T inhibit lysosomal degradation of uracil permease, while the mutant 46–61 shows a mild inhibitory effect (Fig. 8).

3.9. Double mutants of ubiquitin S20F-I61T, A46S-L50P and L50P-I61T negatively affect K-48 linked polyubiquitination

K48 linked polyubiquitination on substrate proteins and their subsequent degradation changes the proteome makeup of a cell from to time, working as a dominant mode of regulation. One of the possible causes behind the effect of UbEP42 derived double mutations on various functions of ubiquitin is that these mutations might impede the formation of K48 linked polyubiquitin chains. To test this possibility, SUB60 cells transformed with YEp96 plasmids expressing UbEP42 derived double mutants were subjected to western blotting using antibody specific for K48 linked Polyubiquitin. Results indicate that three double mutants,

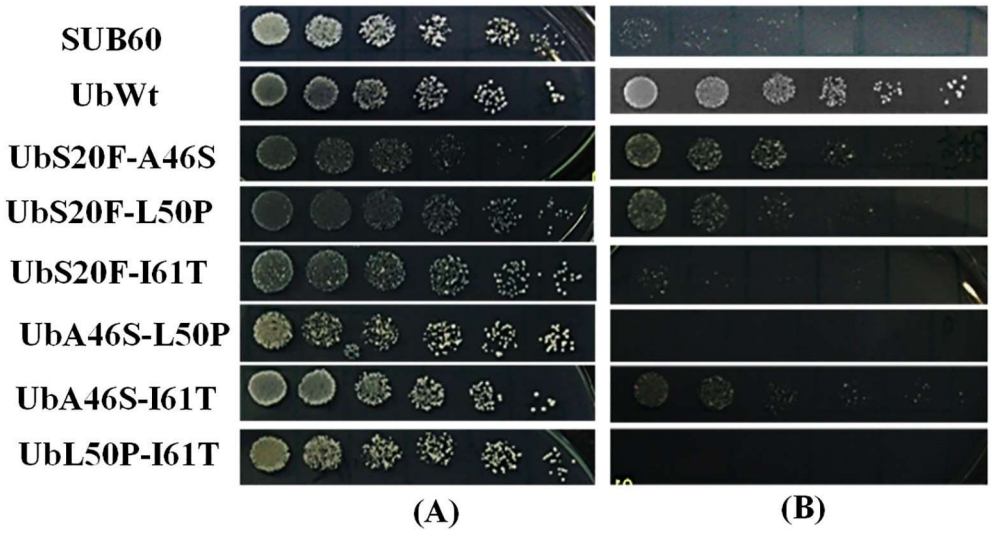

Fig. 5. Drop test for testing sensitivity to cycloheximide of SUB60 transformed with YEp96/UbWt, YEp96/UbS20F-A46S, YEp96/UbS20F-L50P, YEp96/UbS20F-I61T, YEp96/UbA46S-L50P, YEp96/UbA46S-I61T and YEp96/UbL50P-I61T in SD medium in (A) Without induction and in the absence of cycloheximide, (B) under induction by 25 μM $CuSO_4$ and in the presence of cycloheximide.

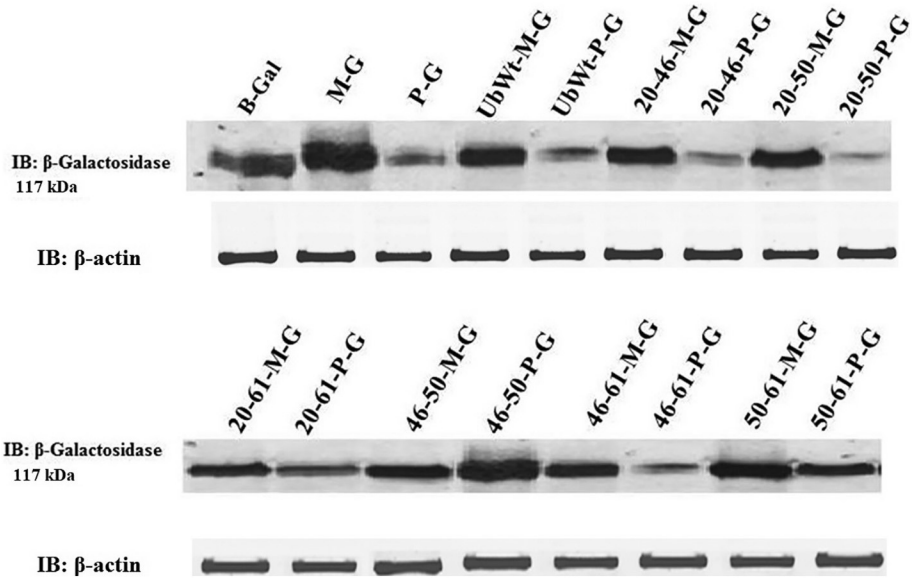

Fig. 6. Effect of UbEP42 derived double mutations on *ubiquitin fusion degradation* (UFD) pathway in SUB60 cells. Effect of the double mutations on the degradation of proteins by UFD pathway was monitored by assaying the activity of β galactosidase. SUB60 transformed by plasmids YEp96/UbWt, YEp96/S20F-A46S, YEp96/S20F-L50P, YEp96/S20F-I61T, YEp96/A46S-L50P, YEp96/A46S-I61T, YEp96/L50P-I61T were cotransformed by pUB23 carrying the chimeric gene for Ub-Met-β galactosidase (indicated as M-G) and pUB23 carrying the chimeric gene for Ub-Pro-β galactosidase (indicated as P-G) respectively. SUB 60 cells transformed only by pUB23 with M-G and with P-G as positive controls for respectively. Ub-Met-β -galactosidase fusion is a substrate which undergoes deubiquitination and subsequently degradation by N-end rule pathway. Expression of UbS20F-I61T, UbA46S-L50P, UbL50P-I61T blocked the degradation of Ub-Pro-β galactosidase, the substrate of UFD pathway. Expression of UbS20F-A46S, UbS20F-L50P, UbA46S-I61T had no bearing on UFD pathway mediated degradation of Ub-Pro-β galactosidase.

namely S20F-I61T, A46S-L50P and L50P-I61T have a negative effect on K48 linked polyubiquitination of proteins, which could be the reason behind the detrimental effect of these double mutations on the other functions studied here (Fig. 9).

3.10. Endosomal sorting of Carboxypeptidase S is inhibited by the double mutant forms S20F-I61T, A46S-L50P, L50P-I61T

Carboxypeptidase S is a single pass membrane protein found in the membrane of vacuoles in yeast cells. It is a proteolytic enzyme. It is known to be a cargo of multivesicular bodies (MVBs), and sorting of membrane proteins through MVBs is known to be dependent on monoubiquitination as well as K63 linked polyubiquitination [51]. Specifically, K-63 linked polyubiquitination is believed to play a role in internalization of membrane proteins through the ESCRT pathway, while monoubiquitination seems to be involved not just in internalization of membrane proteins, but also their subsequent sorting into MVBs.

Since the UbEP42 derived double mutations have shown effects to varying degrees on SUB60 cells, we decided to study their effect on endosomal sorting of carboxypeptidase S. For this, a plasmid bearing genetic fusion of carboxypeptidase S and GFP (GFP-CPS) was obtained and was transformed into SUB60 cells, that were earlier transformed with YEp96/UbWt and YEp96 expressing the UbEP42 derived double mutants. These cells were then grown in SD medium till log phase, treated with FM464 dye and fixed for confocal microscopy. FM464 dye gives red fluorescence and specifically stains vacuolar membrane [52]. The sorting of GFP-CPS and its localization to vacuole was studied by observing the yellow fluorescence obtained by the colocalization of GFP-CPS and FM464 in vacuolar membrane and MVBs. SUB60 cells expressing UbS20F-I61T, UbA46S-L50P, UbL50P-I61T showed failure of sorting of CPS into endosomes, while sorting remains unaffected in the expressing cells expressing UbS20F-A46S, UbS20F-L50P, UbA46S-I61T and was comparable to UbWt control (Fig. 10).

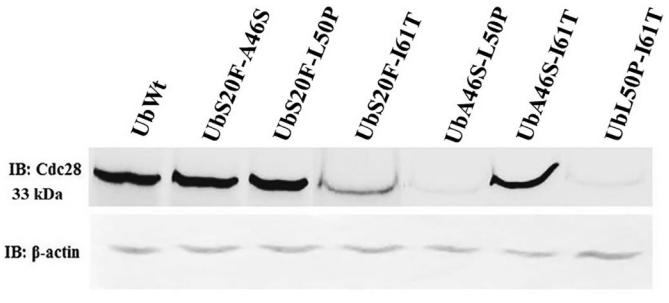

Fig. 7. Effect of UbEP42 derived double mutations on cell cycling, Cdc28 levels in SUB60 cells. Western blot analysis of Cdc28 shows band intensities in cells expressing UbS20F-A46S, UbS20F-L50P, UbA46S-I61T remained comparable to that of UbWt. The band of Cdc28 in cells expressing UbS20F-I61T showed considerable reduction in its intensity, while those of UbA46S-L50P, UbL50P-I61T showed negligible intensities corresponding extremely diminished levels of Cdc28.

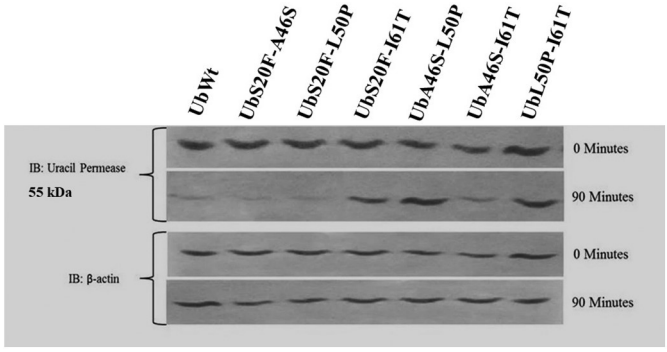

Fig. 8. Effect of UbEP42 derived double mutations on lysosomal degradation of uracil permease in SUB60 cells. Expression of UbS20F-A46S, UbS20F-L50P did not have any effect over the degradation of uracil permease by lysosomes. Expression of UbA46S-I61T had a mild negative influence over the degradation. UbS20F-I61T, UbA46S-L50P, UbL50P-I61T hindered the lysosomal degradation of uracil permease to a great extent.

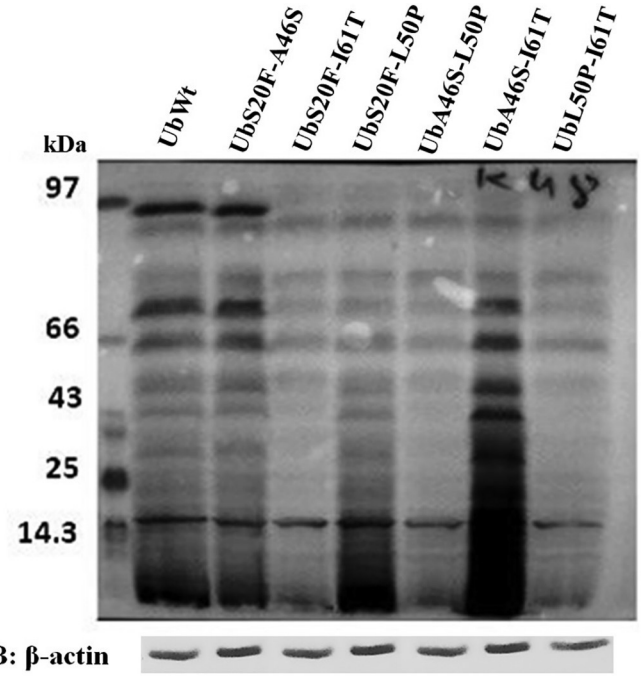

Fig. 9. Effect of UbEP42 derived double mutations on K48 linked polyubiquitination. Western blotting carried out with anti-K48 polyubiquitin antibody. Cells expressing UbS20F-A46S, UbS20F-L50P, UbA46S-I61T showed K48 linked polyubiquitination to the same extent as those of UbWt. In contrast, in cells expressing UbS20F-I61T, UbA46S-L50P, UbL50P-I61T K48 linked polyubiquitination is much reduced.

The functional aspects of the six double mutant forms of ubiquitin along with wild type ubiquitin are presented in the form of a Table for comparison (Table 1).

4. Discussion

Single mutations are introduced in proteins to understand the contribution of individual residues to the structure, stability and function of a protein. While double mutations of proteins have the potential to produce varied effects depending on the structural relationship present between the two residues, revealing insights into the structural details of a protein and the related functional consequences, besides revealing how the two residues individually contribute to the proteins structure and function. Hence as it was observed with many proteins the effects of double mutations may be additive, partially additive, synergistic, antagonistic, or show no change [53,54].

Ubiquitin being an essential and indispensable protein in eukaryotes, nature permitted evolution of very few sequence homologs. In order to understand the importance of conserved residues certain site directed mutations were generated and characterized in our laboratory. Combinations of double mutations located in the parallel β-bulge were studied previously [33,34]. However, site directed mutations in a protein like ubiquitin with no enzymatic function of its own may not always give significant insights.

As an alternative, *in vitro* evolution ubiquitin and selection of mutations on the basis of lethal effects produced appeared a promising strategy. As mentioned in the Introduction among the four genes encoding ubiquitin, *UBI1*, *UBI2*, *UBI3* and *UBI4* in *S. cerevisiae* [28,38], the genes *UBI1*, *UBI2*, *UBI3* are required for normal growth and survival of the yeast, while *UBI4* is required for its survival under stress conditions. Hence, SUB60 strain of *S. cerevisiae* lacking UBI4 is stress-hypersensitive. However, SUB60 strain can grow under conditions of stress, provided ubiquitin is expressed extrachromosomally [3,55]. The strategy can be effectively used to test the potential of mutant forms of ubiquitin under various stress conditions was tested for their ability to functionally complement and rescue *UBI4* deletion mutants of *S. cerevisiae*.

UbEP42 evolved using error-prone PCR turned out to be a dosage dependent lethal mutation, with four amino acid substitutions. The four mutations present in UbEP42 were singled out and studied for their functional effects. The results of this study showed that the two single mutations L50P and I61T were lethal to the host cell [37]. The results of the present study establish that they exhibit similar effects when present along with other substitutions. Interestingly, the least harmful of the six double mutants does not contain either of these two substitutions, namely S20F-A46S. Although non-lethal when over-expressed, the mutant increases generation time. It does not make cells sensitive to cycloheximide and shows complementation under heat stress. It does not have any negative influence over UFD pathway, Cdc28 levels, lysosomal degradation of uracil permease and K48 linked polyubiquitination. The most harmful of all six double mutants is the combination of the lethal mutations, namely L50P-I61T. L50P-I61T endosomal sorting of carboxypeptidase S, it makes host cells sensitive to cycloheximide and heat stress than untransformed SUB60 cells. It has increased generation time and slowed growth and shows no complementation in UFD pathway, Cdc28 levels, lysosomal degradation of uracil permease and K48 linked polyubiquitination. The next two most detrimental double mutants are S20F-I61T and A46S-L50P, both of which contain either L50P substitution, or I61T substitution. Lower than wild type K48 linked polyubiquitination in L50P-I61T, S20F-I61T and A46S-L50P suggests that this could be the root cause of their detrimental effect on other functions. None of the double mutants show any effect on sensitivity towards tunicamycin and gentamycin and endosomal sorting of CPS. All double mutants containing L50P are sensitive to cycloheximide, a translational inhibitor that acts on the elongation phase of protein synthesis. Substitutions S20F and A46S appear to compensate the detrimental effect of L50P and I61T to some extent. This is clearly seen in how A46S-L50P and S20F-I61T are less harmful than L50P-I61T, while S20F-A46S and A46S-I61T are the least damaging, the former being so because it contains neither L50P nor I61T. The compensatory effect shown by A46S is particularly intriguing, as it involved substitution of a hydrophobic amino acid like alanine with serine which is not only hydrophilic but differs significantly from alanine in structure. Hence, it can be stated that The double mutants could protect the host as long as their structure closely resembled the wildtype protein. However, they failed when the mutation altered the structure considerable (Fig. 11).

The translational inhibitor cycloheximide acts by preventing the translocation of tRNA and mRNA with respect to the ribosome [48]. All double mutants containing L50P substitution are sensitive to cycloheximide. Hence, the molecular mechanisms behind the increase in sensitivity of cells carrying UbEP42 derived double mutations to different translational inhibitors seems to be complicated and needs further investigation.

There are three possible reasons for double mutants being detrimental to yeast. One reason can be that substitutions L50P and I61T alter the structure of ubiquitin which prevents it from functioning normally, while substitutions S20F and A46S being located in turns are surface residues and may not lead to major structural alterations. Another reason could be that these substitutions are surface residues and may prevent recognition of mutant forms of ubiquitin by one or more of the enzymes involved in the ubiquitination pathway, or by the other proteins such as those involved in non-proteasomal functions of ubiquitin. Out of the four residues that are substituted in UbEP42 mutation, S20 and A46 are surface residues, while L50 and I61 are buried residues. Hence, L50 and I61 are unlikely to play a role in the direct recognition of ubiquitin by the ubiquitination and deubiquitination enzymes and other interacting proteins. Previous studies in our laboratory have shown that L50P and I61T substitutions alter the structure of ubiquitin, and these, as mentioned above, are also the most detrimental of the four substitutions [37]. Hence, it is likely that the detrimental effects of L50P-

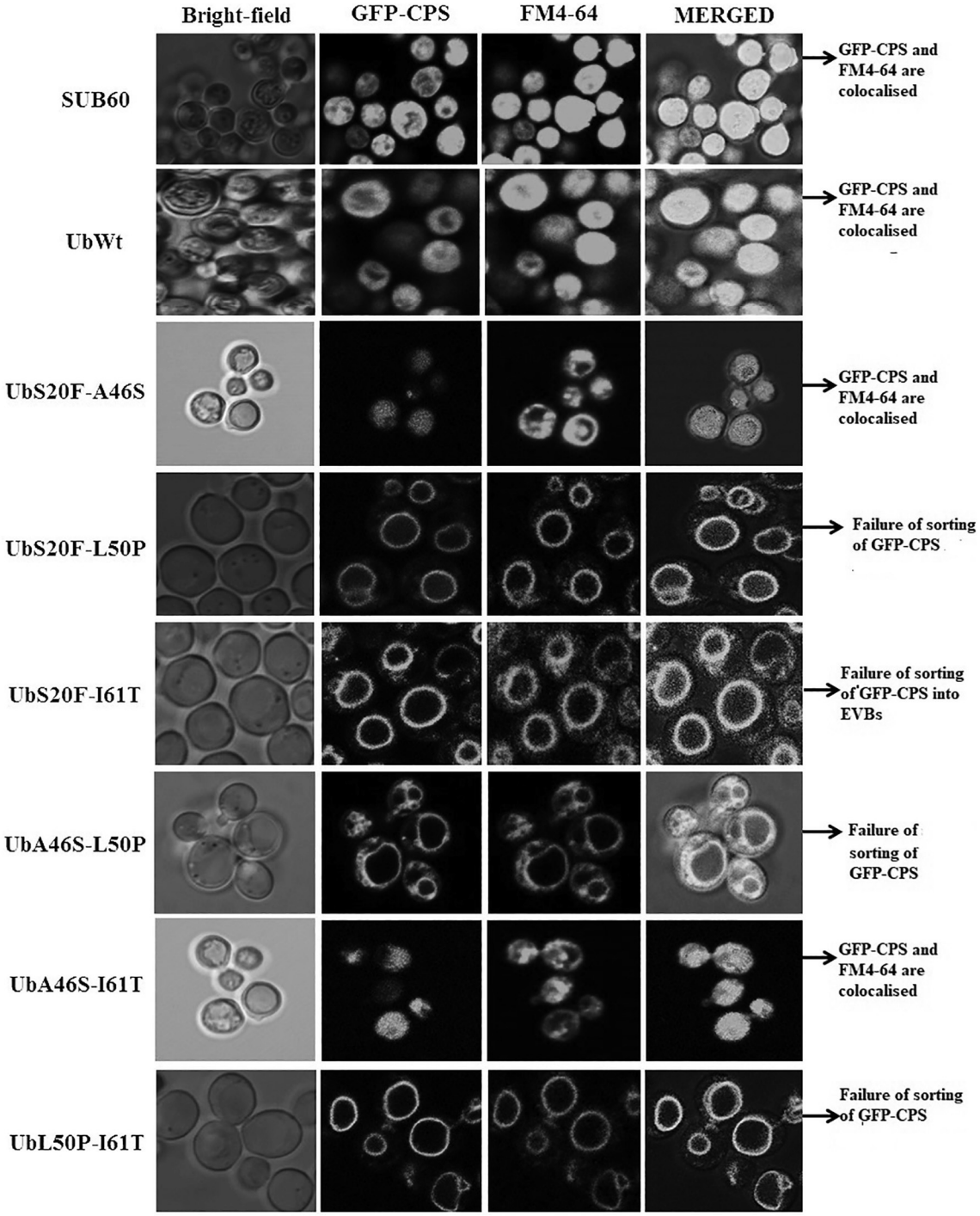

Fig. 10. Effect of expression of UbWt, UbEP42 derived double mutants of ubiquitin on the sorting of carboxypeptidase S monitored using confocal microscopy. *Images* of SUB60 cells transformed with YEp96 expressing wild type ubiquitin and the UbEP42 derived double mutants. The cells expressing wildtype and double mutant forms of ubiquitin were observed under: (A) bright field, (B) RFP filter (FM4-64), (C) GFP filter (GFP), (D) *images resulting from overlay* of (B) and (C) to observe colocalization. It can be seen from the images that the transformants of SUB60 cells expressing UbS20F-I61T, UbA46S-L50P, UbL50P-I61T showed failure of sorting of CPS into endosomes, while sorting remains unaffected in the expressing cells expressing UbS20F-A46S, UbS20F-L50P, UbA46S-I61T and was comparable to UbWt control.

I61T, S20F-I61T and A46S-L50P are an indirect consequence of structural alterations produced in ubiquitin, and not due to a more direct change in the surface of ubiquitin leading to failure of recognition by interacting proteins. The less detrimental effects of S20F and A46S suggest that despite being surface residues, they may not play a direct role in the recognition of ubiquitin by the interactors. In fact, a previous study identified the ubiquitin surface residues essential for life in yeast, and neither S20 nor A46 were found to be among them [56]. The fact that A46 is a surface residue might also be the reason why A46S substitution, despite substituting a hydrophobic residue with a hydrophilic one, is not very unfavourable, as the surface residues of cytosolic proteins usually tend to be hydrophilic.

Also, worth noting is the finding that L50P-I61T, S20F-I61T and A46S-L50P substitutions affect K48 linked polyubiquitination and sorting of carboxypeptidase S, which depends on monoubiquitination and K63 linked polyubiquitination [51]. The findings suggest that the

Table 1
Summary of the results of functional characterization of double mutants of ubiquitin. Bold font was used to signify that there is a change with respect to cells expressing ubiquitin wild type (UbWt).

	20–46	20–50	20–61	46–50	46–61	50–61
Over-expression	Not lethal	Lethal	Mildly lethal	Lethal	Not lethal	Lethal
Generation time	Increases when induced	Increases when induced	Increases when induced	Increases when induced	No effect	Increases when induced
Viability	Does not decrease	Decreases	Decreases	Decreases	Does not decrease	Decreases
Heat stress	Less effect	More sensitive than SUB60	More sensitive than SUB60	More sensitive than SUB60	Less effect	More sensitive than SUB60
Cycloheximide	Shows complementation	Shows complementation	Sensitive	Sensitive	Shows complementation	Sensitive
UFD pathway	Shows complementation	Shows complementation	No complementation	No complementation	Shows complementation	No complementation
Cdc28	Shows complementation	Shows complementation	No complementation	No complementation	Shows complementation	No complementation
Uracil permease	Shows complementation	Shows complementation	No complementation	No complementation	Shows less complementation	No complementation
K48 poly-ubiquitination	Shows complementation	No complementation	No complementation	No complementation	Shows complementation	No complementation
Endosomal sorting of CPS	Not affected	Failure of sorting	Failure of sorting	Failure of sorting	Not affected	Failure of sorting

structural alteration of ubiquitin caused by these double mutations might not affect monoubiquitination as much as they affect K48 linked polyubiquitination.

In summary, the two single mutations S20F and A46S did not show any negative effects either on the structure or functions associated with ubiquitin as they did not disturb the structure locally. The two residues mutated do not show any direct interaction in the wild type protein. So occurring together in S20F-A46S did not produce any negative effect. Ala46 associates with Ile61 and Leu67 in the native structure of wild type ubiquitin. Therefore, A46S compensates the negative effect produced when I61T is alone and when present together A46S-I61T the double mutation does not produce any negative effect. A46S and L50P are too close in their location in the ubiquitin protein and when they occur together in A46S-L50P they probably accentuate structural distortion and cause greater negative effect. S20F fails to have any bearing on the lethal effect produced by I61T in S20F-I61T. The mutations L50P and I61T produced lethal effect even in isolation. The combination of the two mutations is a potent lethal mutation. Interestingly, the double mutations S20F-L50P and A46S-I61T showed compensatory effects, reducing the negative impact L50P and I61T had when they were present alone. In conclusion, the double mutant forms of ubiquitin S20F-I61T, A46S-L50P and L50P-I61T are detrimental to cells. Expression of these three double mutant forms at sublethal levels produced greater nega- tive effects over the host SUB60 cells compared to untransformed SUB60 cells. It may be envisaged that ubiquitin bearing either of these double mutations competes with wild type ubiquitin in the cells, inter- fering with the functional dynamics of the protein and preventing proper functioning of the pathways dependent on the ubiquitin system. Since they are detrimental to cells under several conditions, these dou- ble mutants could be used to manage certain diseases that are caused due to gain of function of ubiquitination process.

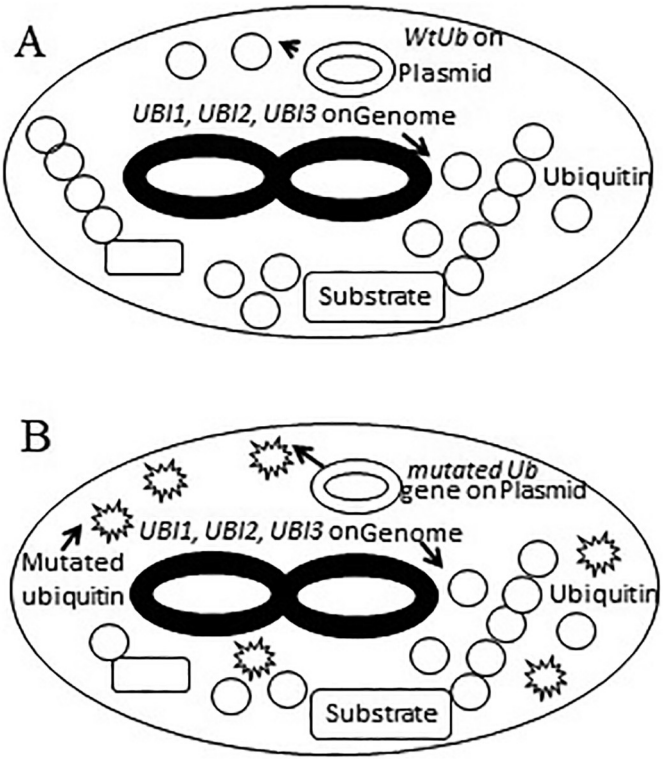

Fig. 11. Illustration of complementation of ubiquitin expressed by the ubiquitin gene and its mutant forms from the plasmid in *UBI4* deletion mutant strain SUB60. A. Polyubiquitination of substrate protein by ubiquitin molecules produced by genomic *UBI1*, *UBI2*, *UBI3* and ubiquitin gene on the plasmid. This is also the case where the mutant forms are not lethal and can add to Polyubiquitin chains. (B) Low levels of ubiquitination seen due to contamination of cytoplasmic ubiquitin pool by the lethal mutant forms of ubiquitin encoded by the gene on the plasmid. (Since the possibility of addition of lethal mutant forms to substrate protein is less likely, it is not shown in the illustration.)

CRediT authorship contribution statement

CRP was responsible for the concept and design of the research problem, procuring funds in the form of a major research projects from UGC and CSIR, design of the experiments, data analysis and writing of the manuscript. MS, PY and AD have contributed equally towards carrying out experimental work and acquisition of data. HB helped in conducting some preliminary experiments.

Declaration of competing interest

The authors have declared no conflict of interest.

Acknowledgements

This work was supported by two major research project grants awarded to C. Ratna Prabha, Grant No. F.33-225/2007 (SR) from the University Grants Commission (UGC), India, and Grant No. 37 (1569)/

12/EMR-II from Council of Scientific and Industrial Research (CSIR), India. MS was employed as a project fellow in the UGC project. PY was supported by UGC scholarship. AD was supported by university research fellowship of The M. S. University of Baroda, India and later on by senior research fellowship of Council of Scientific and Industrial Research (CSIR), India. CRP thanks Prof. Mark Searle and Prof. Daniel Finley for providing the plasmids and strains necessary for the study.

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