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Regulatory mechanisms involved in the control of ubiquitin homeostasis

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Ubiquitin (Ub) modification plays an essential role in the regulation of various cellular processes. Ub performs a remarkable array of cellular tasks through the production of a large number of ubiquitinated proteins; such tasks require many Ubs. Ubs are expressed abundantly from several Ub encoding genes, though not in excess. Rather, Ub expression is tightly regulated through various control mechanisms. Recent studies have shown that the cellular Ub level is regulated by balanced activities of deubiquitinating enzymes and their regulators. Here, we review the current understandings of the regulatory mechanisms that control Ub expression and its metabolism and maintain Ub homeostasis.

Keywords: deubiquitinating enzyme/endosome/homeostasis, stress response/ubiquitin.

Abbreviations: Dub, deubiquitinating enzyme; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin ligase; *gad*, gracile axonal dystrophy; MVB, multivesicular body; Rfu1, Regulator for free ubiquitin chains 1; Ub, ubiquitin; Ub_3, free triubiquitin; Ub_4, free tetraubiquitin; *UBI1–3*, *UBI1*, *UBI2* and *UBI3*; UPS, ubiquitin-proteasome system.

Ubiquitin

Ubiquitination is a reversible post-translational modification of cellular proteins and is known to play central roles in the regulation of various cellular processes, such as protein degradation, protein trafficking, cell-cycle regulation, DNA repair, apoptosis and signal transduction (*1*, *2*). Ubiquitin (Ub) is a highly conserved 76 amino acid protein that covalently attaches to the lysine residues of target proteins via its carboxy-terminal glycine residue, forming an iso-peptide linkage, in an ATP-dependent fashion. The ubiquitination process is catalyzed by the sequential actions of three enzymes; a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2) and a ubiquitin ligase (E3). Since Ub itself contains seven lysines, it can attach repeatedly to other Ubs, allowing the formation of polyubiquitin chains. Therefore, Ub exists intracellularly either as a monomer, a substrate-conjugated polyubiquitin or monoubiquitin, or free (or unanchored) Ub chains, and there is a dynamic equilibrium among the three forms in the cell. The ubiquitination process can be reversed by deubiquitinating enzymes (Dubs), which are Ub-specific proteases. It is estimated that ~600 E3s and 100 Dubs exist in mammalian cells (*3*, *4*).

Among the various functions of Ub, the most characterized function is serving as a tag for selective proteolysis by the 26S proteasome. Multiple Ubs are covalently added to a substrate successively by E1, E2 and E3 enzymes, producing a substrate conjugated with polyubiquitin. The ubiquitinated substrates are recognized and degraded by the 26S proteasome after the polyubiquitin chain is processed off and recovered by Dubs.

In addition, ubiquitination is also critical in the vacuolar sorting process of both endocytic and biosynthetic membrane proteins (*2*, *5*). At the plasma membrane, Ub serves as a signal for endocytosis, and at the endosome, Ub serves as a signal to sort cargo proteins into the multivesicular body (MVB), which is a critical step to their transport to lysosomes. Ub is removed from the cargo by Dubs before its entry into the MVB.

Importance of Adequate Cellular Level of Ubiquitin

Ub is an abundant protein in eukaryotic cells constituting ~0.1–5% of total proteins, therefore it is assumed that it is redundantly expressed (*6*). However, due to its pervasive use and large number of substrates to be ubiquitinated in a cell, Ub does not seem to be produced in excess, rather the free pool of Ub is maintained at an adequate level depending on the cell conditions.

In yeast as well as in most higher eukaryotes, Ub is initially expressed in the form of different precursors: polyubiquitin, a linear fusion protein consisting of four or more Ub copies in a head-to-tail configuration, and fusion proteins between Ub and usually Ub_{L40} and Ub_{S27}, that are large and small essential ribosomal polypeptides, L40 and S27, respectively (*7*, *8*) (Fig. 1). These Ub precursors are cleaved by Dubs to release identical functional monomeric Ub units. In yeast, the single polyubiquitin gene, *UBI4*, is not required under vegetative conditions, suggesting that ribosome fusion Ub genes, *UBI1*, *UBI2 and UBI3* (*UBI1–3*) provides the bulk of Ub in the cell (*9*). However, cells lacking *UBI4* become sensitive to

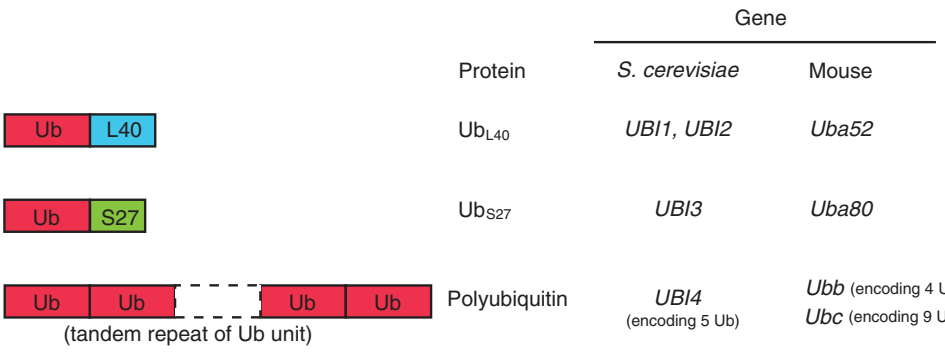

Fig. 1 Three Ub precursors and multiple Ub encoding genes in yeast *Saccharomyces cerevisiae* and mouse (see text for details).

various stresses including high temperature, starvation and amino acid analogs such as canavanine or L-threo-α-amino-β-chlorobutyric acid, and also defective in sporulation (*9*).

Mammals have four Ub genes, two of which encode polyubiquitin and the other two encode fusions with ribosomal proteins (*8*) (Fig. 1). The two polyubiquitin-encoding genes, *Ubb* and *Ubc*, express usually four and nine tandem repeats of Ub, respectively. Thus, the polyubiquitin genes seem redundant; however, the importance of the polyubiquitin-encoding genes was highlighted in the knockout mouse of either *Ubb* or *Ubc* (*6*, *10*, *11*). In the case of *Ubc*, disruption of *Ubc* in mice is embryonically lethal, possibly due to the lack of fetal liver proliferation at midgestation (*6*). Analysis of $Ubc^{-/-}$ mouse embryo fibroblasts showed 40% reduction in Ub level compared with the control. On the other hand, mice lacking *Ubb* are born normally at the expected Mendelian frequency (*10*). However, they are infertile due to the failure of progression of meiosis in germ cells (*10*). Consistently, significantly low Ub levels were found in the testis and germinal vesicle oocytes in 5-month-old mice whereas other organs were not significantly affected. Furthermore, the *Ubb* null mice develop adult-onset obesity due to the degeneration of hypothalamic neurons involved in the control of energy balance and feeding. Although the Ub level was not reduced in the whole brain, it was reduced in the hypothalamus by 30%. Therefore, a modest reduction in Ub level seems to cause infertility and neurodegeneration in mice.

Other than the mutations in Ub-encoding genes, mutations in several Dubs cause reduction of Ub and various defects. In yeast, deletion of Dub encoding genes, including *DOA4* and *UBP6*, can reduce the amount of monomeric Ub (*12*–*15*). These mutants are sensitive to canavanine, and the defects are compensated by expression of excess Ub. In mice, UCH-L1 is an abundant brain-specific Dub, constituting ~1–5% of the total proteins in the brain (*16*). The mutation in UCH-Ll is responsible for gracile axonal dystrophy (*gad*) in mice (*17*). The mice develop synaptic dysfunction and degeneration of neurons. In the brain of *gad* mice, monomeric Ub level is reduced by 20–30% compared with control mice, suggesting that a low level of Ub is a possible cause of the disease (*16*). Since UCH-L1 binds Ub, it is suggested that UCH-L1 stabilizes Ub or prevent Ub from degradation (*16*). Similarly, ataxia (ax^J) mutation, a spontaneous recessive mutation, is caused by reduced expression of Usp14, a homolog of yeast Ubp6 (*18*, *19*). The ax^J mice develop neurological dysfunctions including progressive motor system abnormalities, ataxia, loss of movement and premature death. In the ax^J mice, monomeric Ub level is reduced by 30–40%.

Curiously, not only a small amount of Ub but also Ub surplus is not beneficial to cells. In yeast, overexpression of Ub renders cells sensitive to certain kinds of stresses such as treatment with cadmium, arsenite and paromycin (*20*). In addition, overexpression of Ub worsens cell growth when it is introduced in mutants of ubiquitin-proteasome system (UPS)-related genes, such as *cdc48* temperature-sensitive mutant (*21*). Such mutants exhibit accumulation of ubiquitinated proteins, which could consequently lead to further accumulation of cytotoxic ubiquitinated proteins (*21*, *22*).

Regulatory Mechanism of Ubiquitin Homeostasis

Since keeping adequate amount of Ub is essential for a balanced cell function, cells have different regulatory systems to maintain Ub homeostasis by utilizing various machineries.

Transcriptional regulation of Ub-encoding genes

One of the regulatory mechanism for Ub level is operated at the level of transcription of Ub-encoding genes. In budding yeast, among the four Ub-encoding genes, *UBI1-4*, transcription of *UBI4*, a polyubiquitin gene, is heat inducible (*9*). Similarly, in higher organisms, transcription of polyubiquitin gene is stress inducible (*23*, *24*). In yeast, *UBI4* contains classical heat-shock elements (HSE) and stress-responsive elements in the promoter. Since multiple Ubs are produced efficiently by a single round of transcription–translation from a polyubiquitin gene, it is beneficial that a cell increases polyubiquitin gene expression under a state of emergency such as stress conditions. However, the mRNA levels of *UBI1-3* encoding ribosome-Ub fusions are likely to be repressed by stresses or conditions that induce *UBI4*, and there is evidence that the *UBI1-3* mRNA expression patterns are similar to those of ribosome subunits (*25*). Therefore, the exact net effect of these

opposing regulatory mechanisms on Ub production remains unclear. In yeast, upon heat shock, the level of monomeric Ub slightly increases for a short period and then decreases, probably due to the massive ubiquitination reactions at heat shock [(*9*) and Y. Kimura, unpublished results].

Regulation by the change of proteasome composition

As described, Ubp6 and its mammalian homolog Usp14 are Dubs associated with the proteasome (*26*). By binding reversibly to the proteasome via its Ubl domain, Ubp6 disassembles polyubiquitinated substrate proteins that are taken to the proteasome, and recovers Ub moiety from proteasomal degradation. Its Dub activity is enhanced by binding to the proteasome (*27*). The reduction of Ub observed in deletion or mutations of yeast Ubp6 and mouse Usp14 is explained by the lack of Ub recovery and that the unrecovered Ub is degraded along with the substrate by the proteasome (Degradation of Ub section) (*27*). Interestingly, Ubp6 inhibits the proteasome non-catalytically and decreases the overall flux of ubiquitinated proteins through the proteasome (*28*).

The regulation of Ubp6 expression is related to the level of Ub (*29*). In response to Ub deficiency, transcription of *UBP6* is increased, resulting in the production of more Ubp6. The increase in Ubp6 leads to an increase in Ubp6-associated proteasomes, which serves to retrieve Ub. Therefore, the versatility of proteasome content acts as a regulatory mechanism for Ub homeostasis. The mechanism of the transcriptional increase of Ubp6 upon Ub deficiency is unknown: it is not mediated by Rpn4, a transcription factor that regulates the expression of a set of proteasome subunits. Thus, the existence of an alternative transcriptional pathway is possible, which senses and responds to Ub deficiency.

Regulation by deubiquitinating enzyme and its regulators

Recent studies identified another mechanism involved in the regulation of monomeric Ub level: Ub level is regulated by the balanced action of Dub and its regulators (*21*, *30*) (Fig. 2). Rfu1 (regulator for free ubiquitin chains 1), a previously uncharacterized protein, was isolated initially as a multi-copy suppressor of *cdc48-3* temperature-sensitive mutant (*21*). Interestingly, cells that lack Rfu1 showed accumulation of monomeric Ub and reduced level of free (or unanchored) Ub chains, whereas overexpression of Rfu1 was associated with the opposite effects; reduction of monomeric Ub and accumulation of free Ub chains. These results suggest that Rfu1 inhibits the production of monomeric Ub and promotes the formation of free Ub chains. It turned out that the target of Rfu1 was Doa4, which is an endosome-localized Dub (*31*). Doa4 deubiquitinates cargo proteins at the endosome to retrieve Ub before cargo proteins are delivered to MVB (*31–33*). In addition, Doa4 is involved in Ub homeostasis, since lack of Doa4 was associated with accumulation of free Ub chains or small Ub species and reduction of monomeric Ub, which is the opposite effect of Rfu1 depletion (*12*). Subsequently, it was shown that Rfu1 interacts with Doa4 both *in vitro* and *in vivo*, and that recombinant Rfu1 inhibits the Dub activity of Doa4, indicating that Rfu1 is an inhibitor of Doa4 (*21*). Interestingly, Doa4 is recruited to the endosome and its activity is stimulated by another factor, Bro1, a class E Vps protein (*30*, *34*). In the absence of Bro1, Doa4 localization of the endosome is lost, and the Ub profile of the Δ*bro1* mutant is quite similar to that of Doa4-negative cells (*35*). Therefore, Doa4 is regulated by an activator (Bro1) and an inhibitor (Rfu1), indicating that there must be balanced regulation of Doa4 between Rfu1 and Bro1. It is speculated that Rfu1 may act on Doa4 to inhibit its activity on the endosome after recruitment of Doa4 to the endosome by Bro1.

Cellular stresses such as heat shock causes accumulation of misfolded proteins and these proteins should be ubiquitinated and degraded by the 26S proteasome. It was discovered that free Ub chains rapidly disappear at heat shock (Fig. 3) (*21*). At the same time, it was shown that Rfu1 decreases whereas Doa4 increases, producing more Doa4, which is free of Rfu1 (*21*). Since the lack of Doa4 as well as overexpression of

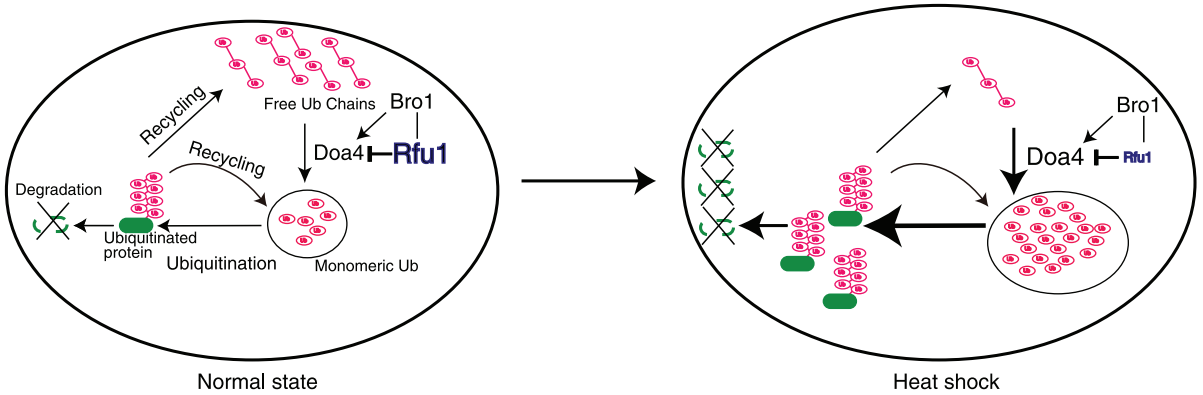

Fig. 2 Model of mechanisms involved in Ub homeostasis through Doa4, Bro1 and Rfu1, at normal state (left) and at heat shock (right). Under normal conditions, Rfu1 inhibits Doa4 activity, and excess Ub may be stored in the form of free Ub chains. Since Doa4 is activated by Bro1, Doa4 is controlled by a balance between activators and inhibitors. Heat shock results in a decrease in Rfu1 and increase in Doa4, favoring production of monomeric Ub from free Ub chains by Doa4. Physical interaction between Rfu1 and Bro1 is detected.

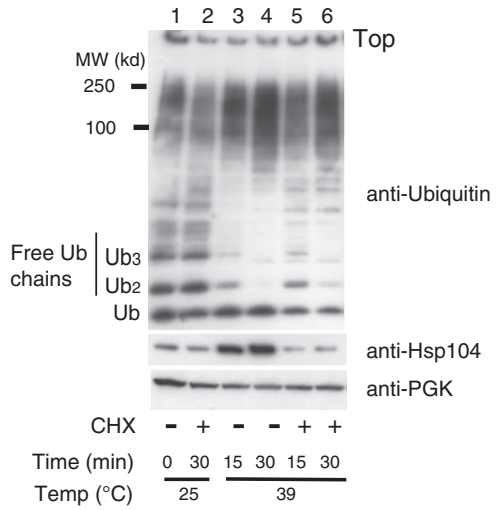

Fig. 3 Disappearance of free Ub chains upon heat shock in the absence and presence of CHX. Cells in early log phase at 25°C were treated or untreated with CHX and immediately placed at 25 or 39°C for the indicated time intervals. Top panel, anti-Ub; middle, anti-Hsp104; bottom, anti-phosphoglycerate kinase (PGK) as control for protein loading.

Rfu1 inhibits the heat shock-induced disappearance of free Ub chains, it was suggested that Doa4 and Rfu1 act on free Ub chains to produce monomeric Ub at heat shock. Thus, free Ub chain may function as a Ub reservoir that allows maintenance of monomer Ub at adequate levels under normal conditions and a rapid supply for ubiquitination of substrates under stress conditions (Fig. 2). The disappearance of free Ub chains occurs in the presence of cycloheximide (CHX), indicating that it does not require new protein synthesis (Fig. 3). Therefore, this regulatory system would be beneficial for a cell to produce monomeric Ub quickly to cope with emergency states such as heat shock.

Other factors involved in Ub homeostasis

In addition to Doa4 and Ubp6, several Dubs are likely to be involved in the regulation of Ub homeostasis. In yeast, Ubp14, a homolog of mammalian isopeptidase T, acts preferentially on free Ub chains, but cannot act on polyubiquitin conjugated with protein (*36*). Cells lacking Ubp14 show accumulation of free Ub chains, however, the mutants have a normal level of monomeric Ub (*37*). Cells lacking Ubp3, Ubp8 and Ubp10 accumulate free tetraubiquitin (Ub_4), free triubiquitin (Ub_3) and both Ub_3 and Ub_4, respectively (*15*).

Ufd3 (Doa1), a homolog of PLAP (phospholipase A2-activating protein) in mammals, was originally isolated as a factor required for the degradation of a ubiquitin-fusion degradation (UFD) substrate (Ub-Pro-β-galactosidase) in yeast (*38*, *39*). In addition to its role in protein degradation in the UPS, Ufd3 functions in the DNA damage response, and in the targeting of ubiquitinated membrane proteins to MVB (*40*, *41*). Ufd3 binds to Ub, Cdc48 and Hse1, a component of the proteins required for MVB sorting (*39–44*). Consistent with its function in MVB sorting, some Ufd3 localize to the endosomes (*40*). Interestingly, cells lacking Ufd3 show depletion of monomeric Ub and accumulation of Ub_3 (*38*, *41*). Since no enzymatic activities have been reported for Ufd3, the reason why deletion of *UFD3* results in the loss of Ub homeostasis is unknown at present. Recent studies showed that interaction of Ufd3 with Cdc48 is required for the maintenance of the Ub level (*44*). Since *UFD3* genetically interacts with various Dub encoding genes, including *UBP4*, *7*, *8*, *10*, *12* and *14*, Ufd3 may regulate these Dub activities through Cdc48 (*41*). Moreover, it is tantalizing that several factors known to be involved in Ub homeostasis, including Doa4, Rfu1, Bro1 and Ufd3 are localized in the endosomes. Hence, it is conceivable that the endosome may function not only as protein sorting factory but also as regulator of Ub homeostasis.

In a mutant of Rsp5, an E3 in yeast, reduced level of Ub is observed upon heat shock and it was shown that the reduced level of protein synthesis is sustained in *rsp1* mutant at heat shock (*45*).

Degradation of Ub

Ub is considered a stable protein based on its heat-stable physicochemical property and its globular structure, and indeed there have been reports that the half life of Ub is rather long [see review (*46*)]. For example, pulse-chase studies show that Ub turns over with a half-life of 28–31 h in cultures of human lung fibroblasts (*47*). One study using erythrocyte-mediated microinjection of ^{125}I-proteins showed that the Ub half life is 320 h in human fibroblasts, whereas that of BSA and lysozyme are only 20 and 22 h, respectively (*48*). However, there are several reports that Ub appears to be metabolized rather rapidly in the cell. A pulse-chase experiment showed that Ub half-life is 9 h in mouse leukemia cells (*49*). In yeast, CHX treatment experiments indicated a long Ub half-life, but only 2 h when estimated by the promoter-shut off experiment using galactose-inducible promoter (*13*, *50*). Such difference in Ub half-life time probably reflects differences between organisms or differences in susceptibility to change to experimental conditions. Finally, a recent study using in-cell nuclear magnetic resonance spectroscopy suggested destabilization of Ub in the cell (*51*).

With regard to degradation of Ub, Ub is degraded by the 26S proteasome and three different degradation processes have been described (*52*, *53*) [reviewed in (*46*)]. First, experiments using ^{125}I-Ub and proteasome inhibitor indicated Ub is degraded along with its conjugated substrate. When the ubiquitinated substrate is processed by the 26S proteasome, Ub are recovered by Dubs such as Ubp6 and Rpn11. However, it is possible that Ub at the most proximal part of the polyubiquitin chain is degraded along with the substrate. Second, monomeric Ub with an extension tail longer than 20 residues at the C-terminus are effectively degraded by the 26S proteasome without further modification (*52*, *53*). It is proposed that the tail allows the Ub-tail to reach the catalytic site of the proteasome. Such an unusual Ub derivative is indeed naturally produced; UBB^{+1}, which is produced by a misreading of *Ubb*

transcript. UBB^{+1} is implicated in the pathogenesis of an early onset form of Alzheimer's disease (*54*). In the third process, monomeric Ub is degraded after Ub itself is ubiquitinated (*53*).

The vacuole or lysosome, another major degradation machinery in the cell, may be involved also in the degradation of Ub. Ub localization in the vacuole/lysosome is reported in various species (*55*, *56*). Moreover, in yeast *doa4* cells in which Ub is not recovered and transported to the vacuole along with cargo proteins, Ub is rapidly degraded (*13*). However, the introduction of mutation in *PEP4*, which encodes a vacuolar protease, prevents the rapid degradation of Ub (*13*).

Conclusion

Various and complex regulatory mechanisms operate in the cell to maintain stable Ub levels. Thus, there are probably other yet unidentified mechanisms involved in the control of Ub homeostasis, *e.g.* one that involves Ufd3. Identification of various mechanisms involved in Ub homeostasis would enhance our understanding of the pathogenesis of various conformational diseases such as neurodegenerative diseases since the UPS plays an important role in the prevention of such diseases.

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Conflict of interest

None declared.

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