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Invited Mini Review

Regulation of polyubiquitin genes to meet cellular ubiquitin requirement

*Seung-Woo Han, Byung-Kwon Jung & Kwon-Yul Ryu**
Department of Life Science, University of Seoul, Seoul 02504, Korea

Ubiquitin (Ub) is one of the proteins that are highly conserved from yeast to humans. It is an essential core unit of the well-defined post-translational modification, called ubiquitination, which is involved in a variety of biological processes. In metazoans, Ub is encoded by two monoubiquitin genes and two polyubiquitin genes, in which a single Ub is fused to a ribosomal protein or Ub coding units are arranged in tandem repeats. In mice, polyubiquitin genes (*Ubb* and *Ubc*) play a pivotal role to meet the requirement of cellular Ub pools during embryonic development. In addition, expression levels of polyubiquitin genes are increased to adapt to environmental stimuli such as oxidative, heat-shock, and proteotoxic stress. Several researchers have reported about the perturbation of Ub pools through genetic alteration or exogenous Ub delivery using diverse model systems. To study Ub pool changes in a physiologically relevant manner, changing Ub pools via the regulation of endogenous polyubiquitin gene expression has recently been introduced. Furthermore, to understand the regulation of polyubiquitin gene expression more precisely, *cis*-acting elements and *trans*-acting factors, which are regulatory components of polyubiquitin genes, have been analyzed. In this review, we discuss how the role of polyubiquitin genes has been studied during the past decade, especially focusing on their regulation. [BMB Reports 2021; 54(4): 189-195]

INTRODUCTION

Ubiquitin (Ub) is a highly conserved small eukaryotic protein composed of 76 amino acids, which can be mono- or polyubiquitinated on the substrate by Ub-activating enzyme (E1), Ub-conjugating enzyme (E2), and Ub ligase (E3) (1, 2). Monoubiquitination is known to regulate histone modification, receptor endocytosis, etc. (3). Polyubiquitination plays different roles depending on the type of Ub chains (4). It is involved in a variety of cellular processes, such as proteasomal degradation, stress response, signal transduction, and membrane protein trafficking (5-8). After exerting its role, Ub conjugated on the substrate can be removed by deubiquitinating enzymes (DUBs) and becomes free Ub (9). Under certain circumstances, Ub itself can also be degraded by the proteasome (10). This Ub recycling is important for maintaining Ub homeostasis inside cells.

In mice, Ub is encoded by four different genes: *Uba52* and *Uba80* (*Rps27a*), which are Ub-ribosomal fusion genes, and *Ubb* and *Ubc*, which are polyubiquitin genes (Fig. 1). Similarly, there are also four different genes, *UBA52*, *UBA80* (*RPS27A*), *UBB*, and *UBC*, in humans (11-13). Ub-ribosomal fusion proteins or Ub polymers translated from these genes are converted into Ub monomers by DUBs, which constitute free Ub pools (14). Polyubiquitin genes are upregulated under stress conditions such as oxidative, heat-shock, and proteotoxic stress, thereby increasing the levels of free Ub pools (Fig. 1) (15, 16). This is important in maintaining cell viability when the cellular stress response pathway is activated (17). If the free Ub level does not increase under stress conditions, for example, because of disruption of the polyubiquitin gene, cell viability can be reduced (18).

It is also important to control the levels of Ub under normal conditions as well as stress conditions. Under normal conditions, the expression of both polyubiquitin genes is tightly regulated, so that it is not upregulated or downregulated significantly, to maintain the basal expression levels. If the levels of Ub decrease under normal conditions, problems may occur in proliferation, differentiation, and even in development (19-22). It has also been reported that chronic Ub overexpression induces synaptic dysfunction in neurons (23, 24). Therefore, the regulation of polyubiquitin gene expression under normal and stress conditions is important, but the relevant molecular mechanism is not known well. Some known facts are that the intron region is important for the regulation of basal expression levels of *UBC*, and the promoter region is important for the upregulation of *UBC* under stress conditions (25-28). Recently, it has been reported that using the inducible CRISPR-activation system, a modified version of the CRISPR/Cas9 system, in which guide RNA was targeted to the intron region of *UBC*,

*Corresponding author. Tel: +82-2-6490-2674; Fax: +82-2-6490-2664; E-mail: kyryu@uos.ac.kr

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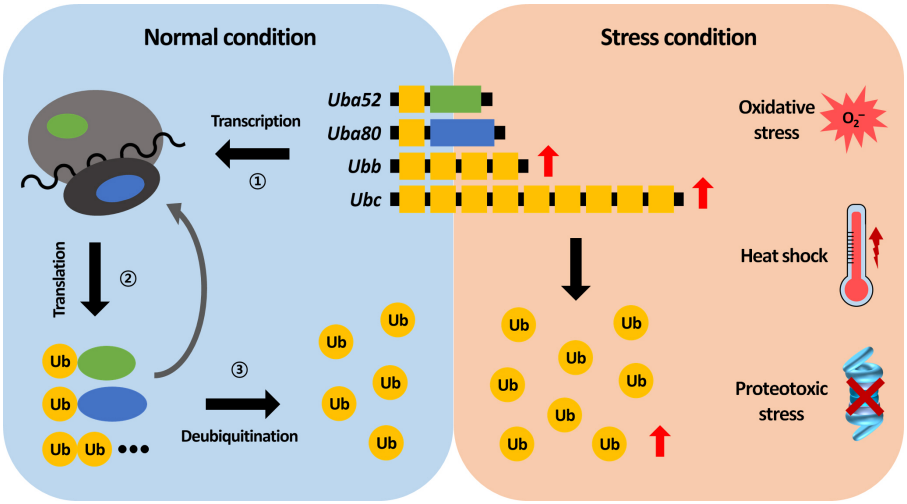

Fig. 1. Expression of ubiquitin genes under normal and stress conditions. Ubiquitin (Ub) is encoded by four different genes: *Uba52*, *Uba80*, *Ubb*, and *Ubc*. Two monoubiquitin genes (*Uba52* and *Uba80*) and two polyubiquitin genes (*Ubb* and *Ubc*) are structured as shown. Under normal conditions, four ubiquitin genes are continuously transcribed, translated, and deubiquitinated to produce available free Ub. Under stress conditions, the expression of polyubiquitin genes is upregulated. As a result, cellular free Ub pool levels increase under stress conditions. For simplicity, only mouse ubiquitin genes are shown here.

upregulation of *UBC* could be achieved temporally under normal conditions (29).

Based on these previous studies, we suggest that the regulation of endogenous polyubiquitin gene expression at the transcriptional level is possible under both normal and stress conditions, which is essential to change the levels of Ub pools. In this review, we have mostly focused on the research trend of transcriptional regulation of polyubiquitin genes during the past decade.

The importance of free Ub pool levels

The cellular Ub pools are composed of free Ub and Ub conjugates, in which Ub is conjugated to the substrate by E1-E3 enzymes. Free Ub pools are maintained from a Ub monomer separated from Ub conjugates by DUBs and also through *de novo* synthesis of Ub by mono- and polyubiquitin genes. Newly synthesized Ub may exist as fusion proteins or polymers, but is converted to free Ub by DUBs right after synthesis (30). Among the important roles of Ub, the most well-known role is to form a K48-linked Ub chain on the substrate, which serves as a signal to be targeted to the 26S proteasome for degradation under both normal and stress conditions (5, 31). Ub also plays an essential role in cell proliferation, differentiation, and stress response. There are many types of Ub chains, for example, K63-linked and K29-linked (10, 32, 33). Although they exert different functions in cells, Ub chains are all formed from free Ub. Therefore, it is important to maintain the homeostasis of available free Ub pools. Either decreased or increased free Ub levels can cause adverse effects on cellular function or survival. Under normal conditions, disruption of Ub homeostasis through knockout of the polyubiquitin gene or ectopic expression of Ub induces dysregulation of proteasomal degradation or UPS (ubiquitin-proteasome system), causing various problems in tissues or cells (18, 23, 24, 34).

In fact, in neural stem cells (NSCs) with free Ub levels reduced because of *Ubb* knockout or knockdown, the degradation of the Notch intracellular domain (NICD) is delayed, leading to the activation of Notch signaling and upregulating the expression of Notch target genes (35, 36). Activation of Notch signaling in the early stages of neuronal development suppresses neurogenesis and promotes premature gliogenesis, causing abnormal differentiation of NSCs, degeneration of defective neurons, and reactive gliosis (35). Reduced free Ub levels also affect the proliferation of NSCs, resulting in a decrease in their numbers (35). In general, Ub deficiency is thought to cause a decrease in cell proliferation by interfering with the degradation of cyclin and CDK inhibitor, which are targets of the proteasome (31).

Cell proliferation decreased in *Ubc* knockout mouse embryonic fibroblasts (MEFs) (37). *Ubc* knockout embryos are lethal as early as 12.5 days post coitum (dpc) because of defects in fetal liver development (37). *Ubc* knockout embryos show smaller livers than do wild-type (WT) controls, suggesting that normal fetal liver development has not occurred, because of the decrease in cell proliferation during embryonic development. In *Ubb* knockout mice, spermatogenesis is arrested in the early pachytene stage of meiosis I, which hampers maturation of germ cells and results in smaller testes than in the WT controls, leading to an infertility phenotype (38). On the other hand, overexpression of Ub can also have an adverse outcome. Investigation of the levels of UPS substrates in the hippocampus of transgenic mice with chronic Ub overexpression has shown that protein levels, but not mRNA levels, of the glutamate ionotropic receptor (GRIA1-4) decreased (24). In addition, analysis of ubiquitinated proteins revealed that ubiquitination of GRIA increased. These results suggest that excessive Ub expression can cause abnormally rapid degradation of proteins through UPS, which leads to dysfunction of neurons (24).

Maintenance of basal expression levels of polyubiquitin genes

In metazoans, polyubiquitin genes consist of multiple Ub coding units, which are arranged in a head-to-tail tandem array. The polyubiquitin gene *UBC* or *Ubc* has 2 to 3 times more Ub coding units than does the other polyubiquitin gene, *UBB* or *Ubb*. They are composed of the promoter, the non-coding exon (exon 1/2), the intron, and the coding exon (exon 2/2) that encodes Ub (Fig. 2). The putative enhancer can also be found in the intron region, not only in the upstream of the promoter region. For precise understanding of the regulation of polyubiquitin gene expression, analysis of *cis*-acting regulatory elements, located in the intron and the promoter region, and *trans*-acting transcription factors have been carried out. Various transcription factor binding sites, such as NF-κB, Sp1, heat-shock factor 1 (HSF1), and AP-1, have been found in the regulatory region of *UBC*, including the TATA box (13, 39). The mechanism for regulating *UBC* expression may differ depending on the cell type (40). For example, in muscle cells, activation of the mitogen-activated protein kinase (MAPK) signaling pathway by treatment with dexamethasone (glucocorticoid) and Sp1 transcription factor binding are involved in the regulation of *UBC* basal expression (41-43). In addition, the intron region is involved in the regulation of *UBC* basal expression (25, 26). The basal expression levels of *UBC* significantly decreased upon removal of the intron region of *UBC* (25). The transcriptional activity of *UBC* decreased when the Yin Yang 1 (YY1)-binding sequence present in the intron was mutated (26). Therefore, YY1 transcription factor binding to the intron region plays an important role in the regulation of *UBC* basal expression.

When Ub levels are lowered because of disruption of one polyubiquitin gene, compensatory expression of the other polyubiquitin gene occurs to rescue cellular Ub levels. However, because of different cell or tissue spectra of two polyubiquitin gene expressions, such compensation cannot fully rescue Ub levels (Fig. 3). When Ub levels were increased through Ub overexpression, both polyubiquitin gene expressions were downregulated in mice and humans (24, 44). Intriguingly, when Ub was overexpressed, *UBC* pre mRNA levels were rather increased over those in the control; however, the mRNA levels tended to decrease (44). These results suggest that Ub overexpression contributes to the downregulation of *UBC* expression by affecting the post-transcriptional processing such as splicing.

Upregulation of polyubiquitin gene expression under stress conditions

Polyubiquitin genes are stress-inducible genes and are upregulated under various stress conditions to increase cellular Ub levels. This upregulation occurs in diverse species, such as yeast, chicken, mouse, and human (15-17). Under oxidative or proteotoxic stress, the proteasome activity is compromised, and many misfolded proteins are generated (45, 46). If these misfolded proteins are not removed by the proteasome in a timely manner, insoluble protein aggregates can be formed that may cause cytotoxicity (47-49). Therefore, when the stress response is induced, it is important to upregulate the

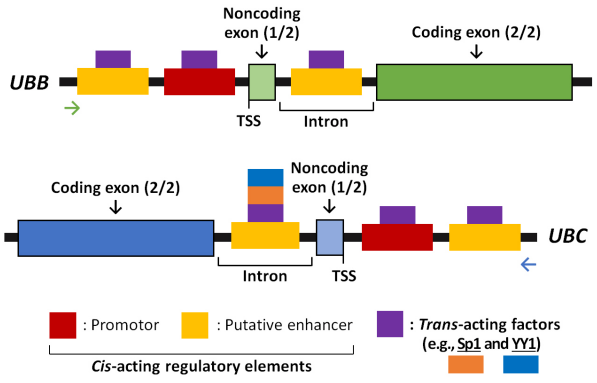

Fig. 2. Structures of human polyubiquitin genes. *UBB* and *UBC* share genetic structures that consist of a promoter, a noncoding exon, an intron, and a Ub-coding exon. *Trans*-acting factors of *UBB* and *UBC* bind *cis*-acting elements located in the putative enhancer as well as in the promoter region. The putative enhancer can be found in the regulatory region including the intron. As an example of *trans*-acting factors, Sp1 and YY1 transcription factors binding to the *UBC* intron region are shown. Promotor and putative enhancer locations are displayed based on the candidate *cis*-acting regulatory elements (cCRE) from the Encyclopedia of DNA Elements (ENCODE) database. TSS, transcription start site.

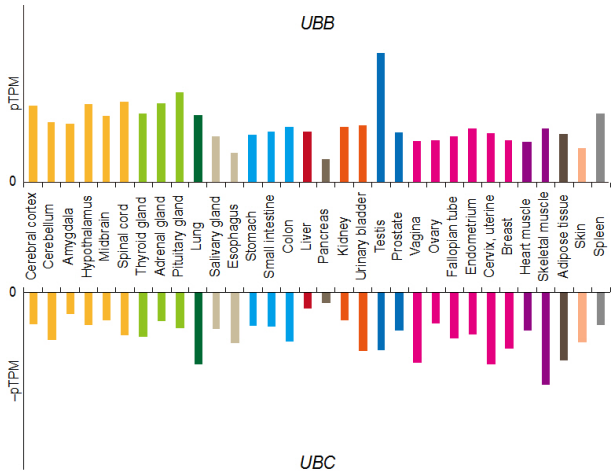

Fig. 3. Tissue expression patterns of human polyubiquitin genes. Based on the Genotype-Tissue Expression (GTEx) and Human Protein Atlas database, protein-coding transcripts per million (pTPM) values of *UBB* and *UBC* from 32 tissues are presented. Gene information and raw data are available at https://www.proteinatlas.org/ENSG00000170315-UBB/tissue and https://www.proteinatlas.org/ENSG00000150991-UBC/tissue.

expression of polyubiquitin genes to meet the required free Ub pool levels, so that the increasing amount of misfolded proteins can be degraded without delay (16, 46). In fact, in cells treated with the proteasome inhibitors or exposed to oxidative stressors, *UBC* expression has been upregulated (28).

When *Ubb*$^{+/GFP\text{-}puro}$ and *Ubc*$^{+/GFP\text{-}puro}$ heterozygous MEFs, which synthesize GFP by the promoters of endogenous *Ubb* and *Ubc*, are exposed to proteasome inhibitor (MG-132), which causes proteotoxic stress, and arsenite, which causes oxidative stress, the GFP signal increased in a time- and concentration-dependent manner (50). Therefore, the increase in free Ub pool levels under stress conditions is achieved by upregulation of polyubiquitin genes. When *Ubc* knockout MEFs were exposed to oxidative stress, Ub levels were not increased and the viability of cells was significantly reduced (18). Similarly, reduced viability was observed when a polyubiquitin gene *UBI4* was disrupted in yeast (17). These results suggest that the upregulation of polyubiquitin genes under stress conditions and the corresponding increase in Ub levels are essential for stress tolerance.

The stress-inducible polyubiquitin gene *UBC* has heat-shock elements in the promoter region, which play an important role in the upregulation of the polyubiquitin gene under stress conditions (51, 52). In the promoter region of *UBC*, HSF1 binding allows upregulation of *UBC* expression. Under HSF1 deficiency by treatment with *HSF1* shRNA, the upregulation of *UBC* expression was greatly reduced even under stress conditions (27, 28, 39). The expression of HSF1 was increased under oxidative stress and proteasome inhibition, not only under heat-shock stress (53, 54). Thus, HSF1 plays a role to upregulate *UBC* expression in response to various cellular stresses or environmental stimuli.

When MEFs are exposed to oxidative stress, MAPK and nuclear factor erythroid 2-related factor 2 (Nrf2)-Kelch-like ECH-associated protein 1 (Keap1) pathways are activated (55). Stabilized Nrf2 moves into the nucleus as a transcription factor and binds to an antioxidant response element (ARE) to upregulate the expression of antioxidant response genes to resist oxidative stress (56). In *Ubc* promoter, there is a region containing the consensus sequence of ARE, and when this region is deleted, the transcriptional activity decreases even in the presence of stabilized Nrf2 (18). In addition, when MEFs were exposed to arsenite, *Ubc* mRNA levels increased significantly, but much less in Nrf2 knockout MEFs. These results provide evidence that under oxidative stress, Nrf2 may also affect the upregulation of *Ubc*. However, this phenomenon has not been observed in other cells, such as HeLa or NIH3T3 cells (28).

A novel strategy to induce changes in Ub pools

Free Ub pools are maintained through the regulation of ubiquitin gene expression and by Ub recycling. The expression levels of polyubiquitin genes are tightly regulated under normal conditions without increasing or decreasing significantly, thereby maintaining the basal levels. However, their expression levels increase dramatically under stress conditions (16, 37, 51, 52). It was not clear whether the expression of endogenous polyubiquitin genes can also be increased under normal conditions. To answer this question, ectopic Ub overexpression has been used to increase Ub levels (37, 57, 58). Conversely, a method used to reduce Ub levels is a knockdown through shRNA or use of cells isolated from knockout mice (36-38, 59, 60). These methods may alter the Ub levels whatever are the expression levels of endogenous genes, although overexpressed Ub has been shown to downregulate the expression of polyubiquitin genes.

Recently, with the development of the CRISPR/Cas9 system, which is widely used as a genome engineering tool in biological research, it has been suggested that Ub levels could potentially be controlled through transcriptional regulation (61, 62). In the CRISPR/Cas9 system, an endonuclease Cas9 binds the target sequence by guide RNA and induces double-strand break (63-65). By introducing double knockout of two polyubiquitin genes using this system, compensatory expression was avoided and more efficient free Ub depletion was achieved (34). In fact, double knockout of *UBB* and *UBC* in HEK293T and HeLa cells showed a dramatic decrease in free Ub levels with reduced proliferation and proteasome activity.

dCas9, a modified protein from which the nuclease activity of Cas9 has been removed, can carry out guide RNA-mediated binding to the target sequence, but does not cause double-strand break. When the transcriptional activator is fused to dCas9, the activator can be recruited only to the promoter of a specific gene, targeted by the guide RNA (65, 66). The transcriptional activator mainly used in this CRISPR-activation system is VP64, a tetramer of the minimal activation domain of VP16. VP16 is a transcription factor of herpes simplex virus type 1 and activates transcription by inducing chromatin remodeling through interaction with other transcription factors (67). The activation system consisting of a simple combination of guide RNA and dCas9-VP64 targeting the *UBC* intron region did not induce *UBC* upregulation (29). These results suggest that *UBC*, with high basal expression levels, may already have a relaxed chromatin structure for transcriptional activation (66). Therefore, to upregulate *UBC*, a modified guide RNA-derived system using an RNA aptamer was introduced (66). *UBC* was successfully upregulated by means of this system, in which the activation domain of HSF1 and p65, the activation domain of NF-κB, were added through RNA aptamer that was part of the modified guide RNA. Thus, modified guide RNA targeting the intron region of *UBC*, dCas9-VP64, and p65-HSF1 were required for the upregulation of *UBC* expression. Because *UBB* was not affected, this modified guide RNA-derived system is quite specific to *UBC* (29). In addition, regulation using this system was inducible using doxycycline and completely reversible (68). These findings suggest that even under normal conditions, it is

possible to induce changes in the free Ub pools by upregulation of endogenous polyubiquitin genes (29).

Concluding remarks

So far, we have mentioned that the upregulation of polyubiquitin gene expression under stress conditions is important to confer resistance against stress, otherwise cells may exhibit the reduced viability. Not only under stress conditions, regulation of their expression is also important under normal conditions. It is problematic if the state of decreased or increased Ub levels persists because of failure in balancing the regulation of their expression. There is a limitation in that the strategies used in the past studies to change Ub pool levels are irreversible. Under normal conditions, Ub pools do not seem to be regulated beyond the threshold levels. Furthermore, when Ub is overexpressed, polyubiquitin genes are downregulated, and when one polyubiquitin gene is disrupted, the other polyubiquitin gene is upregulated as a compensatory expression. These changes seem to maintain the free Ub levels inside cells. However, the molecular mechanism that can explain the dependency on Ub levels for the regulation of their expression is not known yet. One hypothesis is that there is a sensor that can bind free Ub directly or indirectly, and this sensor regulates the transcription factor complex assembly in the promoter region of polyubiquitin genes when it is released from free Ub.

Recently, using the CRISPR/Cas9 system, double knockout of *UBB* and *UBC* has been shown to be a way to deplete free Ub pools more efficiently, because compensatory expression can be avoided. In addition, transcriptional regulation using a dCas9-fused effector may be a promising candidate to regulate Ub levels. Especially, the CRISPR-activation system can be advantageous for conferring oxidative stress resistance, and this system regulates endogenous gene expression, which is cell friendly and physiologically relevant. It has been reported that continuous overexpression of Ub can prepare cellular resistance against expected stress conditions (69). However, persistent Ub overexpression may have adverse effects, such as destroying the synaptic function or reducing muscle development (24, 70). Therefore, if polyubiquitin genes can be upregulated temporarily by means of the reversible CRISPR-activation system, this problem could be overcome; so this system is useful under stress conditions. Furthermore, the potential of this system in Ub research is that, if *UBB* can also be upregulated in a similar way, it can be used to upregulate both polyubiquitin genes, *UBB* and *UBC*, for maximal increase of Ub levels, or upregulate one of two polyubiquitin genes in a tissue-specific manner, for efficient increase of Ub levels.

During the past decade, although many studies have been conducted on the regulation of polyubiquitin gene expression, there are still areas that need to be explored. Considering the diverse functions of Ub, it is important to study the mechanism by which polyubiquitin genes are regulated. It seems that the development of various high-throughput sequencing techniques provided the foundation for studying the mechanism of gene regulation. Understanding the regulatory mechanism of intracellular Ub levels should be beneficial for studying Ub pool dynamics. Furthermore, therapeutic strategies for diseases such as dementia and cancer can be developed using Ub as a target protein.

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CONFLICTS OF INTEREST

The authors have no conflicting interests.

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