Review

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The evolving world of ubiquitin: transformed polyubiquitin chains

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Abstract: The regulation of diverse cellular events by proteins that have undergone post-translational modification with ubiquitin is well documented. Ubiquitin can be polymerized and eight types of polyubiquitin chain contribute to the complexity and specificity of the ubiquitin signal. Unexpectedly, recent studies have shown that ubiquitin itself undergoes post-translational modification by acetylation and phosphorylation; moreover, amyloid-like fibrils comprised of polyubiquitin chains have been discovered. Thus, ubiquitin is not only conjugated to substrate proteins, but also modified and transformed itself. Here, we review these novel forms of ubiquitin signal, with a focus on fibril formation of polyubiquitin chains and its underlying biological relevance.

Keywords: amyloid-like fibril formation; post-translational modification; ubiquitin.

Introduction

Ubiquitin was first identified in 1975 (1) and its essential role in ATP-dependent proteolysis was discovered in 1978 (2). Myriads of subsequent studies have documented how ubiquitin-mediated protein degradation plays a valuable role in many cellular events, such as gene transcription and cell cycle progression; furthermore, the 2004 Nobel Prize in Chemistry was awarded for work on the discovery of ubiquitin-mediated protein degradation. Non-proteolytic functions of ubiquitin – for example, in immune response and DNA repair – have also been revealed (3). Ubiquitylation (the covalent modification of a protein with ubiquitin) has become a common post-translational modification of physiological significance equivalent to that of phosphorylation, acetylation, and methylation.

Ubiquitin itself is a small protein of 76 amino acid residues (8.6 kDa) that are highly conserved in all eukary-otes (4): for example, there are only three conservative amino acid differences between human and yeast ubiquitin (5). A recent study found that an uncultivated thermophilic archaeon (Candidatus '*Caldiarchaeum subterraneum*') also possesses a ubiquitin-like protein modifier and its system (6). As its name suggests, ubiquitin is present in all types of cell and organization of their organisms (1). Intriguingly, its intracellular concentration is extremely high (approx. 85 μM) (7) and the amount of (un-)conjugated ubiquitin molecules is tightly controlled in cells (8).

Eukaryotes utilize ubiquitin in a monomeric and/or polymeric form as a reversible protein tag to regulate proteolytic functions and non-proteolytic events. To provide a signal as such, ubiquitin is covalently conjugated to intracellular substrate proteins in successive enzymatic reactions brought about by ubiquitin-activating (E1), ubiquitin-conjugating (E2), and ubiquitin ligase (E3) enzymes (9, 10). Ubiquitin is first activated by formation of an E1-ubiquitin thioester in an ATP-dependent manner and then transferred to the catalytic cysteine residue of an E2 enzyme. Lastly, an E3 enzyme catalyzes the formation of an isopeptide bond between the lysine residue on the target protein and the C-terminal glycine residue of ubiquitin. In some cases, a covalent bond is formed between the N-terminus of the substrate protein and the C-terminus of ubiquitin (N-terminal ubiquitylation) (11). Downstream proteins containing a ubiquitin-binding domain (UBD) interact with conjugated ubiquitin molecules in cells, and these interactions function to control various cellular events (12). In contrast, deubiquitinating enzymes (DUBs) can remove the ubiquitin tag from a substrate protein (13), and this reaction is counterbalanced by the action of the E1-E2-E3 machinery (8).

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Importantly, anchored ubiquitin tags have various linkage types and lengths. Ubiquitin is attached to target proteins not only as a single moiety (monoubiquitylation), but also as several independent ubiquitin molecules (multi-monoubiquitylation) or a polymeric ubiquitin chain. In the latter chain formation, the C-terminus of a given ubiquitin molecule is covalently conjugated to either the N-terminal residue (M1) or one of seven lysine residues (K6, K11, K27, K29, K33, K48, and K63) of another ubiquitin molecule (3). Interestingly, the topology of ubiquitin chains depends on its chain linkage; in other words, different linking of ubiquitin chains results in distinct conformations. The ubiquitin monomer possesses two solvent-exposed hydrophobic patches (centered on I36 and I48) (14), and ubiquitin-binding proteins distinguish between different types of ubiquitin chain by interacting with topological features of the hydrophobic patches (12, 15–17). All eight linkage types have been structurally characterized by using X-ray crystallography, small angle X-ray scattering (SAXS), and nuclear magnetic resonance (NMR) spectroscopy. M1-, K27-, K29-, and K63-linked diubiquitin form extended structures (18–21), whereas K6-, K11-, K33- and K48-linked diubiquitin form compact structures owing to the interactions between the two hydrophobic patches (22–27). In addition, a recent study suggested that each type of ubiquitin chain possesses the linkage-dependent dynamics as well as a linkage-specific conformation (28–30). In solution, each ubiquitin chain does not adopt a single conformation, but is in equilibrium among its own different conformations: extended or compact structures. The distribution of conformations is different depending on the linkage type of ubiquitin chains. Intriguingly, ubiquitin-binding proteins may recognize a pre-existing conformation of each ubiquitin chain (28).

This diversity means that ubiquitylation contributes to the regulation of many cellular events. For example, monoubiquitylation regulates endocytosis and DNA repair (31, 32), and K48-linked poly-ubiquitylation predominantly mediates ATP-dependent proteasomal degradation (33). In contrast, K63-linked poly-ubiquitylation regulates non-proteolytic protein function, subcellular localization, and protein-protein interactions (3, 12), although some reports indicate that it might also be related to the proteolytic system or lysosomal degradation (34). K11-linked polyubiquitylation has been involved in cell cycle regulation (24). M1-linked poly-ubiquitylation plays an essential role in NF-κB activation related to inflammatory, anti-apoptosis, and immune pathways (35, 36). The roles of the other four types of ubiquitin chain (K6, K27, K29, and K33) have been less apparent, but data on their specific functions are just starting to emerge (37). Overall, it is clear that ubiquitylation acts as a code to store and transmit information by means of the specific recognition of polyubiquitin chains and/or substrate proteins by downstream proteins (17).

Both homo-typic (possessing a single type of linkage) and hetero-typic (possessing a mixed linkage or branched) ubiquitin chains have been identified recently (38–42). In addition, ubiquitylation mixed with sumoylation (the covalent modification of a protein with small ubiquitin-like modifier, SUMO) has also been observed (43). Most recently, post-translational modifications (acetylation and phosphorylation) of ubiquitin itself have been discovered (44–47). Furthermore, our previous study revealed the length-dependent fibril formation of poly-ubiquitin chains (48). Namely, ubiquitin is not only conjugated to substrate proteins, but also modified and transformed into fibrils. On the one hand, the quantitative mass spectroscopic studies have revealed that approximately 80% of ubiquitin chains comprise K48 and K63 linkages (7, 49). On the other hand, the intracellular proportion of these novel and minor types of ubiquitylation is limited. Nevertheless, they seem to be indispensable for specific biological events; for example, the percentage of M1-linked ubiquitylation in total linkages is 0.01–0.02% (44, 49), but the M1-linked ubiquitylation is an important regulator of NF-κB signaling (35, 36). Here, we review the recently discovered post-translational modifications and the transformation of ubiquitin to polyubiquitin fibrils (Figure 1). In particular, we focus on fibril formation of polyubiquitin chains and discuss its possible roles in intracellular protein aggregation.

Post-translational modifications of ubiquitin

Ubiquitin is not only modified by ubiquitin itself. According to the PhosphoSite Plus database (50), ubiquitin has been reported to be acetylated at K6, K11, K27, K29, K33, K48, and K63; phosphorylated at T7, T12, T14, S20, S57, Y59, S65, and T66; and sumoylated at K11 and K63. However, the physiological relevance and biological functions of these modifications have mostly remained unclear. Recently, acetylated ubiquitin at K6 or K48 (AcK6 or AcK48) and phosphorylated ubiquitin at S65 have been functionally identified. Other recent review articles provide a detailed discussion of such post-translational modifications (51, 52); therefore, they are briefly described here.

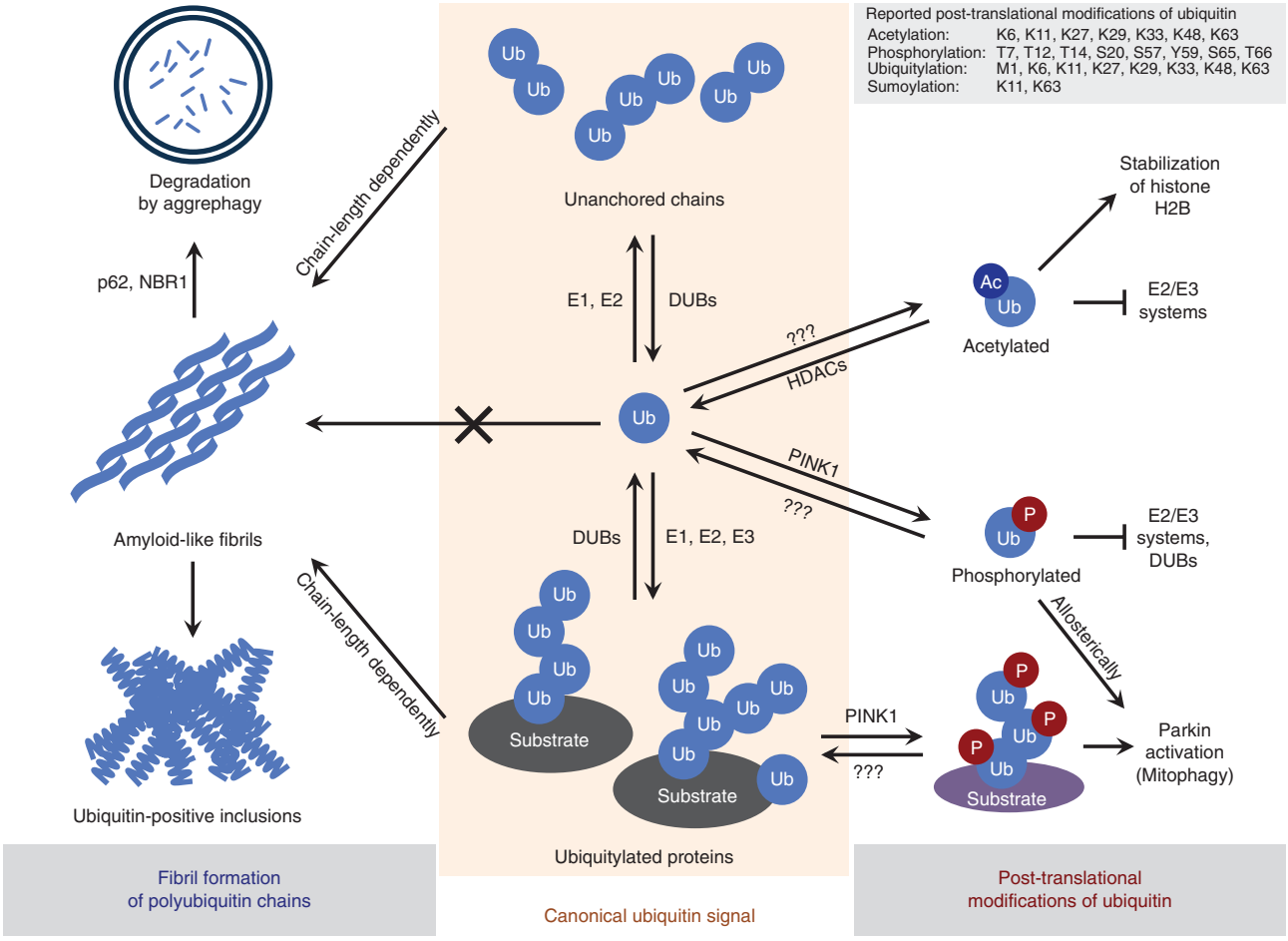

Figure 1: Conventional and novel types of ubiquitylation.
Ubiquitin molecules are covalently conjugated to substrate proteins in monomeric or polymeric forms. Conjugated (poly-)ubiquitin molecules can be cleaved and recycled by DUBs. The intracellular reservoir of ubiquitin consists of monomeric ubiquitin and unanchored short chains for maintaining adequate levels of ubiquitin molecules. Not only eight types of homo-typic ubiquitin chains, but also branched or mixed ubiquitin chains have been identified. In this manuscript, the two types of novel ubiquitin signal have been reviewed: right, post-translational modification of ubiquitin; left, fibril formation of polyubiquitin chains. The post-translational modifications reported in the PhosphoSite Plus database are shown in the upper right box. Acetylation of ubiquitin at K6 inhibits ubiquitin chain elongation, which contributes to stabilization of histone H2B in cells. Phosphorylation of ubiquitin at S65 impairs both elongation and hydrolysis of ubiquitin chains. In addition, phosphorylated (poly-)ubiquitin at S65 activates parkin and this contributes to the efficient mitophagy. On the other hand, polyubiquitin chains are thermodynamically destabilized in a chain-length-dependent manner and can form fibril by heat or mechanical forces. Their fibrillar aggregates are selectively degraded by autophagy, but impairment of autophagy results in accumulation of ubiquitin-positive inclusions. Question marks represent that no factor has been identified in the indicated pathway.

Acetylation of ubiquitin

In 2015, the functions of AcK6 or AcK48 were reported (44). Lysine acetylation is a reversible post-translational modification and regulates protein functions by neutralizing the positive charge of lysine residues (53). K6 and K48 are located close to the hydrophobic patch centered on I44, which forms the interface with ubiquitin-binding proteins. This suggests that AcK6 and AcK48 might alter the charge of the hydrophobic path and that these modifications might affect recognition of ubiquitin. In fact, acetylation represses the elongation of ubiquitin chains (K11-, K48-, and K63-linked), but it does not significantly affect the ability of acetylated ubiquitin molecules to conjugate to the substrate protein (monoubiquitylation). In other words, acetylation of ubiquitin does not inhibit activation of ubiquitin by E1 enzymes nor its subsequent transfer to E2 enzymes. In contrast, the acetylation inhibits polyubiquitin chain elongation mediated by the E2 enzymes (CDC34, UBE2K, UBE2S, UBC13-UEV1a, RAD6, and UBCH5). The two acetylation sites (K6 and K48) are close to the interaction surface of the acceptor ubiquitin with the E2 enzyme (UEV1a) (54). In addition, the interaction

surfaces of the acceptor ubiquitin with the E2 enzymes (CDC34, UBE2K, UBE2S, and UBCH5) are also located near the K6 residue (55–58). Because formation of isopeptide link between a donor ubiquitin and an acceptor ubiquitin is mediated by the interaction of the E2 enzyme with the acceptor ubiquitin (54), the decreased affinity of the E2 enzymes for ubiquitin by the acetylation inhibits elongation of ubiquitin chains (44).

The repression of chain elongation in cells would result in the inhibition of signal transduction specific to polyubiquitin chains. So far, the only reported example is monoubiquitylation of histone H2B, which is found to be stabilized by acetylated ubiquitin in cells. Although it remains unclear whether this stabilization is caused by the repression of proteolysis-related polyubiquitylation or by other mechanisms, acetylated ubiquitin seems to have a role in stabilizing monoubiquitylation of the endogenous substrate protein in cells.

Mass spectrometry has indicated that the proportion of AcK6 and AcK48 molecules in total ubiquitin is very low at approximately 0.03% and 0.01%, respectively (44). The mass spectrometry has also showed that M1-linked ubiquitin chains constitute 0.02% of total ubiquitin (44). As discussed above, M1-linked ubiquitylation is an important regulator of NF-κB signaling in spite of its low abundance in cells (35, 36). Therefore, acetylation of ubiquitin might be directed at specific targets in cells although its intracellular proportion seems to be limited. Further studies need to focus on identifying both endogenous substrates of acetyl-ubiquitylation (i.e. the conjugation of acetylated ubiquitin) and the related biological significance of these modifications.

Phosphorylation of ubiquitin

As indicated above, ubiquitin has been reported to be phosphorylated at eight residues (T7, T12, T14, S20, S57, Y59, S65, or T66), but three different research groups have simultaneously found that phosphorylation at S65 plays an essential role in the selective degradation of damaged mitochondria by autophagy (mitophagy) (45–47). During PINK1- and parkin-mediated clearance of damaged mitochondria, it is important to accelerate the E3 ubiquitin ligase activity of parkin because at steady state this enzyme forms an auto-inhibited conformation (59–61). Dysfunction-related mutations in PINK1 or parkin cause an intracellular accumulation of damaged mitochondria, followed by an abnormal generation of reactive oxygen species (ROS), which can trigger autosomal recessive Parkinson's diseases.

So far, it has been shown that both phosphorylation of the N-terminal ubiquitin-like (Ubl) domain of parkin by PINK1 (62, 63) and allosteric association of phosphorylated ubiquitin with parkin (45–47) are required for enzymatic activation. The structure of the Ubl domain is similar to that of ubiquitin (64), and both the Ubl domain and ubiquitin are phosphorylated at S65 by PINK1; however, the structural roles of these two phosphorylation events are different, although both of them can induce conformational rearrangements of parkin. On the one hand, phosphorylation of the Ubl domain has been proposed to induce release of the domain from the core structure including the enzymatic center. Because the Ubl domain interacts with the E2-binding site in the RING1 domain in an auto-inhibited state (59–61), its release may enhance the enzymatic activity (65). On the other hand, phosphorylated ubiquitin interacts with another surface in the RING1 domain, which induces conformational changes of a helix near the RING1 domain, suggesting that binding of phosphorylated ubiquitin would induce release of the Ubl domain and subsequent phosphorylation of the Ubl domain (65). Thus, although there is little structural difference between the Ubl domain and ubiquitin, phosphorylation of Ubl causes inhibition of the domain-domain interaction, while phosphorylation of ubiquitin generates a novel protein-protein interaction.

The proportion of phosphorylated ubiquitin is 0.05% of total ubiquitin in intact cells, but it rises to approximately 3% when mitochondria are depolarized (47). These cellular amounts would seem to be insufficient for activation of parkin; however, the local concentration is relatively high on mitochondria: ~20% of ubiquitin on depolarized mitochondria is phosphorylated (66). In addition, overexpression of phosphorylation-deficient ubiquitin (S65A mutants) results in delayed activation of parkin (46, 47), suggesting that phosphorylation of ubiquitin and the association of phosphorylated ubiquitin with parkin are necessary for the clearance of damaged mitochondria. Not only monoubiquitin but also K48- and K63-linked polyubiquitin chains are reported to be phosphorylated, which also enhances the enzymatic activities of parkin (67).

Phosphorylation of ubiquitin at S65 has been shown to have a novel biological role in the clearance of damaged mitochondria and has also been shown to alter physicochemical and biochemical properties of ubiquitin itself (68). Although there is no clear conformational difference between unphosphorylated and phosphorylated ubiquitin, phosphorylation results in significant changes in the electrostatic surface potential of the molecule and generates a new minor conformation containing a different

hydrophobic patch (68). This phosphorylation has no influence on the E1-mediated formation of an E2-ubiquitin complex, but it inhibits both discharge of the E2 enzyme to form ubiquitin chains and hydrolysis of the chains by DUBs, thereby affecting chain synthesis and cleavage. Although the physiological relevance of the changes induced by phosphorylation has not been fully elucidated, phosphorylation of ubiquitin affects ubiquitin-specific interactions with ubiquitin-binding proteins and some of the related enzymatic reactions are impaired; in particular, inhibition of the hydrolytic cleavage of the phosphorylated ubiquitin chains by DUBs might contribute to the stabilization of specific signals on mitochondria.

Polyubiquitin chains: new physicochemical properties and unexpected transformations

In addition to the post-translational modifications of ubiquitin as described above, novel physicochemical properties, as well as the 'transformation', of ubiquitin chains, have been recently identified and described (48).

Length-dependent physicochemical properties of ubiquitin chains

On the one hand, ubiquitin is well known to possess exceptional physical and chemical stability. On the other hand, little is known about the physicochemical properties of polyubiquitin chains. As discussed above, ubiquitin moieties are oriented in different ways depending on the linkage point of the polyubiquitin chain. Previous structural studies have shown that the tertiary structure of ubiquitin moieties in polyubiquitin chains almost matches that of monoubiquitin. Although structural studies have suggested that a chain length of two to four ubiquitin units may be sufficient for specific recognition by ubiquitin-binding proteins (3), polyubiquitin chains of more than four units in length are commonly observed in cells. An early study indicated that longer chains might be bound more tightly by ubiquitin-binding proteins, and polyubiquitin signals with more than four units seem to provide an efficient protein degradation signal (69). Nevertheless, there is little structural evidence to show how polyubiquitin chains longer than tetra-ubiquitin are recognized. In addition, excessively long polyubiquitin chains would seem to be paradoxical, given that the biosynthesis of such long chains demands a great deal of cellular energy in form of ATP.

Notably, despite the exceptional structural rigidity and high solubility *in vitro*, ubiquitin chains have been identified as a major component of protein inclusion bodies in various intractable diseases, including cancer and neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis (70–76). In some cases, ubiquitylated aggregate-prone proteins might contribute to formation of ubiquitin-positive aggregates, i.e. the aggregate-prone proteins form aggregates and attached ubiquitin chains may be just entrapped by the aggregates. For example, α-synuclein is one of the aggregate-prone proteins and its intracellular aggregates contain ubiquitylated α-synuclein (77). Alternatively, dysfunction of the proteolysis system is suggested to cause accumulation of ubiquitin-positive inclusions. Indeed, protein aggregates including ubiquitin chains are thought to be the result of failure to eliminate ubiquitylated substrates by the 26S proteasome or autophagy. In neurodegenerative diseases, some stress factors such as ROS and neurotoxic compounds may lead to dysfunction of the proteolysis system (78). However, the detailed formation mechanism and function of the ubiquitin-positive inclusion bodies remain unclear. This implies that polyubiquitin chains have as yet unidentified features.

Differential scanning calorimetry analysis has revealed the novel thermodynamic properties of polyubiquitin chains that are significantly different from that of monoubiquitin. The denaturation point of ubiquitin monomer is close to the boiling point of water and it refolds easily after heat denaturation (79). However, these properties are found to be weakened simply by its covalent linkage to another ubiquitin or another protein molecule (48). A longer ubiquitin chain is further destabilized regardless of the linkage type, and no ubiquitin polymers show thermal reversibility. Although polyubiquitin chains are a type of repeat protein, other repeat proteins have been reported to show increasing thermodynamic stability with increasing numbers of units (80, 81). In these cases, the interactions between monomeric units contribute to the thermodynamic stabilization. Such hydrophobic interactions between units are also observed in K48-linked ubiquitin chains (26, 82); nevertheless, these chains are destabilized in a chain-length-dependent manner. This length-dependent destabilization seems to be specific to ubiquitin chains, suggesting that the covalent conjugation of ubiquitin may destabilize the ubiquitin molecule that is attached. Indeed, recent computational studies have discussed the possibility that ubiquitylation may induce

thermodynamic destabilization of the attached protein (83, 84).

Fibril formation of polyubiquitin chains

The length-dependent thermal destabilization of polyubiquitin chains has been shown to result in amyloid-like fibril formations (48). Whereas no aggregate of monoubiquitin is observed after heat denaturation, polyubiquitin chains form fibrillar insoluble aggregates after heat denaturation. Regardless of the chain length or the linkage type, all ubiquitin chains form fibrils after heat denaturation. The fibrils show morphology very similar to that of amyloid-like fibrils reported previously (85); in addition, the fibrils form β-rich secondary structure and are stained by thioflavin T. Previously, it has been reported that the thermodynamic destabilization of a protein is correlated with its propensity to aggregate in the case of other fibrillogenic proteins such as α-synuclein and tau (86, 87). This suggests that the length-dependent thermodynamic destabilization of polyubiquitin chains might induce fibril formation.

In addition, it is well known that hydrodynamic forces (agitation or shear forces) induce the formation of amyloid fibrils in fibrillogenic proteins such as Aβ, insulin, lysozyme, and β-lactoglobulin (88). Indeed, polyubiquitin chains also form fibrils upon moderate agitation or shear forces in a Couette cell, regardless of the chain length or linkage type (48). As expected, the responses of polyubiquitin chains to such mechanical forces correlate with their thermodynamic stability: i.e. a longer chain forms fibrillar aggregates faster or with smaller forces. Elongated molecules may be more easily affected by external mechanical stress because they undergo larger anisotropic Brownian motions.

In the agitation experiments, M1-linked ubiquitin chains were found to form aggregates more easily than K48-linked ones (48). In contrast, previous atomic force microscopy (AFM) studies have shown that the force required to unfold K48-linked ubiquitin chains is smaller than that required to unfold M1-linked ones (89, 90). On the one hand, the mechanical forces in those AFM experiments were used to stretch a single ubiquitin chain molecule. On the other hand, not only energy for partial deformation of the molecules, but also their inter-molecular interactions are required for the formation of fibrillar aggregates. It is possible, therefore, that the inter-molecular interactions occur more frequently or easily when M1-linked ubiquitin chains are agitated as compared with K48-linked ones. Further studies should focus on the mechanism by which mechanical forces induce the formation of ubiquitin chains.

Formation of intracellular aggregates of ubiquitin

Polyubiquitin chains of all linkage type are found within the ubiquitin-positive inclusion bodies in livers with impaired proteasome activity (48) or those that are autophagy-deficient (48, 91). These inclusion bodies are also frequently observed in neurodegenerative diseases (70–76), suggesting that the length-dependent properties of ubiquitin chains may be related to aggregate formation. Indeed, the length-dependent propensity of polyubiquitin chains to aggregate *in vitro* has also been observed in living cells (48). Monoubiquitin expressed in cells does not form aggregates; however, recombinantly expressed M1-linked hexa-ubiquitin forms aggregates in the cytosol. In cells, there are likely to be several intracellular forces due to cytoplasmic streaming or macromolecular crowding. Polyubiquitin chains are subject to such intracellular forces and, as a result, may form aggregates in cells. Notably, simple overexpression of polyubiquitin chains seems to be sufficient for intracellular aggregate formation. In contrast, the cellular ubiquitin pool includes unanchored polyubiquitin chains (~3-mer) that can generate free ubiquitin molecules (8, 92) and intracellular forces may constitutively affect their endogenous chains. However, the intracellular concentration of such unanchored chains may not be enough for aggregation, and they dynamically undergo both cleavage by DUBs and re-synthesis (8), suggesting that substantial formation of aggregates of polyubiquitin chains may hardly occur in healthy cells. Instead, an increase in the concentration of polyubiquitin chains owing to proteasome dysfunction or dysregulated deubiquitylation may lead to their aggregate formation.

If intracellular aggregates of polyubiquitin chains continue to accumulate in cells, the cells will die due to endoplasmic reticulum stress and/or abnormal inhibition of inherent cellular functions (93). In particular, the accumulation of polyubiquitin aggregates in neurons leads to neurodegeneration (94). Indeed, on the one hand, ubiquitin-positive aggregates are known to be hallmarks of neurodegenerative diseases, as described above (70–76). On the other hand, the ubiquitin-positive aggregates are thought to be selectively eliminated by autophagy (aggrephagy) (94, 95). Ubiquitin-adaptor proteins such as p62 and NBR1 recognize ubiquitin on the aggregates and the core Atg proteins form an autophagosome, which then

fuses with a lysosome, leading to the degradation of its constituents (95). The process of aggrephagy is indispensable for eliminating of polyubiquitin aggregates and its impairment causes the accumulation of ubiquitin-positive aggregates (48, 94). In contrast, it seems to be difficult to degrade the aggregates via the proteasome due to their size and volume; moreover, they impair the ubiquitin-proteasome system (96). Recently, it has also been found that dysfunction of the proteasome leads to the activation of autophagy (97, 98).

Polyubiquitin aggregates not only are autophagic cargo, but also have been proposed to serve as an initiation signal for aggrephagy (48). Solution NMR studies have indicated that the ubiquitin-adaptor proteins p62 and NBR1 recognize the fibrillar form of polyubiquitin chains. Although the structures of the fibrils may be partially or entirely different from those of native ubiquitin chains, ubiquitin-adaptor proteins have the ability to bind to the fibrillar aggregates *in vitro* and they are co-localized with them in cells (48). Furthermore, over-expression of polyubiquitin chains results in S351-phosphorylation of p62 and co-localization of endogenous LC3 (48), indicating that polyubiquitin aggregates activate aggrephagy (95, 99).

Thus, the intracellular aggregate formation of polyubiquitin chains has two different aspects: first, the abnormal accumulation of aggregates displays cytotoxicity, leading to neurodegeneration; second, the aggregates function as signals to eliminate protein aggregates efficiently. Previously, it was reported that ubiquitylation is involved in the sequestration of misfolded proteins (100, 101). The insolubilization caused by aggregate formation would prevent any undesired activities of the substrate proteins in cells (102, 103). Therefore, the protein aggregates sequestered by ubiquitylation need to be rapidly degraded by autophagy before they form large inclusions. Notably, the dysfunction or inactivation of autophagy has been observed in senescent cells (104, 105), which may result in the cytotoxic accumulation of ubiquitin-positive aggregates.

Expert opinion

Approximately 30 000 kinds of proteins have been identified in the human proteome (106) and they maintain cellular homeostasis with other components such as peptides, nucleotides, and lipids. Because ubiquitylation controls the activity, lifetime, and localization of the proteins, it plays an important role in the homeostasis. The diverse ways in which polyubiquitin chains can be linked, coupled with a sufficient number of specific E3 ligases, seem to have the ability to regulate many types of cellular events. Recently, however, it has been found that not only is ubiquitin used for conjugation and/or polymerization, but it is also acetylated and phosphorylated; furthermore, it can form fibrils (Figure 1). Increasing evidence indicates that ubiquitin shows a wider variety of signaling and that eukaryotes use ubiquitin in more different ways than previously thought.

On the one hand, acetylation and phosphorylation alter the inherent function of the ubiquitin signal: acetylation of ubiquitin represses elongation of the ubiquitin chain, while phosphorylation inhibits the formation of ubiquitin chains as well as the hydrolysis of chains by DUBs. Phosphorylation also provides a novel biological function as an allosteric effector. On the other hand, the fibril formation of polyubiquitin chains seems to be an inherent property of ubiquitin chains themselves. Wild-type polyubiquitin chains show chain-length-dependent destabilization and have the ability to form fibrils. This feature contrasts with other amyloid-prone proteins, many of which are truncated and/or carry mutations (86, 107), and might account for the pathological ubiquitin-positive aggregates observed in human sporadic proteinopathies without genetic mutations.

Collectively, these recent new findings indicate that ubiquitin acquires its multifunctional features by diverse types of polymerization, post-translational modification, and transformation. Nevertheless, several questions about the ubiquitin signal remain. Is the acetylation or phosphorylation of ubiquitin a reversible reaction in cells? If so, how is their quite low abundance in cells maintained? Do other post-translational modifications of ubiquitin have biological significance? What is the structure of polyubiquitin fibrils? What are the biological functions of ubiquitin chains with minor linkage-types (K6, K27, K29, and K33)? How is ubiquitin itself degraded? What is the intracellular localization of each ubiquitin signal? Future studies should aim to answer these questions and reveal the underlying mechanisms.

Conflict of interest statement: The authors declare no competing financial interests.

List of abbreviations

AcK6	acetylated ubiquitin at K6
AcK48	acetylated ubiquitin at K48
Aggrephagy	selective autophagic degradation of aggregated proteins

Atg	autophagy-related
C-terminal (C-terminus)	carboxyl-terminal (carboxyl-terminus)
DUBs	deubiquitinating enzymes
E1	ubiquitin-activating enzymes
E2	ubiquitin-conjugating enzymes
E3	ubiquitin ligase enzymes
Mitophagy	selective degradation of damaged mitochondria by autophagy
NBR1	next to BRCA1 gene 1 protein
NF-κB	nuclear factor κ-light-chain-enhancer of activated B cells
PINK1	PTEN-induced putative kinase 1 RING
PRM	parallel reaction monitoring
p62	sequestosome 1
RING	really interesting new gene
ROS	reactive oxygen species
SUMO	small ubiquitin-like modifier
Sumoylation	post-translational modification by SUMO
UBD	ubiquitin-binding domain
Ubiquitylation	post-translational modification by ubiquitin
Ubl	ubiquitin-like

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