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Review

Mechanisms of cross-talk between the ubiquitin-proteasome and autophagy-lysosome systems

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ABSTRACT

The ubiquitin proteasome system (UPS) and macroautophagy (hereafter called autophagy) were, for a long time, regarded as independent degradative pathways with few or no points of interaction. This view started to change recently, in the light of findings that have suggested that ubiquitylation can target substrates for degradation via both pathways. Moreover, perturbations in the flux through either pathway have been reported to affect the activity of the other system, and a number of mechanisms have been proposed to rationalise the link between the UPS and autophagy. Here we critically review these findings and outline some outstanding issues that still await clarification.
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1. Introduction

The UPS and autophagy are two cornerstones of cellular catabolism that are involved in most aspects of normal physiology and development, and are also implicated in a broad array of pathological states, including cancer, neurodegeneration and aging. Protein degradation controls processes like the cell cycle, signaling, DNA transcription, repair and translation, by downregulating their critical regulatory elements. Additionally, the UPS and/or autophagy are involved in the degradation of virtually every type of surplus, dysfunctional or damaged cellular component, ranging from soluble proteins to whole organelles. This allows recycling of both matter and energy and therefore serves to save valuable resources. Thus, autophagy and the UPS are critical in the maintenance of cellular homeostasis, suggesting that their activities need to be carefully orchestrated. Yet, the two pathways differ so significantly with respect to their mechanistic details (autophagy is a vesicular trafficking pathway, while the enzymatic reactions of the UPS occur directly in the cytosol), substrates (the activity of UPS is restricted to soluble proteins, while autophagy is practically omnivorous), machinery, specificity, kinetics, elements of control, etc., that this leaves very little room to suspect any cross-talk. Indeed, for a long time these processes were viewed as independent of each other [1,2]. Here, we review the evidence generated during recent years that challenge this view and offer a glance into a complex and often an unexpected interplay between these two cellular waste conveyors.

2. Basic mechanics of the UPS and autophagy

Proteins are targeted for destruction by the UPS via a series of enzymatic reactions that tag them with homopolymers of a small, 76-amino acid residue, protein called ubiquitin [3,4]. Polyubiquitylation marks the UPS clients for transportation by a poorly understood shuttling machinery to a specialized organelle called the proteasome, where proteins are degraded to oligopeptides, which are released into the cytoplasm or nucleoplasm, where they can be digested into amino acids by soluble peptidases. The specificity and selectivity of the ubiquitylation process is achieved by a combination of three types of enzymes [5]. E1 enzymes, two of which are known in mammals, initiate the reaction by activating ubiquitin and transferring it onto E2 ubiquitin-conjugating molecules, of which around 40 are thought to be encoded in the mammalian genome. A substrate is selected in our cells by one of several hundred E3 ligases, which bind the

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ubiquitin-carrying E2 enzyme, resulting in the transfer of the ubiquitin onto lysine residues of the target substrate [6,7]. As a result of such a reaction, the substrate becomes monoubiquitylated in one or more places. These initial modifications are not yet sufficient for proteasomal targeting. Since ubiquitin itself contains lysine residues in positions 6, 11, 27, 31, 33, 48 and 63, all of these sites could become acceptors of another ubiquitin moiety in a subsequent round of ubiquitylation, which would lead to the generation of different types of polyubiquitin chains. It is thought that chains of at least four ubiquitins [8] interconnected via K48 residues, which are characterized by a closed conformation [9], are optimal for delivery to the proteasome. The proteasome is a barrel-shaped proteolytic organelle found throughout the cell that consists of a 20S central complex and two 19S lid complexes. The 19S complexes bind cargo-loaded shuttling proteins, deubiquitylate the substrates and control access to the six proteolytic sites of the inner core of 20S subunit [10,11]. The catalytic activities of the proteasome have different specificities, and are considered trypsin-, chymotrypsin- and peptidyl-glutamyl peptide-hydrolyzing-like [12]. The narrow size of the proteasomal catalytic pore suggests that protein substrates need to be partially-unfolded prior to entry into the 20S subunit. Thus, protein complexes and aggregates can only be digested if disassembled, which makes them poor proteasome substrates [13].

In contrast to the UPS, autophagy is restricted to the cytoplasm, but is capable of degrading a much wider spectrum of substrates, which, on average, tend to be longer-lived and bulkier. These include functional or misfolded soluble proteins, protein complexes, oligomers and aggregates. Although limited, there appears to be a certain overlap in function between the two degradative pathways, as both seem to be capable of degrading soluble misfolded polypeptide chains [14]. Additionally, autophagy can degrade whole cellular organelles. Terms like pexophagy, mitophagy or ribophagy have been coined to describe autophagosomal degradation of peroxisomes, mitochondria or ribosomes, respectively. Interestingly, also, proteasomal subunits were found to be degraded by lysosomes [15]. This provides a possibility that the autophagy-lysosome system could affect the activity of the UPS by controlling the numbers of proteasomes, a hypothesis that, to our knowledge, has not yet been investigated.

Autophagy is initiated by the formation and elongation of a double-layered isolation membrane (the origin of which remains an intensely debated topic) also called a phagophore, that enwraps and sequesters portions of cytoplasm containing autophagic substrates, to form autophagosomes. The formation of autophagosomes is regulated by a set of Atg genes, where Atg stands for autophagy-related, the nomenclature taken from yeast where they were originally identified [16]. These can be grouped, according to their function, into the Atg1 complex (Atg1, Atg13 and Atg17 controlling autophagosomal induction), the PI3K complex III (including phosphatidyl inositol 3-phosphate kinase vps34, Beclin 1 (Atg6 orthologue) and UVRAG (UV radiation resistance associated gene)) regulating vesicle nucleation, and two interconnected ubiquitin-like conjugation systems that mediate vesicle elongation and sealing. The first of these conjugation systems involves the formation of Atg5-12 conjugate, mediated by the E1-like enzyme, Atg7, and the E2-like enzyme, Atg10. The second involves conjugation of Atg8 (in mammalian cells also known as microtubule-associated protein 1 light chain 3, LC3) to the lipid, phosphatidylethanolamine, regulated by Atg7, along with Atg3, as the E2-like enzyme [17]. Following the formation of autophagosome, Atg5-12 conjugate is removed from the vesicle, while LC3 remains attached. Thus, LC3 serves as a reliable autophagosomal marker that can be used to estimate both the rates of autophagosome formation and degradation [18]. Autophagosomes are transported along microtubules in a dynein-dependent manner and fuse with endosomes or directly with lysosomes where autophagosomal contents are degraded by lysosomal hydrolases [19].

3. Ubiquitin as a unifying factor linking the UPS and selective autophagy

Autophagy is often thought of as a non-specific process that degrades cytoplasmic proteins and organelles in bulk, a situation likely to occur when cell survival depends on autophagy during periods of starvation [20]. However, as early as the 1970s, the first evidence of selective autophagy was suggested for organelles such as the endoplasmic reticulum or mitochondria, although further understanding of such selectivity was impossible until more recent insights into the molecular mechanisms of selective autophagy [21]. While this process is still poorly understood, it is postulated that during selective autophagy, certain autophagic substrates may be specifically targeted for destruction, rather than being randomly taken up along with bulk cytoplasm. The relevance of this issue to the topic of our discussion becomes evident when we learn that it is ubiquitylation, just like in the ubiquitin proteasome pathway, that serves as the signal for selective autophagy. Thus, it might be tempting to speculate that ubiquitin coordinates the catabolism of cellular targets by both the UPS and autophagy. Indeed, many proteins are known to be substrates of both degradative systems, and in certain conditions ubiquitylated proteasomal substrates, which are normally degraded by the UPS, can also be digested by autophagy, and vice versa [22–24]. Moreover, impairment of proteasome activity was found to activate autophagy, which was thought to be a compensatory mechanism allowing the cell to reduce the levels of UPS substrates (see below) [25–28]. However, the overall contribution of autophagy to the degradation of the total pool of cellular ubiquitylated proteins remains unknown, and so it is unclear whether ubiquitylation is an important mechanism for autophagic targeting of many proteins.

In addition, although ubiquitylation may appear to be a universal tag targeting substrates for destruction via both catabolic systems, the exact type of modification recognised by each pathway appears to be different. While K48-linked polyubiquitin chains are employed by the UPS, substrates recognised by autophagosome-lysosome pathway are thought to be modified either by K63-linked chains (adopting a more open conformation than K48 chains), or may just be monoubiquitylated [29]. Thus, despite the use of ubiquitin in both catabolic pathways, the structural complexity of different polyubiquitin chains may be sufficient to maintain selectivity and specificity of the UPS and autophagy towards their substrates. However, some potential overlap may result from incomplete specificity of the different adaptor molecules that have been proposed to retrieve ubiquitylated substrates for each degradative pathway. In this category, there are several proteins that appear to serve as linkers between ubiquitylated cargo and the phagophore, including p62 (also called SQSTM1/A170), NBR1 (neighbour of BRCA1 gene 1), HDAC6 (histone deacetylase 6) and Alfy [30]. These proteins have the capacity to interact directly or indirectly with both ubiquitin and components of autophagic machinery, thus providing the type of link that would be required from an adaptor molecule. The most established of these adaptors, p62, is itself an autophagy substrate that forms homo-oligomers to which ubiquitylated proteins are recruited via its UBA (ubiquitin-associated) domain [30–33]. It was proposed that these complexes serve to sequester ubiquitylated substrates that are recognised by the autophagic machinery (p62 interacts directly with LC3 via a dedicated LIR motif [33]), and then engulfed and degraded [30,31]. The UBA domain of p62 appears to have a slightly higher affinity for monoubiquitin or polyubiquitin chains with open conformations (K63-linked), compared to those with a closed

conformations (K48-linked) [34]. This, on the one hand, may suggest a preference of autophagy for substrates tagged with single ubiquitin, short chains, or with longer K63 chains. On the other hand, this might allow K48 chain-tagged substrates to still be recruited into autophagosomes, especially in circumstances when the UPS is compromised, and when the concentration of K48-polyubiquitylated proteins is sufficient to allow such chains to interact effectively with p62 [23,34]. Indeed, a mild accumulation of primarily K63-linked polyubiquitin-tagged proteins was observed in p62-deficient mouse tissues. The interpretation of this effect is complex, since p62 also appears to serve as an adaptor for proteasomal degradation of certain ubiquitylated proteins and is further complicated by suggestions of ubiquitin-independent roles for p62 in the degradation of some autophagy substrates [22,35]. Nevertheless, these studies are consistent with the idea that p62 can serve as an adaptor required for autophagic degradation of ubiquitylated proteins [30,36].

The idea of the unifying role of ubiquitin in the UPS and selective autophagy has recently been discussed in detail elsewhere [14,30,37], and so we will next focus on the more specific question of how changes in the activity of one of the degradative pathways affect the flux through the other system.

4. Impairment of the UPS is compensated by upregulation of autophagy

One of the proposed links between the UPS and autophagy is based on the observation that impairment of the UPS leads to increased autophagic function [25–27]. This is commonly considered to be a compensatory mechanism, allowing cells to reduce the burden of accumulated UPS substrates. Indeed, treatment of both cells and mice with rapamycin to upregulate autophagy has been demonstrated to protect against cell death caused by proteasome inhibition [38] and upregulation of autophagy has been shown to protect against genetic loss of proteasome activity in *Drosophila* [25]. Unfortunately, there is little consensus on the exact mechanism(s) of this cross-talk, as several potential explanations have been suggested. One such proposed mechanism involves activation of endoplasmic reticulum (ER) stress, due to the accumulation of misfolded proteins that leads to the induction of the unfolded protein response (UPR). The UPR is an ER-to-nucleus signaling pathway that results in the transcriptional activation of variety of genes, including those involved in protein folding and degradation in the ER. The activation of this pathway has been shown by a number of studies to result in the activation of autophagy (reviewed in [39]). There are discrepancies in the exact mechanics of this phenomenon, and it is likely to depend on the cell type and stimulus for the UPR. Investigations into the direct link between proteasome inhibition, UPR and autophagy have been carried out in two studies using the proteasome inhibitor bortezomid. These studies both demonstrate the importance of the transcription factor ATF4 in the upregulation of autophagy genes following proteasome inhibition. However, the study of Zhu et al. suggests that the mechanism for increased ATF4 level is the activation of the PERK arm of the UPR requiring the phosphorylation of eIF2α [40], whereas Milani et al. suggest that direct stabilisation of the ATF4 protein due to the loss of proteasome activity, independent of the upstream activity of PERK, results in its increased activity [28]. Additionally these studies diverge on the downstream targets of ATF4 action, showing an increase in either ATG5 and ATG7 transcription [40], or LC3 expression [28]. Additionally, another study has suggested that compensatory autophagy upregulation following treatment with MG132, or bortezomib, is mediated by the IRE1 arm of the UPR and its downstream target c-Jun NH2-terminal kinase (Jnk1) [26,41]. More recent studies have demonstrated that Jnk1, in turn, may induce autophagy by phosphorylation of Bcl-2, thereby disrupting its autophagy–inhibitory interaction with Beclin 1 [26,41].

Independently of the UPR, proteasome inhibition in dopaminergic neurons has been shown to induce autophagy via a mechanism requiring p53 [42]. Following proteasome inhibition, levels of p53 are increased, [42], and multiple pathways have been elucidated through which increases in p53 are suggested to upregulate autophagy, including activation of APMK and subsequent inhibition of mTOR and induction of damage-regulated autophagy modifier (DRAM) (reviewed in [43]).

The protective effect of the compensatory upregulation of autophagy in proteasome-inhibited cells has also been suggested to be dependent on HDAC6 [25,27]. However, the role for HDAC6 in this process is not thought to be through signaling to increase autophagic flux, but rather through ensuring efficient delivery of substrates to the autophagic machinery for degradation. HDAC6 was earlier found to regulate the formation of perinuclear ubiquitylated inclusion bodies, called aggresomes [44]. The concentration of misfolded proteins into these aggresomes has been hypothesised to allow them to be degraded more efficiently by autophagy [27]. Additionally, HDAC6 is thought to mediate the transport of components of the autophagic machinery to the aggresome [27].

Overall, while there is a general consensus about a compensatory role of autophagy following proteasomal inhibition, the exact mechanisms of this link requires further clarification (Fig. 1). These different mechanisms may not be mutually-exclusive and may also be of different importance in different cell types or at different time-points after the proteasome is inhibited.

5. Effect of autophagy on the UPS

Genetic studies in mice demonstrated that inactivation of autophagy by the knockout of essential autophagic genes (Atg5 or Atg7) results in the accumulation and aggregation of ubiquitylated proteins [45,46]. There are several possible interpretations of this result. One of them is in line with the idea that ubiquitylated proteins could be degraded by autophagy, although it is currently unknown whether the type of polyubiquitin chains accumulating in autophagy-deficient tissues is consistent with the proposed specificity of autophagy for K63-linked polyubiquitin chains, the extent to which autophagy contributes to the degradation of the total pool of cellular ubiquitylated proteins, or whether the accumulation of ubiquitylated autophagic substrates can alone explain the profound accumulation of ubiquitin seen in autophagy-deficient mice. Another possibility is that autophagosomal clients that initially are not ubiquitylated, remain for long enough in autophagy-deficient cells to eventually become modified with ubiquitin. Finally, autophagy impairment could impact on the flux through the UPS. Indeed, we and others support this last hypothesis, as we found that impaired autophagy also leads to the impaired degradation of specific UPS clients [47–49]. Our data suggested that the decreased UPS flux in autophagy-compromised cells was not due to impaired catalytic activity of proteasomes isolated from them. Instead, we found that the block in the UPS function is mediated by accumulation of p62, as its knockdown rescued the levels of UPS substrates in autophagy-deficient cells. In addition, overexpression of p62 alone was sufficient to inhibit the UPS, an effect partially dependent on its UBA domain. Since p62 competes with other ubiquitin-binding proteins involved in proteasomal degradation, like p97/VCP (valosin-containing protein), for binding to ubiquitylated proteins, we proposed that elevated levels of p62 may deny such shuttling proteins access to ubiquitylated UPS substrates (Fig. 2) [47,48]. These findings help to explain how knockout of p62 rescues the increased levels of soluble and aggregated

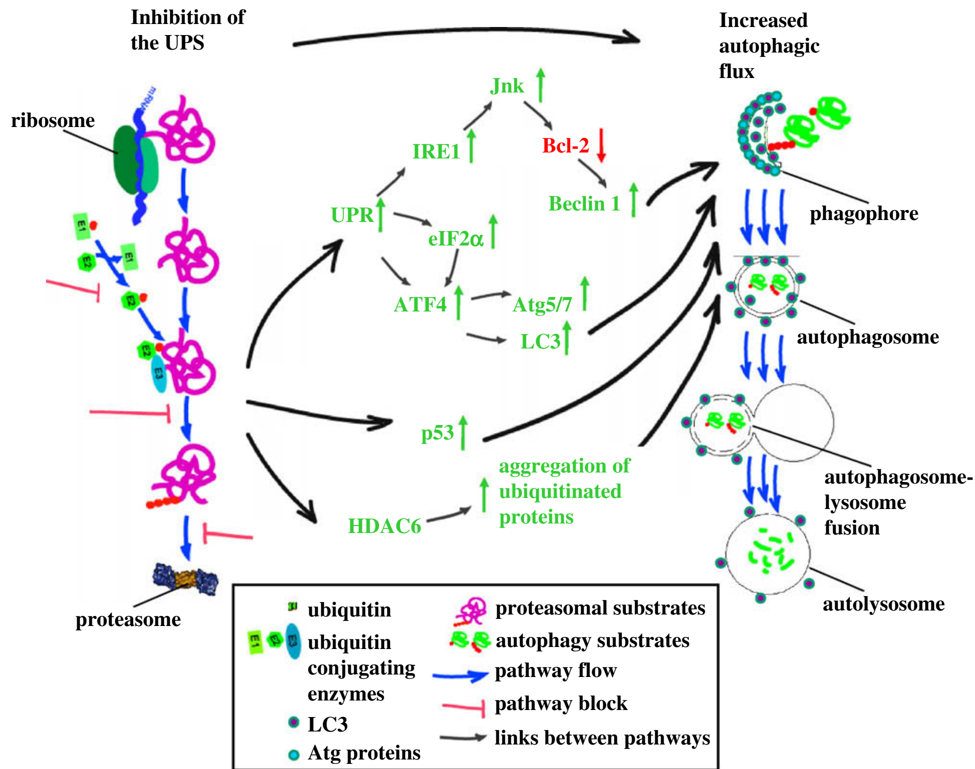

Fig. 1. A diagram illustrating possible mechanisms of compensatory autophagic upregulation following UPS inhibition. Unfolded protein response, elevated levels of p53 and the increased aggregation of ubiquitylated proteins mediated by HDAC6, have all been implicated in the cross-talk between the UPS and autophagy.

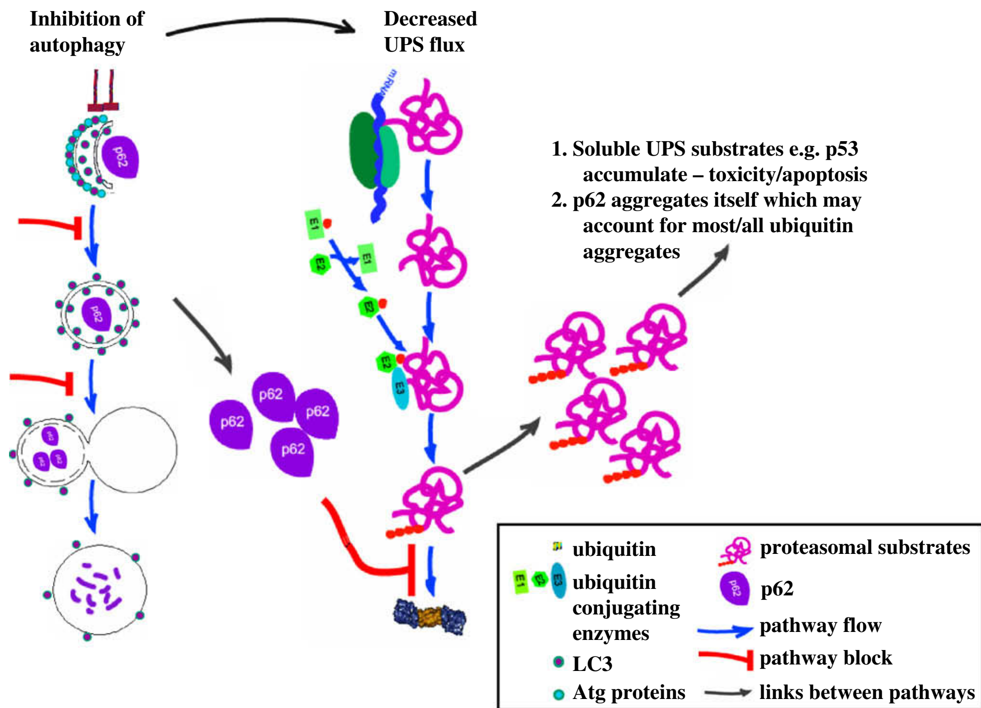

Fig. 2. Inhibition of autophagy impairs the UPS function. p62, which accumulates due to autophagy blockade, binds ubiquitylated proteins and prevents their delivery to and degradation by the proteasome. Toxicity due to elevated levels of certain UPS substrates, like p53, and accumulation of ubiquitinated p62-positive aggregates are the components of the autophagic deficiency phenotype.

ubiquitylated proteins observed in autophagy-deficient tissues [32]. Thus, p62 has been implicated in two different, but not mutually-exclusive, mechanisms of cross-talk between the UPS and autophagy. In the physiological state, where autophagy operates at normal rates, p62 could serve to deliver ubiquitylated proteins for autophagosomal destruction [30,31,33]. In contrast, in situa-

tions where autophagy becomes impaired (which occurs in a variety of pathological conditions, including certain neurodegenerative conditions, such as lysosomal storage disorders), p62 becomes a Trojan horse due to its binding (probably non-selectively because of elevated levels) to ubiquitylated proteins and preventing their delivery to the proteasome for degradation. The lack of compensation for autophagy dysfunction by the UPS is in agreement with the fact that p62, when accumulates, oligomerizes and therefore would be too bulky to be a good substrate for the proteasome with its narrow catalytic pore.

A special case of coordination between the two degradative systems comes from Goldberg and colleagues, who demonstrated that both the UPS and autophagy contribute to muscle atrophy in physiological conditions, like fasting, as well as in diseases characterised by muscle wasting [50]. In this case, coordinate upregulation of both catabolic pathways was induced by the FoxO3 transcription factor downstream of the IGF-1/PI3K/Akt signaling axis. It would be interesting to investigate if coordinated induction of both degradative pathways could be achieved in other tissues, like the brain.

6. Concluding remarks

Extensive effort has been invested during the last decade into studies of the fine molecular detail of both the UPS and autophagy. This has allowed us to begin the ascent to another level, where we aim to learn how different degradative pathways are integrated as components of cellular catabolism. This may be important when we want to manipulate one of the pathways for therapeutic goals. Furthermore, it will be interesting to test if the development of the pathology caused by primary deficiency in one degradative system could be largely affected by secondary changes in the other pathway.

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