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REVIEW

p62-mediated phase separation at the intersection of the ubiquitin-proteasome system and autophagy

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ABSTRACT

The degradation of misfolded proteins is essential for cellular homeostasis. Misfolded proteins are normally degraded by the ubiquitin-proteasome system (UPS), and selective autophagy serves as a backup mechanism when the UPS is overloaded. Selective autophagy mediates the degradation of harmful material by its sequestration within double-membrane organelles called autophagosomes. The selectivity of autophagic processes is mediated by cargo receptors, which link the cargo to the autophagosomal membrane. The p62 cargo receptor (SQSTM1) has a main function during the degradation of misfolded, ubiquitylated proteins by selective autophagy; here it functions to phase separate these proteins into larger condensates and tether them to the autophagosomal membrane. Recent work has given us crucial insights into the mechanism of action of the p62 cargo receptor during selective autophagy and how its activity can be integrated with the UPS. We will discuss these recent insights in the context of protein quality control and the emerging concept of cellular organization mediated by phase transitions.

KEY WORDS: Quality control, Proteostasis, Selective autophagy, Cargo receptor, Degradation, Lysosome, SQSTM1, Aggrephagy

Introduction

The degradation of cytoplasmic proteins in eukaryotic cells is regulated by two main pathways: the ubiquitin-proteasome system (UPS) and the autophagy-lysosomal system. Short-lived and misfolded proteins are preferentially degraded by the UPS, while long-lived proteins and proteins that cannot be unfolded, or even entire organelles, can be degraded by autophagy (Dikic, 2017). For a long time, protein degradation accomplished by the UPS and autophagy have been largely viewed independently, but it is becoming increasingly clear that the two pathways are highly interconnected with regard to cellular protein quality control as they use ubiquitylation as a common degradation signal (Kirkin et al., 2009b). In this Review, we discuss the current knowledge about the interplay between the UPS and autophagy in the degradation of cytoplasmic proteins. We will focus on recent insights of the sequestosome 1 (SQSTM1) cargo receptor (hereafter referred to as p62) that have given us clues about how the two pathways may be connected. In particular, we will discuss the most recent findings of how p62 mediates the phase separation of ubiquitylated proteins into larger condensates that can sequester them and perhaps also serve as nucleators for autophagy.

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The UPS

As briefly introduced above, two main pathways regulate protein degradation of cytoplasmic proteins in eukaryotic cells – the UPS and the autophagy-lysosomal system. Misfolded proteins are normally degraded by UPS by marking them with the conjugation of ubiquitin, a covalent posttranslational modification (Ciechanover, 2005). The activation of ubiquitin moieties is ATP-dependent and driven by an ubiquitin-activating enzyme (E1). First AMP-ubiquitin is transferred to a cysteine residue in the E1 and from there to a cysteine of an ubiquitin-conjugating enzyme (E2). The E2 then teams up with an ubiquitin-ligase (E3) to ubiquitylate the substrate that is subsequently degraded by the proteasome (Streich and Lima, 2014; Schulman and Harper, 2009; van Wijk and Timmers, 2010; Ye and Rape, 2009). Substrates can be modified with a single ubiquitin or with ubiquitin chains, where the first substrate-attached ubiquitin serves as substrate for further ubiquitin conjugation. Dependent on the Lys of the ubiquitin that is used for chain elongation, diverse ubiquitin chains can be generated (Komander and Rape, 2012). Historically, proteasomal degradation is thought to be driven by the recognition of Lys 48 (K48)-linked ubiquitin chains, but most chain types can direct proteins for degradation by the proteasome (Komander and Rape, 2012). Certain chain types, including K63-chains have been mainly associated with autophagy and signaling; however, evidence exists that they can also be targeted by the proteasome (Dikic, 2017; Komander and Rape, 2012; Yau and Rape, 2016). At the proteasome, the ubiquitylated substrates are recognized by receptors, and then deubiquitylated and unfolded in order to funnel them into the catalytic chamber of the protease. Substrates that cannot be unfolded can, thus, not be degraded by the proteasome (Finley, 2009).

Macroautophagy

Macroautophagy (hereafter referred to as autophagy) is an evolutionarily conserved and highly regulated pathway that plays a key role in quality control by mediating the degradation of cellular material within the lysosomal system. During autophagy, cytoplasmic material is engulfed by double-membrane organelles called autophagosomes that subsequently fuse with lysosomes; therein their content is degraded and the resulting building blocks are recycled for reuse (Kraft and Martens, 2012). Although the term autophagy was proposed upon detection of double-membrane vesicles in rat hepatic cells decades ago (De Duve and Wattiaux, 1966), its molecular understanding is largely based on the discovery of autophagy-related (ATG) genes in yeast genetic screens and the subsequent identification of homologues in complex eukaryotes (Thumm et al., 1994; Tsukada and Ohsumi, 1993; Harding et al., 1995). Autophagy is regulated by more than 40 known ATG genes, but only a restricted subset of them encodes proteins that are fundamental for all types of autophagy (Xie and Klionsky, 2007). This subset is referred to as the 'core' autophagy machinery and can be divided into five subgroups that are thought to act in a hierarchical manner: initiation, nucleation of the isolation

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membrane (the precursor to the autophagosome), membrane expansion and maturation, fusion with the lysosome and nutrient recycling (Fig. 1). In eukaryotic cells, the initiation of autophagy is controlled by activation of the unc-51-like kinase (ULK) complex, comprising ULK1/2, ATG13, FIP200 and ATG101 (Mizushima, 2010; Hosokawa et al., 2009; Mercer et al., 2009; Hara et al., 2008; Young et al., 2006). The nucleation of the isolation membrane is also regulated by the class III phosphatidylinositol-3 kinase (PI3K) complex comprising VPS34, VPS15, Beclin and ATG14L (Hurley and Young, 2017; Itakura et al., 2008). Membrane delivery to the expanding isolation membrane is still a largely enigmatic process, but appears to be controlled by the transmembrane protein ATG9A (Young et al., 2006). Vesicle expansion involves two sets of ubiquitin-like (Ubl) conjugation systems, including the ATG12–ATG5–ATG16L1 complex and proteins of the Atg8 family. In humans, this family comprises the ubiquitin-like modifiers LC3A, LC3B, LC3C, GABARAP, GABARAPL1 and GABARAPL2. The two conjugation systems are highly interconnected and collectively mediate attachment of the Atg8 C-terminus to phosphatidylethanolamine in the isolation membrane, where it functions as docking site for other factors (Geng and Klionsky, 2008; Mizushima et al., 1998; Ichimura et al., 2000, 2000). The closure of the isolation membrane and the recruitment of the SNARE protein syntaxin 17 (STX17) completes autophagosome maturation. Finally, fusion of completed autophagosomes with lysosomes is mediated by interactions between STX17, SNAP29 and VAMP8 as well as YKT6, SNAP29 and STX7 (Itakura et al., 2012; Matsui et al., 2018). The homotypic fusion and vacuole protein sorting (HOPS) tethering complex and several other accessory molecules, such as BRUCE and PLEKHM1 promote these fusion events (Hegedűs et al., 2013; Takáts et al., 2013; Jiang et al., 2014; Takáts et al., 2014; Ebner et al., 2018; McEwan et al., 2015). During starvation, the level of autophagy can be drastically increased as a general protective response to compensate for the lack of nutrients (Mortimore and Schworer, 1977; Kopitz et al., 1990; Kuma et al., 2004). For this reason, autophagy was long considered to be a nonselective process that is responsible for the indiscriminate degradation of cytoplasmic components and for the recycling of macromolecules to promote cellular adaptation and survival. However, recent data have revealed that autophagy can selectively direct cargos to lysosomal degradation under nutrient-rich conditions by encapsulating cytoplasmic material in a selective and exclusive manner (reviewed in Zaffagnini and Martens, 2016). Furthermore, the finding that tissue-specific knockout of autophagy genes in mice leads to neurodegeneration or liver cancer (Hara et al., 2006; Komatsu et al., 2006; Takamura et al., 2011) and the fact that cells with defective autophagy are unable to clear certain intracellular pathogens (Randow and Youle, 2014; Deretic et al., 2013; Nakagawa et al., 2004) highlight the essential function of selective autophagy in maintaining cellular homeostasis. Selective autophagy can distinguish between diverse targets and different terms were coined to describe such processes. Among the most studied types of selective autophagy are mitophagy, xenophagy, pexophagy and aggrephagy, in other words the selective disposal of old and/or damaged mitochondria (Rogov et al., 2014; Kanki et al., 2009; Narendra et al., 2008; Novak et al., 2010; Okamoto et al., 2009), intracellular pathogens (Gutierrez et al., 2004; Nakagawa et al., 2004; Thurston et al., 2009; Yoshikawa et al., 2009; Zheng et al., 2009), surplus peroxisomes (Farré et al., 2008; Hutchins et al., 1999; Iwata et al., 2006) and the disposal of aberrant and misfolded cytosolic protein aggregates, respectively (Komatsu et al., 2007; Bjørkøy et al., 2005; Kirkin et al., 2009a; Pankiv et al., 2007; Szeto et al., 2006). In the following sections, we will focus on aggrephagy and, in particular, on the role of p62 in this process.

The p62 cargo receptor in aggrephagy

In contrast to non-selective bulk autophagy, selective autophagy requires (i) the specific recognition of the cargo material and (ii) its largely specific encapsulation by an isolation membrane (reviewed in Zaffagnini and Martens, 2016). The selective nature of autophagy is mediated by cargo receptors that recognise the cargo destined for degradation and link it to the growing isolation membrane through

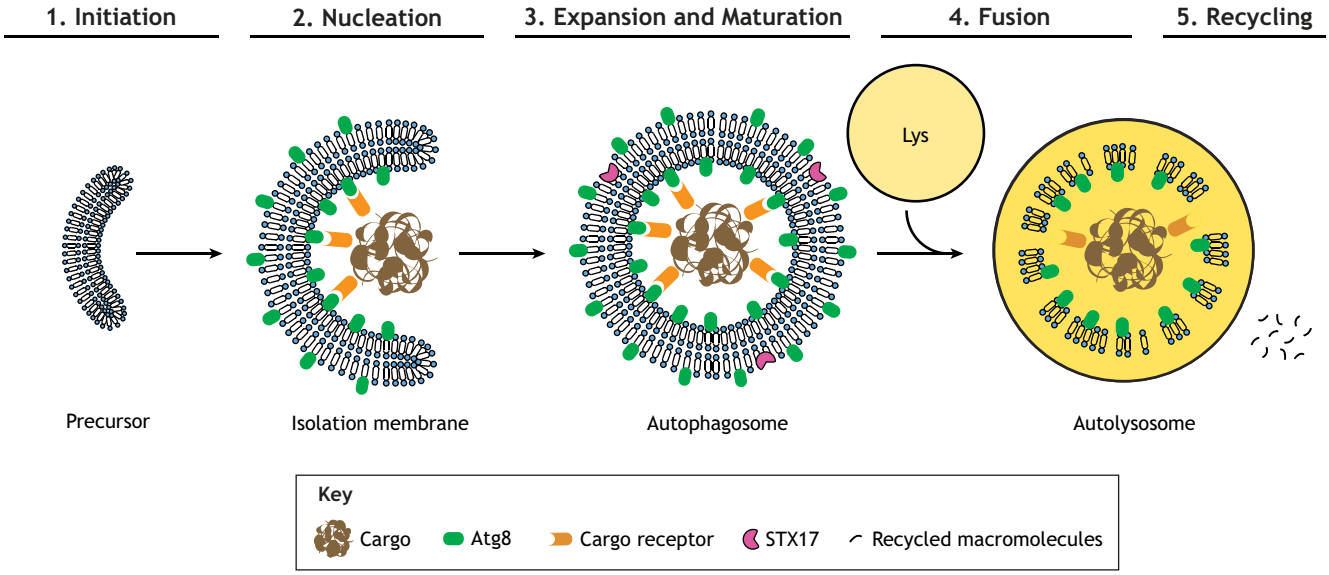

Fig. 1. Brief overview of autophagy. Autophagy maintains cellular homeostasis by mediating the degradation and recycling of cytoplasmic material. Autophagy can be divided in several hierarchical steps. 1) Initiation, which is controlled by the activation of the ULK complex. 2) Nucleation of an isolation membrane that engulfs the cargo material. 3) Vesicle expansion and maturation, which entails the expansion of the isolation membrane and its closure to form an autophagosome. 4) Fusion of the mature autophagosome with a lysosome, resulting in the degradation of the inner autophagosome membrane and the cargo. 5) Recycling of the macromolecules that result from the breakdown of the cargo and are transported back to the cytosol.

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their interaction with membrane associated Atg8 (Rogov et al., 2014; Zaffagnini and Martens, 2016) (Fig. 2). Many cargo receptors, including p62 and the related NBR1, as well as NDP52 (officially known as CALCOCO2) and optineurin (OPTN), recognize their cargos owing to their modification with ubiquitin (reviewed in Dikic, 2017).

Following its detection in ubiquitin-positive protein aggregates, p62 was the first mammalian cargo receptor identified for selective autophagy (Bjørkøy et al., 2005; Pankiv et al., 2007; Ichimura et al., 2008). Its importance for cellular quality control has been demonstrated by mutations in the p62 (*SQSTM1*) gene that are associated with several diseases, including amyotrophic lateral sclerosis (ALS), frontotemporal dementia (FD), neurodegeneration with ataxia, distal myopathy with rimmed vacuoles and Paget disease of the bone (Fecto et al., 2011; Goode et al., 2016; Haack et al., 2016; Rubino et al., 2012; Bucelli et al., 2015; Hocking et al., 2002). Studies in mammalian cells and whole organisms (mice and *Drosophila*) have shown that p62 is necessary for the formation of the ubiquitin-positive condensates and their subsequent degradation (Komatsu et al., 2007; Nezis et al., 2008). Accordingly, knockout of genes that mediate formation of autophagosomes and their fusion with lysosomes all result in a marked increase of p62-positive condensates (Bartlett et al., 2011). p62 contains a number of domains and motifs that mediate its function as cargo receptor during aggrephagy (Fig. 2). The N-terminal Phox and Bem1 (PB1) domain drives its oligomerization into long helical oligomers that have the appearance of filaments (Ciuffa et al., 2015; Lamark et al., 2003). The PB1 domain also mediates the interaction with other PB1-containing proteins including the NBR1 cargo receptor (Kirkin et al., 2009a; Lamark et al., 2003). The PB1 domain is followed by a ZZ type zinc finger (Znf) domain that binds N-terminally arginylated proteins – i.e. to which Arg residue(s) were added posttranslationally – as well as K48- and K63-linked ubiquitin chains (Zaffagnini et al., 2018; Cha-Molstad et al., 2015). The LC3-interacting region (LIR) is localized in the central part of the molecule within an intrinsically disordered region (Pankiv et al., 2007; Ichimura et al., 2008), and the C-terminal ubiquitin-associated (UBA) domain mediates its interaction with ubiquitin (Seibenhener et al., 2004).

Interactions between the UBA domain and ubiquitin, and the LIR motif and Atg8 are both weak. The low affinity of the UBA domain for ubiquitin is in part due to its homo-dimerization, which is mutually exclusive with ubiquitin binding (Long et al., 2010; Long et al., 2008). The affinity of the UBA domain for ubiquitin can be increased by phosphorylation at Ser403 through the casein kinase 2 (CK2) or TBK1 (Matsumoto et al., 2011, 2015; Pilli et al., 2012). The LIR motif of p62 has the sequence DDDWTHL and binds to the autophagy-related protein LC3B (officially known as MAP1LC3B) in the low micromolar range (Novak et al., 2010; Pankiv et al., 2007; Ichimura et al., 2008). Oligomerization mediated by its PB1 domain allows p62 to avidly and selectively bind to cargos, including misfolded proteins, on which ubiquitin is concentrated (Wurzer et al., 2015). A similar effect is seen for the interaction between the LIR and LC3B, where the PB1-mediated oligomerization results in a very high avidity interaction of p62 with membrane- concentrated LC3B, such that the off-rate of the interaction becomes extremely low (Wurzer et al., 2015). This tight interaction enables p62 to bend membranes around cargo material, a property that is conserved in the yeast Atg19 cargo receptor (Wurzer et al., 2015; Sawa-Makarska et al., 2014).

p62 is not merely required for the tethering of ubiquitylated proteins to the Atg8-coated isolation membrane but also for the

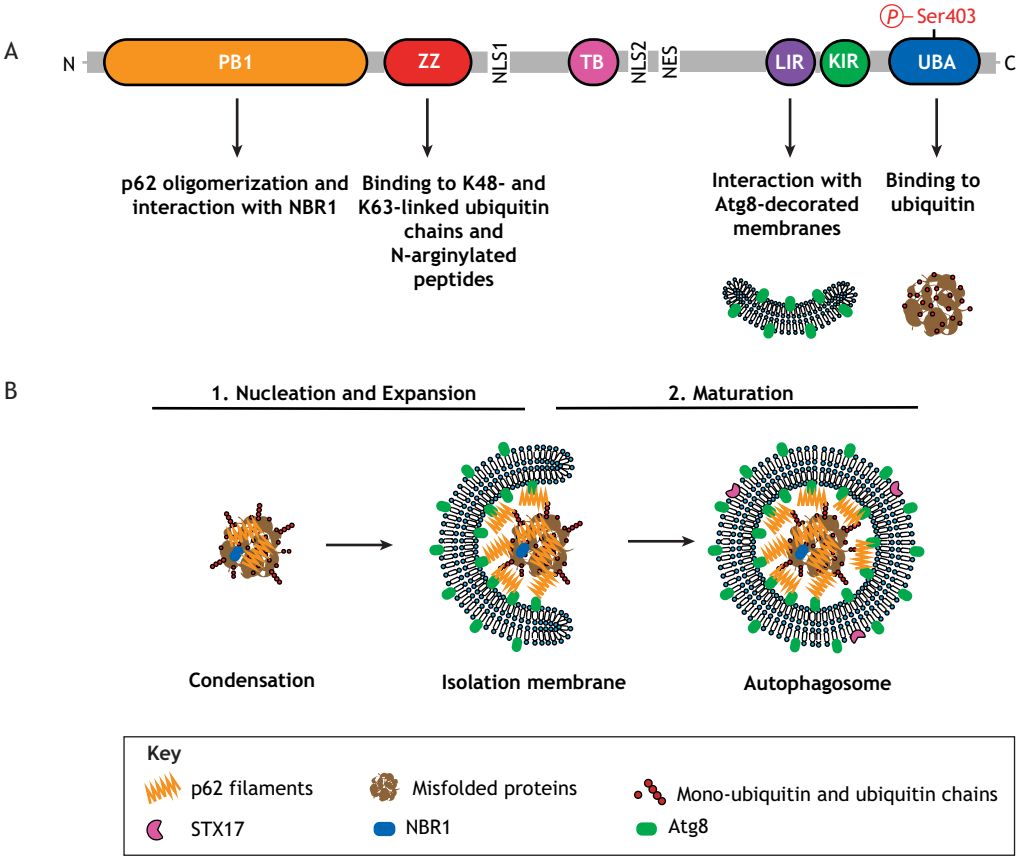

Fig. 2. p62 as a cargo receptor in selective autophagy. (A) Schematic of the domain structure of p62, which mediates its functions as indicated. PB1, Phox and Bem1 domain (aa 21–103); ZZ, ZZ type zinc-finger domain (aa 128–163); TB, TRAF6-binding domain (aa 225–250); LIR, LC3-interacting region (aa 338–341); KIR, Keap1-interacting region (aa 346–359); UBA, ubiquitin-associated domain (aa 386–440); NLS1 and 2, nuclear localization signals 1 and 2; NES, nuclear export signal. (B) Schematic of the mechanism on how cargo is tethered through p62 filaments to the isolation membrane. p62 filaments capture ubiquitylated substrates and link them to the Atg8-decorated isolation membrane to mediate their incorporation into autophagosomes. The NBR1 cargo receptor presumably aids p62 in this process.

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preceding condensation of these proteins into larger structures that subsequently become targets for autophagy. How do the different biochemical activities act together to mediate cargo condensation and isolation membrane tethering, and how can this be coordinated with the activity of the UPS and the autophagy machinery? A number of exciting recent discoveries have given us fascinating clues about these processes and their crosstalk, and are discussed below.

p62-mediated phase separations as nucleators for autophagosomes

It is becoming increasingly clear that many subcellular structures and compartments are the result of phase-separation reactions that condensate the interacting molecules. These condensates can be the result of low affinity, but multivalent interactions and individual molecules can be highly mobile within them (Box 1) (Banani et al., 2017; Shin and Brangwynne, 2017).

When cells are exposed to proteotoxic stress, such as the inhibition of the proteasome, interference with productive translation or the inhibition of chaperones, ubiquitin-positive proteins are concentrated in micrometer-sized condensates (Bjørkøy et al., 2005). Interestingly, the formation of these condensates is largely dependent on p62, since its depletion results in a more dispersed distribution of the ubiquitylated proteins in cells (Demishtein et al., 2017; Bjørkøy et al., 2005; Kageyama et al., 2014). The condensation-promoting activity of p62 requires its PB1 and UBA domains, suggesting that the condensates are the result of the interaction of the ubiquitylated substrates with oligomeric, probably filamentous, p62 (Bjørkøy et al., 2005; Ciuffa et al., 2015; Seibenhener et al., 2004; Zaffagnini et al., 2018). Two recent studies, including one from our group, demonstrated that p62 is entirely sufficient to phase separate ubiquitylated proteins into micrometer-sized condensates *in vitro* (Sun et al., 2018; Zaffagnini et al., 2018). Analogous to the situation in cells, this required the ability of p62 to oligomerize and to bind ubiquitin. The phase separation also required the presence of two or more substrate-attached ubiquitin chains that contain three or more ubiquitins, or very long free ubiquitin chains (Sun et al., 2018; Zaffagnini et al., 2018). On the basis of our electron microscopy data, we suggested that the formation of condensates is the result of p62 filaments that are crosslinked by the substrate (Zaffagnini et al., 2018).

Box 1. Cellular phase-separations

Numerous cellular organelles – such as the nucleolus, P granules, stress granules and PML bodies – are stable entities even though they are not bound by membranes. It has become clear that many of these membrane-less organelles are the result of the phase separation reactions that mediate the condensation of biomolecules, such as proteins, RNA or DNA into larger assemblies (Banani et al., 2017; Shin and Brangwynne, 2017; Brangwynne et al., 2011, 2009). It has emerged that not only organelles but also the formation of more transient cellular condensates, such as signaling puncta, is based on phase separation reactions (Li et al., 2012). Within these condensates, biomolecules can display different mobility, giving various condensates distinct physical properties. Condensates resulting from liquid–liquid phase separations show high mobility of the macromolecules that are concentrated within these structures (Banani et al., 2017; Shin and Brangwynne, 2017). While the exact molecular mechanism(s) underlying the formation of liquid-liquid phase separation are still not entirely clear, it appears that this phenomenon is often based on the interaction of polymers that undergo multivalent, low affinity interactions with each other (Banani et al., 2016). The presence of unstructured regions containing aromatic site chains also promotes liquid–liquid phase separation (Pak et al., 2016). Intriguingly, the filamentous oligomers of p62 and ubiquitin chains have exactly these properties. On the one hand, the ubiquitin-binding UBA domain of p62 binds ubiquitin with micromolar affinity and is linked to the rest of the p62 protein through a long unstructured region. Ubiquitin chains, on the other hand, harbor multiple interaction sites for p62.

Potential regulators of p62-ubiquitin phase separation reactions

Substrates modified with M1-linked (where the N-terminus of ubiquitin is linked to the C-terminal glycine of another ubiquitin), K48-linked and K63-linked ubiquitin chains are all able to trigger phase separation *in vitro* (Sun et al., 2018; Zaffagnini et al., 2018). p62 binds stronger to M1-linked and K63-linked chains compared to K48-linked chains (Long et al., 2008; Wurzer et al., 2015; Seibenhener et al., 2004). Consistently, K48-linked chains were less efficient in triggering phase separation, perhaps preventing p62 from interfering with proteasomal activity under normal conditions. In fact, we found that the formation of condensates *in vitro* is highly dependent on the concentration of the ubiquitylated substrate (Zaffagnini et al., 2018). Taken together, these recent results suggest that, in principle, the substrates for p62 are not fundamentally different from those of the UPS but, rather, that their increased concentration, resulting for example from UPS overload, triggers the p62- mediated phase separation. Indeed, several other lines of evidence suggest that the UPS communicates with the p62 system (Fig. 3). High concentrations of free mono-ubiquitin inhibit condensation, probably by competitively interfering with p62 substrate-binding by interacting with the UBA domain (Zaffagnini et al., 2018). The levels of free monoubiquitin drop substantially when the proteasome is blocked while, at the same time, substrate-attached ubiquitin chains accumulate (Kaiser et al., 2011). Thus, proteasome inhibition decreases the concentration of an inhibitor of the p62–ubiquitin phase separation while, at the same time, it increases the concentration of substrates triggering the reaction. Furthermore, free K63- and, especially, K48-chains but not M1-linked chains inhibit the phase separation (Zaffagnini et al., 2018). Free ubiquitin chains are generated at the proteasome where, at least in yeast, the deubiquitinase Rpn11 cleaves off the ubiquitin chains from the substrate at the base (Lee et al., 2011, Finley, 2009). This activity has been linked to the proteolytic activity of proteasomes and it is, therefore, possible that the levels of free ubiquitin chains signal proteasomal activity to the p62 system (Fig. 3). The mechanistic basis for how free K48- and K63-linked ubiquitin chains inhibit phase separation is currently unclear. However, this effect might be related to their ability to disassemble p62 oligomers owing to their interaction with the zinc-finger domain of p62, which is located close to the PB1 domain (Zaffagnini et al., 2018) (Fig. 2). Therefore, they might sterically interfere with oligomerization and, consequently, with phase separation as shorter p62 filaments are less efficient to elicit the reaction. In general, little is known about the regulation of p62 filament length in cells and this will be an important topic for future research.

In addition, it has been shown that the zinc-finger domain binds N-terminally arginylated proteins (Cha-Molstad et al., 2015, 2017, Yoo et al., 2018). Arginylatied N-termini are N-degrons that trigger rapid substrate ubiquitylation and degradation by the proteasome (Bachmair et al., 1986; Tasaki et al., 2012). Therefore, accumulation of N-terminally arginylated proteins might signal proteasomal overload to p62. Binding of N-terminally arginylated proteins to the zinc finger enhances oligomerization of p62 (Cha-Molstad et al., 2015, 2017; Yoo et al., 2018), which, in turn, should facilitate phase separation of p62 and its ubiquitylated substrates. An interesting but unanswered question is whether p62 is able to simultaneously bind arginylated N-termini and ubiquitylated substrates.

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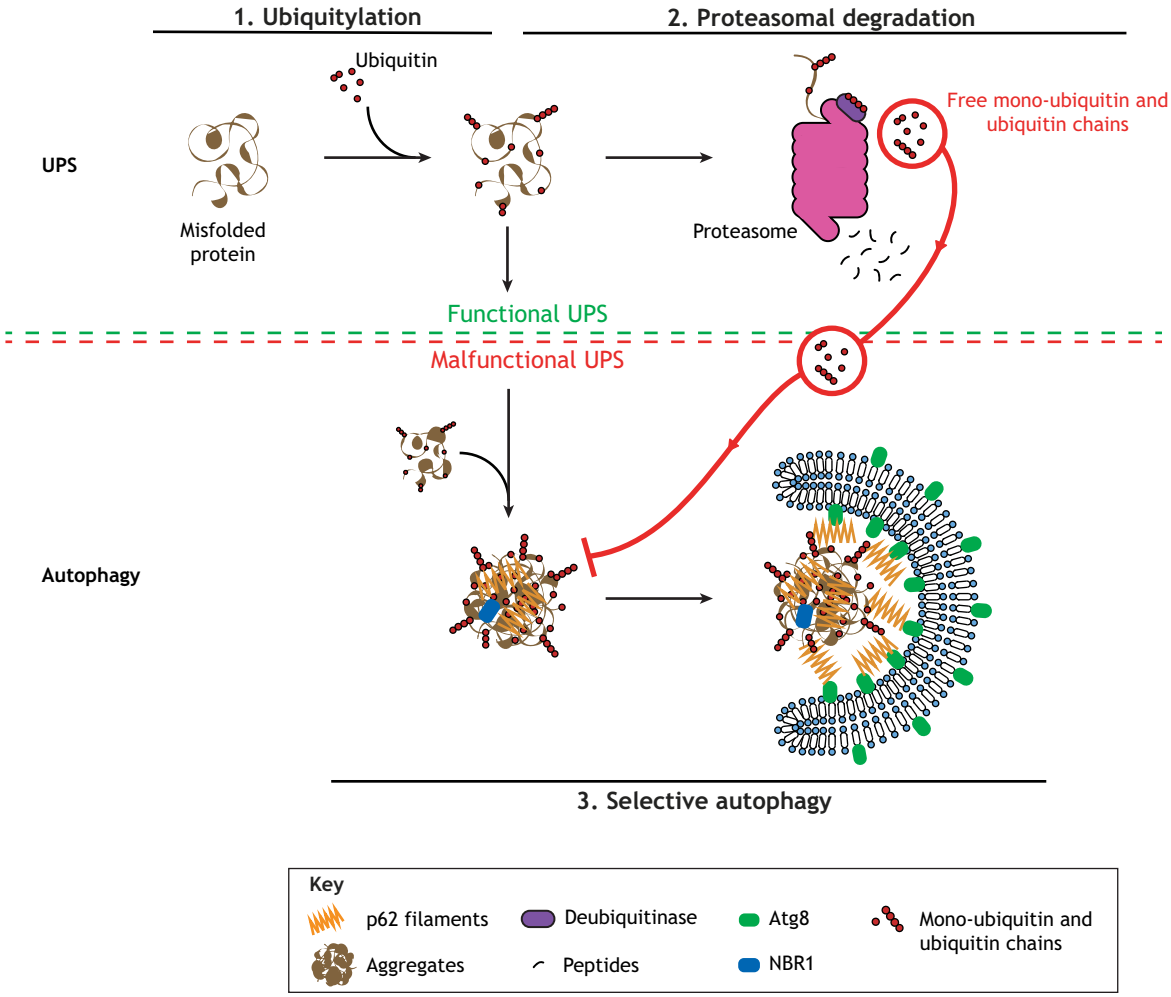

Fig. 3. Crosstalk between the UPS and selective autophagy. Under physiological conditions, misfolded and ubiquitylated proteins (1) are targeted mainly to the proteasome for their degradation (2). In response to proteotoxic stress and as the consequence of proteasome overload or malfunction, misfolded proteins accumulate. In this situation, selective autophagy (3) serves as a backup mechanism and degrades these proteins. p62 filaments act at the intersection between UPS and autophagy by sensing the accumulation of ubiquitylated substrates, and mediate their phase separation into larger condensates. p62 oligomerization is a crucial step in this process that, *in vitro*, is inhibited by free Lys48 (K48)- and K63-linked ubiquitin chains. Free monoubiquitin also inhibits phase separation, probably by binding to the UBA domain of p62. Since free ubiquitin chains and monoubiquitin are generated by active proteasomes, this would provide a mechanism by which the selective autophagy of proteins and protein aggregates can be inhibited by the UPS.

Ubiquitin-binding and oligomerization of p62 are also the subject of regulation through posttranslational modification. Phosphorylation of Ser403 in the UBA domain by TBK1 or casein kinase 2 increases its affinity for ubiquitin and, consequently, the formation of p62–ubiquitin condensates *in vitro* and in cells (Matsumoto et al., 2011, 2015; Zaffagnini et al., 2018; Pilli et al., 2012). Phosphorylation of Ser409 in the UBA domain by ULK1 occurs upon proteotoxic stress and increases the binding to ubiquitin by destabilizing the UBA-dimer interface (Lim et al., 2015). Additionally, the UBA domain is subject to ubiquitylation at Lys 420; and this modification increases its ability to form condensates, probably because it interferes with the inhibitory homodimerization of the UBA domain, thereby activating ubiquitin binding (Peng et al., 2017; Lee et al., 2017). In contrast, ubiquitylation of Lys 7 in the PB1 domain negatively regulates oligomerization and cargo sequestration (Pan et al., 2016). The ubiquitylation of p62 may be affected by the overall levels of free ubiquitin in the cell, which decreases upon proteasome inhibition. It has also been shown that, upon oxidative stress, oxidization of Cys105 and Cys113, which are located in the linker region between the PB1 domain and the zinc-finger domain, enhances oligomerization of p62 and the formation of condensates in cells (Carroll et al., 2018).

In addition to the role of p62 in substrate condensation and isolation membrane tethering in autophagy, p62 has been suggested to be a direct adaptor for the recruitment of substrates to the proteasome in the cytoplasm (Seibenhener et al., 2004). Furthermore, p62 contains a nuclear localization signal (NLS) and a nuclear export signal (NES) and shuttles between the nucleus and the cytoplasm. p62 has not only been suggested to export polyubiquitylated substrates from the nucleus for their degradation by autophagy in the cytoplasm, but also to attach ubiquitylated proteins to the proteasome in the nucleus (Hewitt et al., 2016; Pankiv et al., 2010). Moreover, there are indications that p62 mediates the degradation of proteasomes by autophagy upon starvation (Cohen-Kaplan et al., 2016).

Although the p62–ubiquitin condensates were for a long time considered rather passive aggregates that become linked to the autophagosomal membrane and are subsequently degraded, this view has changed substantially by now. When the *in vitro* reconstituted p62–ubiquitin condensates were analyzed by

fluorescence recovery after photobleaching (FRAP), it turned out that, surprisingly, the ubiquitylated substrates showed fast recovery and, by implication, high mobility within the condensates and considerable exchange with the material in solution (Sun et al., 2018; Zaffagnini et al., 2018). In contrast, p62 displayed very low recovery, demonstrating that it is stably associated with the condensates (Sun et al., 2018; Zaffagnini et al., 2018). Thus, although the formation of condensates is dependent on p62 and ubiquitin, these two interaction partners show a strikingly different behavior within the structures. The reason for the different mobility might be that, owing to their size, the p62 filaments have a much lower diffusion coefficient. In addition, while individual UBA–ubiquitin interactions can be highly transient – which allows the ubiquitylated proteins to diffuse, the p62 filaments have a number of binding sites and, thus, can be fixed because they are engaged in multiple interactions between UBA domains and ubiquitin at any given time. In cells, the situation might be even more complex. Endogenously tagged p62 showed a higher mobility in condensates in cells compared to condensates formed *in vitro* but their FRAP recovery was still relatively low (Zaffagnini et al., 2018). The situation for the ubiquitylated substrates is far less clear because, until now, no imaging experiments with endogenously ubiquitylated substrates have been conducted. Because some of them might be larger and more aggregated than the proteins used for the *in vitro* experiments it is, therefore, possible that their mobility is lower *in vivo* than *in vitro*. However, the condensates *in vivo* might undergo constant ATP-dependent remodeling of the ubiquitin chains by ubiquitin ligases and deubiquitinases (DUBs), chaperones or disaggregases, thereby increasing the mobility of the components in the condensates. The same factors might also regulate the stability and lifetime of the condensates in the cytoplasm. In cells, the p62 condensates have been observed by live cell imaging to undergo fusion followed by relaxation into a spherical shape (Sun et al., 2018). This observation is consistent with a liquid-like behavior of the condensates and, thus, with high mobility of the components within them.

A further aspect is that, *in vivo*, ubiquitin-chain remodeling processes or other ubiquitin-chain types – such as K11- and K33-linked ubiquitin chains, branched ubiquitin chains or heterotypic ubiquitin chains composed of multiple ubiquitin chain linkage types – may be additionally required or regulate efficient phase separation (Nibe et al., 2018; Yau et al., 2017; Meyer and Rape, 2014). As many types of ubiquitin chain have been found to be enriched in insoluble inclusions of autophagy-deficient mice, the relative contribution of specific ubiquitin chains to substrate condensation in cells has still to be elucidated (Riley et al., 2010). Because certain types of ubiquitin chain have a higher affinity for p62 it would, for example, be possible that substrates are specifically targeted to p62 by their modification with K63-linked chains, whereas substrates modified with K48-linked chains are only accepted when they accumulate owing to proteasomal overload.

Thus, multiple connections between aggrephagy and the UPS exist. These include the use of ubiquitin in order to mark the substrate, the recognition of N-terminal arginylation and the fact that p62 can act as an adaptor for the proteasome and as autophagic cargo receptor (Kirkin et al., 2009b; Cha-Molstad et al., 2015; Seibenhener et al., 2004; Babu et al., 2005; Bjørkøy et al., 2005). Furthermore, at least in plants and yeast, proteasomes themselves can become substrates for autophagy (Marshall et al., 2015, 2016; Waite et al., 2016). Additionally, the level autophagy proteins, including p62, can be regulated by the UPS (Platta et al., 2012; Myeku and Figueiredo-Pereira, 2011).

p62–ubiquitin condensates as substrates for autophagy

At least some of the p62–ubiquitin condensates become substrates for autophagy. Indeed, it has been demonstrated that p62 is required to trigger autophagosome formation upon proteotoxic stress, proteasome inhibition and oxidative stress (Carroll et al., 2018; Demishtein et al., 2017; Bjørkøy et al., 2005; Pankiv et al., 2007;

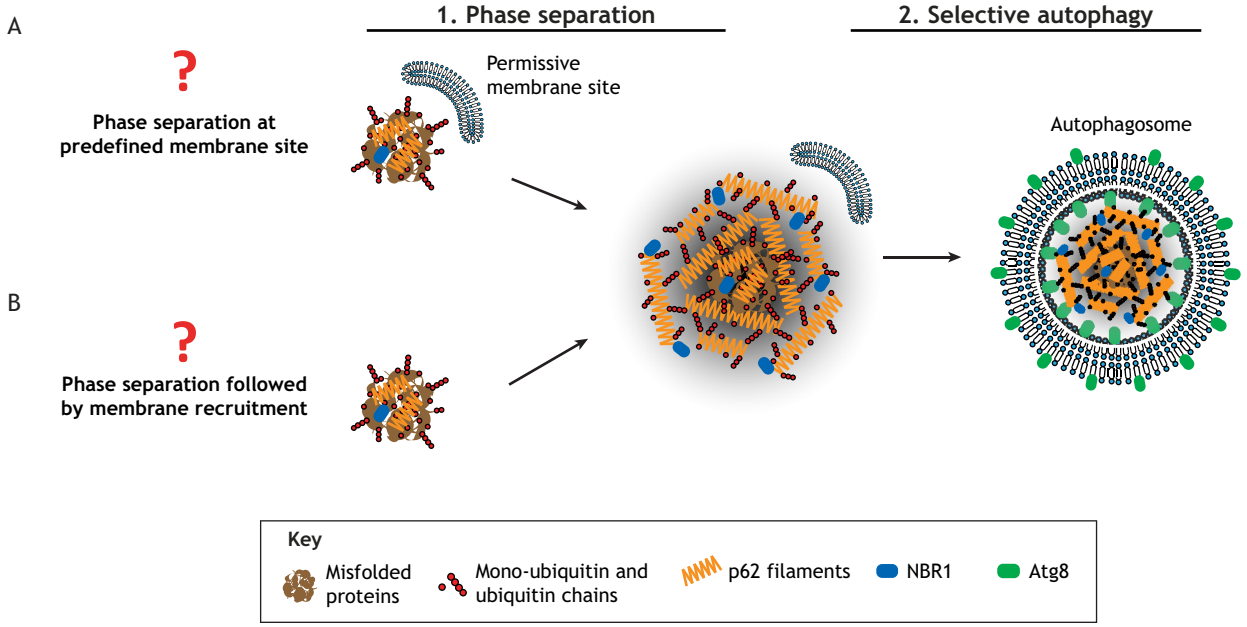

Fig. 4. p62 phase separation in selective autophagy. Degradation of p62–ubiquitin condensates that have formed as a result of phase separation reactions. It is currently unclear whether the phase separation reaction occurs at (A) predefined, membrane proximal sites that later are transformed into autophagosomes or whether the phase separation of p62 and ubiquitylated substrates can occur (B) anywhere in the cells followed by their recruitment to a membrane site that allows the formation of autophagosomes.

Babu et al., 2005). However, it was suggested that the condensates function to sequester ubiquitylated proteins in neuroblastoma cells (Sha et al., 2018). How substrate sequestration and autophagosome formation are coordinated is an important open question (Fig. 4). In cells, these activities are modulated by additional factors including ALFY, WDR81, Huntingtin and the cargo receptor NBR1 (Clausen et al., 2010; Filimonenko et al., 2010; Rui et al., 2015; Liu et al., 2017), which promote p62-mediated substrate condensation and also interact with the autophagy machinery. Among those, NBR1 directly interacts with p62 through its PB1 domain and, *in vitro,* has a direct stimulatory effect on substrate condensation by p62 (Zaffagnini et al., 2018). p62–ubiquitin condensates formed *in vitro* have also been shown to directly recruit LC3B, indicating that p62 is able to interact with LC3B when engaged in the phase separation reaction (Sun et al., 2018; Zaffagnini et al., 2018. Interestingly, the addition of LC3B to phase separation reactions slows down condensation (Zaffagnini et al., 2018). This effect is likely to be triggered by LC3B-mediated masking of the LIR motif of p62, which is required for efficient phase separation (Zaffagnini et al., 2018). The mechanistic basis for the role of the LIR motif in cargo condensation is currently unclear but this observation suggests coordination of substrate condensation and autophagosome formation, such that the recruitment of the autophagy machinery slows down cargo condensation. It is further possible that some structural rearrangement of the p62 filaments occurs when the Atg8-decorated isolation membrane forms at the condensates, such that p62 can efficiently link the condensates to it.

p62 has also been demonstrated to mediate the autophagic degradation of stress granules, which also are condensates originating from phase separation reactions (Buchan et al., 2013; Molliex et al., 2015). In contrast to aggrephagy, these structures form in a p62 independent manner and it is, therefore, unclear whether the coordination of this phase separation reaction and autophagy machinery recruitment follows similar principles.

It is becoming increasingly clear that the cargo material has an active role in the induction and formation of autophagosomes in selective, starvation-independent autophagy (Zaffagnini and Martens, 2016). This activity is conferred by the cargo receptors upon recognition of their cargo. For example, in *S. cerevisiae*, the Atg19 cargo receptor recruits the scaffold protein Atg11 and the Atg12–Atg5–Atg16 complex to the precursor amminopeptidase I (prApe1), cargo in the cytoplasm-to-vacuole targeting (Cvt) pathway (Fracchiolla et al., 2016; Kamber et al., 2015; Shintani et al., 2002; Torggler et al., 2016). Optineurin and NDP52 recruit the autophagy machinery to damaged mitochondria in mammalian cells (Lazarou et al., 2015). Furthermore, ER proteins have been shown to recruit the autophagy machinery through the Atg11 and/or FIP200 (officially known as RB1CC1) scaffold proteins (Khaminets et al., 2015; Mochida et al., 2015; Smith et al., 2018). In aggrephagy, the ALFY protein (officially known as WDFY3) has been shown to bind ATG5 and phosphatidylinositol 3-phosphate (PI3P) (Filimonenko et al., 2010) and might, therefore, promote isolation membrane elongation, whereas Huntingtin interacts with ULK1, which is required for autophagosome initiation (Rui et al., 2015).

However, with regard to p62 as a cargo receptor, important questions remain. For instance, it is unclear whether p62 itself or as a main component of the condensates can also interact with the upstream autophagy machinery. It is also unknown whether p62–ubiquitin condensates are formed at predefined sites that provide membranes or lipids that are permissive for autophagosome formation (Fig. 4A), or whether the membrane source is recruited to the condensates after their formation (Fig. 4B).

Future Perspectives

It has become clear that the UPS and autophagy are the main systems for the degradation of misfolded proteins in the cytoplasm. Moreover, it has now been generally acknowledged that the two systems are highly interlinked and that they use similar signals on their targets. Although it appears that there is no single, specific signal that specifically targets substrates to either the UPS or autophagy, we still do not fully understand how the decision about the fate of the substrate is made. A key factor in this regard is the ability of p62 to phase separate ubiquitylated proteins into larger condensates that subsequently become targets for autophagy. It remains unclear how cargo condensation and the recruitment of the autophagy machinery are mechanistically and temporally linked. Furthermore, if – as has been suggested – p62–ubiquitin condensates additionally function to sequester misfolded proteins in the cytoplasm rather than mediate their immediate degradation, how is the switch from sequestration to recruitment to the autophagy machinery regulated and how can it be coupled to the activity of the UPS? With regard to p62-mediated phase transition, it is possible that it also plays a role in other forms of selective autophagy that are different from aggrephagy – such as xenophagy or mitophagy; perhaps by aiding the concentration of autophagy proteins at the cargo. Given these important questions, exciting times are ahead for the field of research.

Acknowledgements

We thank Andreas Bachmair for comments on the manuscript.

Competing interests

The authors declare no competing or financial interests.

Funding

S.M. is supported by an European Research Council (ERC) grant (grant number: 646653) and by the Austrian Science Fund (FWF) (grant numbers: P30401-B21 and W1261).

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