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Current Opinion in Cell Biology

# Don't forget to be picky – selective autophagy of protein aggregates in neurodegenerative diseases

Anne Simonsen[a,b] and Thomas Wollert[c]

**Abstract**

The homeostasis of cells depends on the selective degradation of damaged or superfluous cellular components. Autophagy is the major pathway that recognizes such components, sequesters them in *de novo* formed autophagosomes and delivers them to lysosomes for degradation. The recognition of specific cargo and the biogenesis of autophagosomes involve a dedicated machinery of autophagy related (ATG) proteins. Intense research over the past decades has revealed insights into the function of autophagy proteins and mechanisms that govern cargo recognition. Other aspects including the molecular mechanisms involved in the onset of human diseases are less well understood. However, autophagic dysfunctions, caused by age related decline in autophagy or mutations in ATG proteins, are directly related to a large number of human pathologies including neurodegenerative disorders. Here, we review most recent discoveries and breakthroughs in selective autophagy and its relationship to neurodegeneration.

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## Introduction

Autophagy was discovered more than 60 years ago as a mechanism by which the cell captures cytoplasm in double membrane vesicles, termed autophagosomes, upon starvation. It took much longer to reveal that autophagy also degrades damaged or superfluous cytoplasmic components, including protein aggregates and mitochondria, in a highly selective manner [1]. An important milestone was the discovery of a direct relationship of selective autophagy and human diseases, including cancer and neurodegeneration [2]. Notably, proteins that are prone to aggregate into amyloid-like filaments, including Huntingtin, α-Synuclein, Tau and the Amyloid-Precursor-Protein fragment Aβ, are causative agents for neurodegenerative diseases and also substrates of selective autophagy [3]. Mechanistic studies of the formation of such aggregates and their degradation by selective autophagy (aggrephagy) have revealed the importance of membrane-less organelles (MLO) and liquid−liquid phase separation (LLPS) [4]. Moreover, autophagy receptors have been found to regulate the formation of LLPs and initiate selective autophagy by recruiting core autophagy components to cargo. The goal of this review is to summarize most recent advances in our understanding of how selective autophagy counteracts amyloid-like aggregation and neurodegeneration.

## Recognition of autophagic substrates by the autophagy machinery

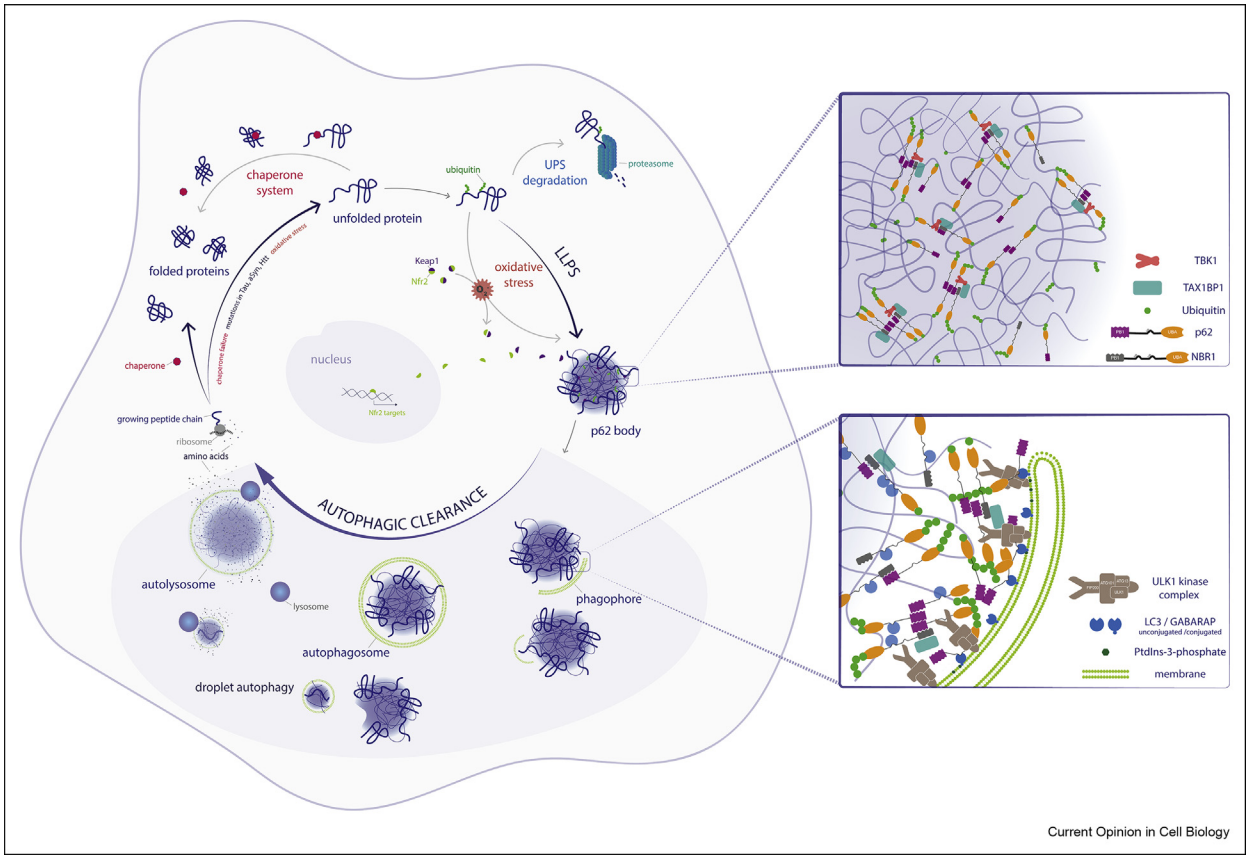
Selective autophagy is orchestrated by a dedicated machinery (Box 1) that assembles in a spatiotemporally well-defined order to enable sequestration of cytoplasmic cargo by a phagophore membrane. The process starts with the recruitment of autophagy receptors to cargo, which can occur in an ubiquitin (Ub) dependent or independent manner [1]. Examples of Ub-dependent autophagy receptors include the Sequestosome-like receptors SQSTM1/p62, NBR1, OPTN, NDP52 and well as human CUET family proteins [5]. These receptors possess canonical Ub-binding motifs such as UBA, UBZ and UBAN domains and their recruitment depends on cargo ubiquitination by upstream acting E3-ligases [6]. Ubiquitin-independent autophagy receptors are often integral membrane proteins being activated in response to organellar stress or dysfunction. Examples include mitochondrial receptors BNIP3 and BNIP3L or ER receptors FAM134B and RTNL3 [1]. Common to all autophagy receptors is the presence of an LC3 interacting region (LIR) that mediates their interaction with ATG8 proteins to promote tethering of cargo to the phagophore membrane by multivalent interactions of

receptors and membrane-bound ATG8 proteins [7]. The autophagy-related kinases ULK1 and TBK1 can phosphorylate residues within and surrounding the core LIR motif to regulate the affinity and specificity of cargo receptors for ATG8 proteins [8,9]. In addition to their interaction with cargo and ATG8 proteins, several autophagy receptors have recently been found to facilitate recruitment of the early autophagy machinery (Box 1), leading to *de novo* phagophore formation and LC3 lipidation in a cargo-dependent manner [10]. However, the specificity and redundancy of cargo receptors in different types of selective autophagy are just starting to be unraveled [11].

## Role of cargo receptors in liquid–liquid phase separation and aggrephagy

Misfolded ubiquitinated proteins are substrates for proteasomal degradation, but can also form phase-separated condensates that become substrates for selective autophagy in a process involving cargo receptors (Figure 1). p62 is key to this process, as it contains a PB1 domain that promotes its self-polymerization to form filamentous polymers [12–14] that again can assemble into gel-like liquid droplets [15], referred to as p62 bodies or p62 gels. Elegant in vitro studies showed that p62 is sufficient to phase separate ubiquitinated proteins into condensates [16,17]. p62 recruits NBR1 via PB1-PB1 interactions, which further promotes condensate formation by high affinity binding to ubiquitin and recruitment of TAX1BP1 [18]. These three cargo receptors are all able to interact with the ULK1 complex subunit FIP200, leading to recruitment of the downstream autophagy machinery and *de novo* phagophore formation around the p62 bodies. This may happen independently of ATG8 lipidation [19], although an LIR-dependent interaction of p62 with ATG8 proteins was found to facilitate engulfment of whole p62-gels by autophagosomes [20]. TAX1BP1 further recruits the kinase TBK1 to p62 bodies, leading to phosphorylation of p62 to enhance its binding to ubiquitin [21].

**Figure 1**




Aggrephagy facilitates clearance of misfolded ubiquitinated proteins. Environmental and cellular stress can cause protein misfolding and their ubiquitination. The autophagy receptor protein p62 contains a self-polymerizing PB1 domain and an UBA domain that together with the autophagy receptors NBR1 and TAX1BP1 and the kinase TBK1 mediate liquid–liquid phase separation of ubiquitinated proteins to form p62 bodies. These autophagy receptors further promote phagophore formation by recruitment of the ULK complex subunit FIP200 and ATG8 proteins, leading to autophagic clearance of parts of or whole p62 bodies.

It is not clear what triggers the oligomerization of p62, but it has been found that oxidative stress leads to oxidation of p62 at cysteine residues (105 and 1113), enhancing its oligomerization and condensation [22]. The ability of p62 to oligomerize and phase separate can also be regulated by chaperones and post-translational modifications of its UBA and PB1 domain. Expression of the prefoldin-like chaperone UXT promoted p62 clustering and autophagic clearance of p62 bodies and delayed SOD1(A4V)-induced degeneration of motor neurons in a *Xenopus* model system [23]. It has also been suggested that the type of ubiquitin chain linked to cargo regulates p62 oligomerization and aggrephagy. Importantly, p62 oligomerization is physiologically important. Upon oxidative stress, the p62-interacting protein Keap1 is recruited to p62 gels, leading to activation of the transcription factor Nrf2 important for the anti-oxidative response [20]. This is further stimulated by NBR1, which is induced in response to oxidative stress and mediates p62 body formation [24]. Impaired turnover of p62-gels leads to *in vivo* Nrf2 hyperactivation and liver injury [20]. Moreover, several mutations of TBK1 or p62 linked to amyotrophic lateral sclerosis (ALS) and fronto-temporal dementia (FTD) disrupt p62 oligomerization and its binding to ubiquitinated cargo, as well as the recruitment of KEAP1 to p62 bodies under oxidative stress, leading to formation of TDP-43 associated stress granules implicated in ALS-FTD neurotoxicity [22]. Moreover, premature lipofuscin accumulation and ubiquitin positive aggregates were detected in the brain of young TAX1BP1 knockout mice, showing *in vivo* relevance of aggrephagy [25].

A recent physical model shows that the surface tension of p62 bodies supports the formation of autophagosomal membrane sheets and that the bending of such sheets can lead to sequestration of a piece of a droplet or an entire droplet [26]. In line with this, aggrephagy cargo segregated by liquid–liquid phase separation in p62 bodies seems to be highly mobile and undergo dynamic exchange with the surrounding cytosol. The exact mechanisms involved in *de novo* phagophore biogenesis during aggrephagy still remains largely unknown, but knowledge from other types of selective autophagy indicates that autophagy receptors share some general functions, including receptor-mediated recruitment of FIP200 to the cargo. Similar to p62, NBR1 and TAX1BP, the cargo receptor NDP52 recruits FIP200 to ubiquitinated cargo [27]. Elegant structural characterization shows that NDP52 promotes membrane-binding of the ULK1 complex by allosteric regulation of the membrane-binding portion of the FIP200 coiled coil domain [28] and that its LIR domain directly interacts with the claw domain of FIP200 [29], indicating that FIP200 compete with ATG8 proteins for binding to autophagy receptors [30]. ATG8 proteins can also be recruited to cargo in a LIR-independent manner to further mediate ubiquitin-independent recruitment of cargo receptors via the LIR motif, thereby amplifying selective autophagy [31]. Another mitophagy receptor, OPTN, forms a complex with vesicles containing ATG9A [32], a lipid scramblase required for phagophore biogenesis [33]. The function of OPTN in Parkin-dependent mitophagy is regulated by its phosphorylation by both TBK1 and ULK1 complex activity [34]. The high structural similarity between the N-terminal domain of FIP200 and the dimerization domain of TBK1 suggest they may function in a similar manner [35]. In addition to ubiquitin, cargo-specific "eat me" signals such as mitochondrial NIPSNAP proteins [36] can contribute to recruitment of cargo specific receptors. Thus, complex multivalent interactions of autophagy receptors with several core autophagy proteins seem to drive *de novo* synthesis of autophagosomal membranes during selective autophagy [31].

Interestingly, phase separated condensates are not only formed by p62, but also by many amyloid-like proteins. Revealing how these condensates influence each other and whether co-condensation of p62 and cargo occurs is of particular interest, since phase condensates act as nucleation centers for the formation of amyloid-like fibrils that are the hallmark of neurodegeneration and proteinopathies [37].

## Membrane-less organelles in neurodegenerative diseases

Liquid–liquid phase separation (LLPS) is a fundamental physical property of macromolecules that is based on dehydration, that is unmixing of an aqueous solution by replacing macromolecule–water interactions by inter-macromolecular interactions [38]. Recent advances revealed that many cellular structures that have initially been described as puncta are in fact biological condensates or membrane-less organelles (MLO). Thus, LLPS emerges as a more general phenomenon that helps structuring the cellular content to facilitate mRNA processing, local translation, and chromatin compaction [39]. MLOs can also serve very distinct and specialized functions. Examples include neurons, which rely heavily on MLOs to structure pre- and postsynaptic densities and to regulate neural plasticity [40].

Most proteins that undergo spontaneous LLPS contain intrinsically disordered regions (IDR) of low complexity. The major driving force for the formation of MLOs are multivalent IDR–IDR, IDR-domain or IDR-nucleic acid interactions based on short linear binding motifs [41]. Posttranslational modifications or mutations within these binding regions can influence their physical properties, leading to a changed propensity to undergo LLPS with sometime detrimental consequences for cells. Tau represents a prominent and well characterized example [42]. Phosphorylation of Tau or the mutation of proline 301 to leucine are associated with the onset of Alzheimer's disease (AD), Parkinson's disease (PD) and

frontotemporal dementia with parkinsonism-17 (FTDP-17) in humans [43]. Both modifications trigger LLPS of Tau, which promotes the nucleation of Tau fibrils [44]. LLPS plays also an important role in the pathogenesis of Huntington's disease (HD), a neurodegenerative disease caused by poly-Q expansion in the exon 1 of Huntingtin. The poly-Q tract induces phase separation of Huntingtin and the resulting liquid droplets serve as nucleation centers for fibrillar Huntingtin [45].

## Autophagic clearance of disease related liquid phases and aggregates

Many neurodegenerative diseases are caused or accompanied by the formation of protein aggregates. The process usually starts in a restricted area of the brain from which aggregates spread in a prion-like manner to healthy neighboring cells [46]. The high connectivity of neural tissues facilitates spreading of toxic protein fibrils, which correlates with the progression of pathology [47]. Autophagy degrades toxic protein aggregates, counteracts fibrillization and limits the spread of toxic fibrils in neural tissue, indicating that autophagy plays an essential role in protecting neurons form accumulating toxic aggregates [48]. Accordingly, several mutations in autophagy factors are related to an increased risk of developing neurogenerative disorders (Table 1).

Although the molecular mechanisms remain to be established, autophagic clearance appears to be most efficient early in neurodegeneration since elongated fibrils can escape autophagy by interfering with the autophagy-lysosomal pathway [49]. How autophagy counteracts fibrilization of proteins is currently unknown, but recent experimental evidence suggests that LLPS is an important factor in this process [50].

Tau, the causative agent of tauopathies, is extensively regulated by posttranslational modifications that impact on its proteasomal and autophagic clearance and its tendency to form insoluble aggregates [51,52]. For example, phosphorylation of Tau at Ser214, Ser262 and Ser305 prevents its aggregation [53], while

**Table 1**

**Disease causing mutations in autophagy and cargo proteins. The table summarizes mutations that are directly related to the onset of neurodegenerative diseases. Data have been compiled using the Human Gene Mutation Database (Cooper et al., Institute of Medical Genetics, Cardiff University, Cardiff, UK) and The Human Variants Database Ganesan K, Kulandaisamy A, Binny Priya S, Gromiha MM (2019) HuVarBase: A human variant database with comprehensive information at gene and protein levels. PLoS ONE 14(1): e0210475.**

| Protein | Disease | Mutation |
|---|---|---|
| Tau | Frontotemporal dementia | R5H, E659V, L583V, G589V, L596K, P618S, S622N, K634M, V654M |
| | Pick's disease | K574T, K686I, S637F |
| | Parkinson's Disease | N613del |
| | Alzheimer's Disease | A90V, A152T |
| α-Synuclein | Parkinson's Disease | A30P, H50Q, G51T, A53T, N203S, S222L, K230E, M711V, A714T, R806H, K904E, S912R |
| | Lewy body dementia | E46K |
| APP | Alzheimer's Disease | D678N, E682K, K687Q, F690_V695del, A692G, E693del, E693G, T714A, T715A, I716F, V717I, T719N, M722K, L723P |
| Parkin | Parkinson's Disease | V15M, R33Q, R42H, A46P, K161N, M192L, C212Y, A230V, I236V, V148E, T240R, C253Y, N254S, R256C, D280N, G284R, C289G, Q311R, G328E, T351P, R366W, A405T, T415N, G430D, C431F, C418R, C441R, R455H |
| Pink1 | Parkinson's Disease | G30R, Q115L, A168P, A217D, A232V, H271Q, R279H, L308Q, G309D, T313M, A340T, L347P, Y431H, N521T, K526N |
| p62/SQSTM1 | Frontotemporal dementia/ALS | A16V, D80E, V90M, R107W, V153I, R212W, G219V, S226P, P228L, P232T, K238del, D258N, S318P,D329G, S370P, A381V,T430P |
| | Amyotrophic Lateral Sclerosis | M1K, R12del, E20del |
| | Alzheimer's Disease | P29S, D260A, L268V, R393W |
| WIPI4/WDR45 | Bata-propeller protein-associated neurodegeneration | M1I, V34M, L37S, N61K, N84G, L98P, F100S, L160R, deletions at various positions |
| OPTN | Amyotrophic Lateral Sclerosis | H3Y, P16A, S73L, R83C, A93P, A136V, V161M, K258N, T282P, V295P, Q314L, I451T, N478G, L494W, L500P, L557T, D564H, T567A, L568S, H571N |
| TBK1 | Amyotrophic Lateral Sclerosis | T4A, V23I, G26D, T31A, R47H, L94S, D118N, G121D, R143C, I207 T/V, G217R, G244V, I246T, R308N, T320I, H322R, R358 C/H/W, K401D, Y424E, R444N, M559R, M598V, M662T, R724C |
| | Frontotemporal dementia | T4A, G26D, R143C, G244V, T320I, I418V, M662T |
| | Alzheimer's Disease (late) | D534H |

phosphorylation at positions Ser202, Thr205 and Ser208 inhibits the Ub/proteasome system and promotes aggregation of Tau [54]. Moreover, hyperphosphorylated Tau has an increased tendency to phase separate and is hyperubiquitinated by the E3-ligase CHIP. Both mechanisms promote the formation of Tau fibrils, the building blocks for neurofibrillary tangles [55]. However, overexpressing CHIP in a Tau-BiFC mouse model, in which the progression of Tau oligomers can be spatiotemporally visualized based on the fluorescent protein [56], leads to a reduction of oligomeric Tau and phosphorylated Tau species in an autophagy dependent manner [57]. Since polyubiquitinated cargo stimulates LLPS of p62 [12] one could speculate that hyperphosphorylated and hyperubiquitinated Tau aggregates are included in p62 bodies to be degraded by selective autophagy. To what extent the formation of co-condensates influence the fate of Tau, notably whether Tau is degraded by autophagy or forms larger Tau-tangles, remains to be investigated.

**Box 1. The core autophagy machinery in selective autophagy.**

Local activation of the ULK kinase complex (consisting of ULK1/2, FIP200, ATG13 and ATG1010) is the earliest event upon initiation of autophagy by various specific and pathway dependent stimuli. ULK1 kinase activity is suppressed through its phosphorylation at Ser757 by mTOR1. Thus, inactivation of mTOR1, for example upon nitrogen starvation, leads to activation of ULK1, while glucose starvation activates AMPK, which activates ULK1 by phosphorylating Ser317 and Ser777 [58]. During selective autophagy, ULK1 needs to be activated although its activity is constantly suppressed by mTOR1. This is facilitated by cargo-bound autophagy receptors, which recruit the ULK1 complex through a direct interaction with its subunit FIP200 [27,30,59]. The ULK1 complex further promotes the recruitment of ATG9 vesicles and the PtdIns-3 kinase complex to cargo, leading to the nucleation of the phagophore membrane. ATG9 functions as a lipid scramblase to promote the translocation of phospholipids from the cytoplasmic to the luminal leaflet of the expanding phagophore membrane [33] and together with the lipid transfer protein ATG2 [60,61], being recruited to ER-associated membranes, it provides lipids and proteins needed for *de novo* autophagosome biogenesis [62]. Following its recruitment by the ULK1 kinase complex subunits ATG13 and ATG101, the PtdIns-3 kinase complex enriches the phagophore with PtdIns-3-phosphate which is recognized by the WD-domain containing protein WIPI2 [63]. The latter initiates the lipidation of ATG8 proteins to the phagophore by recruiting ATG16L1 [64]. The conjugation of ATG8 proteins to lipids is catalyzed by an autophagy specific Ub-like conjugation system, comprising the E1-like enzyme ATG7, E2-like ATG3 and the E3-like ligase ATG12–ATG5-ATG16L1 [65]. Based on their sequence, ATG8 proteins can be grouped into the GABARAP (Gamma-aminobutyric acid receptor-associated protein) and L3C (Microtubule-associated proteins 1A/1B light chain 3) subfamilies. ATG8 proteins fulfil various essential functions in autophagy. They tether cargo to phagophores by binding cargo receptors, promote the maturation of phagophores and coordinate fusion of autophagosomes with lysosomes [5,7,11], but are dispensable for some types of selective autophagy, including the autophagic degradation of mitochondria by mitophagy [19,66]. The final closure of the phagophore membrane is facilitated by the endosomal sorting complexes required for transport (ESCRT) machinery [67].

## Conclusion

The application of physical methods and concepts in cell biology has increased our understanding of fundamental biological processes tremendously. This is particularly true for the field of membrane trafficking and autophagy, where many phenomena and observations could only be explained by combining traditional cell biological and physical techniques. Liquid phase separation is a physical process that all of us know well from daily experience. However, its contribution to structure the complex cellular environment just begin to unfold. In contrast to membrane bound organelles, phase separated structures exchange components with the cytoplasm in a highly dynamic and much less constrained manner, providing another level of subcellular organization and regulation. Several processes in autophagy, including cargo sequestration as well as initiation and expansion of phagophores, involve LLPS. Understanding the consequences of perturbed phase separation or changes in the composition of phase separated structures in autophagy will be key to reveal insights into the origin of many human pathologies including neurodegeneration. Future studies of dynamic and structural aspects of phase separation in autophagy bear the potential to develop new therapeutical concepts to treat neurodegeneration and other human diseases.

## Conflict of interest statement

Nothing declared.

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