



# Causative Genes in Amyotrophic Lateral Sclerosis and Protein Degradation Pathways: a Link to Neurodegeneration

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## Abstract

Amyotrophic lateral sclerosis (ALS) is a disease caused by the degeneration of motor neurons (MNs) leading to progressive muscle weakness and atrophy. Several molecular pathways have been implicated, such as glutamate-mediated excitotoxicity, defects in cytoskeletal dynamics and axonal transport, disruption of RNA metabolism, and impairments in proteostasis. ALS is associated with protein accumulation in the cytoplasm of cells undergoing neurodegeneration, which is a hallmark of the disease. In this review, we focus on mechanisms of proteostasis, particularly protein degradation, and discuss how they are related to the genetics of ALS. Indeed, the genetic bases of the disease with the implication of more than 30 genes associated with familial ALS to date, together with the important increase in understanding of endoplasmic reticulum (ER) stress, proteasomal degradation, and autophagy, allow researchers to better understand the mechanisms underlying the selective death of motor neurons in ALS. It is clear that defects in proteostasis are involved in this type of cellular degeneration, but whether or not these mechanisms are primary causes or merely consequential remains to be clearly demonstrated. Novel cellular and animal models allowing chronic expression of mutant proteins, for example, are required. Further studies linking genetic discoveries in ALS to mechanisms of protein clearance will certainly be crucial in order to accelerate translational and clinical research towards new therapeutic targets and strategies.

**Keywords** Amyotrophic lateral sclerosis · Genes · Ubiquitin · Proteasome · Autophagy · Aggregates

## Abbreviations

MN	Motor neuron	C9ORF72	Chromosome 9 open reading frame 72
ALS	Amyotrophic lateral sclerosis	SOD1	Superoxide dismutase 1
UPS	Ubiquitin proteasome system	TARDBP	TAR DNA-binding protein
ERAD	Endoplasmic reticulum-associated protein degradation	FUS	Fused in sarcoma
CAP	Chaperone-assisted proteasomal degradation	OPTN	Optineurin
CMA	Chaperone-mediated autophagy	SQSTM1	p62/sequestosome1
CASA	Chaperone-assisted selective autophagy	VCP	Valosin-containing protein
Ub	Ubiquitin	UBQLN2	Ubiquilin 2
SUMO	Small ubiquitin-like modifier	VAPB	Vesicle-associated membrane protein-associated protein B/C
SG	Stress granules	TBK1	TANK-binding kinase 1
		FIG4	FIG4 phosphoinositide 5-phosphatase
		SIGMAR1	Sigma non-opioid intracellular receptor 1
		CCNF	Cyclin F
		PDIA	Protein disulfide isomerase family A member

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## Introduction

Amyotrophic lateral sclerosis (ALS) is the most frequent motor neuron disease with an incidence of 2 per 100,000 persons

per year and a prevalence of 5 per 100,000 people [1]. The mean age for ALS diagnosis is 64 years old. Signs of spasticity, resistance to movement, and brisk reflexes occur with upper motor neuron degeneration, while lower motor neurons damage include muscular weakness, muscular atrophy along with fasciculations. The consequences are progressive paralysis and difficulties in swallowing and respiration, leading to death in patients three to 5 years after the diagnosis. Ninety percent of cases are sporadic (SALS), while the other 10% comprise familial amyotrophic lateral sclerosis (FALS). ALS remains incurable, but genetic mutations implicated in the disease point out several impaired cellular and molecular mechanisms and, thus, provide clues for potential therapeutic strategies. These mechanisms include the abnormal response to oxidative stress, glutamate-mediated excitotoxicity, defects in cytoskeletal dynamics and axonal transport, and impaired protein homeostasis [2, 3].

Most neurodegenerative diseases are characterized by an accumulation of misfolded proteins and by the presence of protein aggregates in cells [4]. This is largely due to malfunctions in proteostasis. The term proteostasis refers to the biological pathways involved in the biogenesis of proteins including ribosomal translation, folding and trafficking, and protein degradation in cells. In ALS, the dysfunction of these mechanisms has been suspected for many years. In effect, post-mortem studies of ALS patients have shown protein aggregates in upper and lower motor neurons [5]. Many of these aggregates have been ubiquitin-positive, linking these histological observations to a mechanism of protein degradation, the ubiquitin proteasome system (UPS) [6, 7]. These aggregates have become a cornerstone of the disease. Different types of aggregates have been observed. Ubiquitinated aggregates are classified as either Lewy body-like hyaline inclusions or skein-like inclusions. These last inclusions appear as randomly oriented filaments covered by fine granules. In some other cases, ubiquitination is not present. These aggregates are called Bunina bodies, which are small, eosinophilic, and round hyaline inclusions [8]. In ALS, protein inclusions are mainly ubiquitinated and co-localized with the TDP-43 protein, as initially described in 2006 [9]. These cytoplasmic inclusions are present not only in motor neurons but also in astrocytes in ALS [10, 11]. Subsequently, mutations in the *TARDBP* gene encoding TDP-43 were described in FALS and SALS patients [12, 13]. The aggregation of several proteins in ALS has also been proposed to proceed via the formation of SGs. SGs are part of ribonucleoprotein (RNP) granules, which are cellular sites dedicated to RNA processing [14]. These structures, whose formation is favored by various types of stress, have no membrane and are visible under the light microscope. They contain several species and, in particular, RNA and ribonucleoproteins.

An increasing amount of misfolded or unfolded proteins is toxic for cells, generating stress mechanisms, such as the response to ER stress. These proteins must be efficiently removed

from cells by quality control mechanisms linked to protein degradation pathways. This is particularly true for neurons that are not able to dilute their cytoplasmic contents, including damaged substrates, through cellular division. These mechanisms and pathways also participate in a continuous turnover of proteins and release of amino acids after protein degradation. Certain steps of these degradation pathways are known to be downregulated due to decreased expression levels and activity with age, which pose concern for their implication in several neurodegenerative diseases, such as ALS [15, 16]. Therefore, it has been championed that the presence of protein aggregates in motor neurons and glial cells in FALS and SALS reflects dysfunctions in these quality control protein degradation pathways. The discoveries in ALS patients of mutations in several genes encoding proteins directly linked to these pathways support this hypothesis (Table 1).

In this review, we present defects in proteostasis with respect to ALS, focusing on disturbances in quality control/degradation of proteins as a central mechanism in ALS pathophysiology. We first describe two of the main protein degradation pathways in cells, the UPS and autophagy. Researchers have shown an increased interest in proteostasis studies pertaining to ALS since 1989, when the first article linking ubiquitin and ALS was published (Fig. 1). More than 30 genes associated with familial ALS have been described, 15 of which are directly involved in UPS and/or autophagy mechanisms that will be described further below. Other susceptible genes, which increase the risk of ALS, also play roles in UPS and/or autophagy.

## The Ubiquitin Proteasome System

The UPS was associated with protein degradation in the 1980s [33]. Ubiquitination of proteins in cells occurs through the coordinated activity of a unique ubiquitin activating enzyme (E1), a conjugating enzyme (E2), and a ligase (E3) [34]. E1 activates ubiquitin by an ATP-dependent mechanism. Ubiquitin (Ub) is then transferred to an E2 by a transthioleation reaction. We have identified 38 genes encoding E2 enzymes in the human genome [35]. E3 ligases determine the specificity of the ubiquitination process by two main mechanisms: a E3 recognizes the E2-Ub complex and transfers Ub to the target protein, or Ub is transferred from the E2-Ub complex to an E3, and this E3 transfers Ub to the target protein. The human genome encodes more than 500 E3 enzymes [36]. These E3 ligases are implicated in many cellular pathways by acting on protein homeostasis. But they also directly regulate the function of many proteins involved in key mechanisms, such as DNA repair for example [37].

The consequence of the binding of a lysine-48 linked poly-ubiquitin chain to a target protein is its degradation by the 26S proteasome, a multi-subunit complex containing one 20S domain and two 19S subunits (Fig. 2).

**Table 1** ALS-associated genes implication in protein degradation pathways

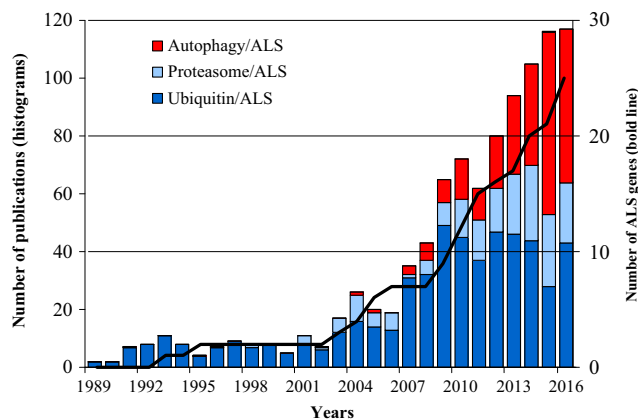
Gene	Year of discovery	Pathway	Aggregate
<i>SOD1</i>	1993	UPS/chaperone network with Hsp70	Yes [17–19]
<i>VAPB</i>	2004	Unfolded protein response	Yes [20, 21]
<i>TARDBP</i>	2008	Autophagy	Yes [8]
<i>FUS</i>	2009	UPS	Yes [22]
<i>FIG4</i>	2009	Autophagy	nc
<i>OPTN</i>	2010	Autophagy	Yes [23, 24]
<i>VCP</i>	2010	UPS/autophagy/ERAD	Yes [25]
<i>C9ORF72</i>	2011	Autophagy	Yes [26, 27]
<i>SQSTM1/p62</i>	2011	Autophagy	Yes [28]
<i>SIGMAR1</i>	2011	ERAD	nc
<i>UBQLN2</i>	2012	UPS/autophagy/ERAD	Yes [29, 30]
<i>TBK1</i>	2015	Autophagy	Yes [31, 32]
<i>PDIA1</i> and <i>PDIA3</i>	2015	ERAD	nc
<i>CCNF</i>	2016	UPS	nc

*SOD1* superoxide dismutase 1, *VAPB* VAMP (vesicle-associated membrane protein)-associated protein B, *TARDBP* TAR DNA-binding protein 43, *FUS* fused in sarcoma, *FIG4* factor-induced gene phosphoinositide 5-phosphatase, *OPTN* Optineurin, *VCP* valosin-containing protein, *C9ORF72* chromosome 9 open reading frame 72, *SQSTM1/p62* sequestosome 1, *SIGMAR1* sigma non-opioid intracellular receptor 1, *UBQLN2* ubiquilin 2, *TBK1* TANK-binding kinase 1, *CCNF* cyclin F, *PDIA* protein disulfide isomerase family A member, *nc* non communicated)

## UPS and ALS

Several studies illustrated or suggested deregulation of UPS in ALS [38–41]. The presence of ubiquitin in intracellular inclusions has been found in motor neurons of FALS and SALS patients [42, 43]. This positive immune-reactivity for ubiquitin has become a neuropathological feature of ALS. Inclusions also contain components of the proteasome [44], and impaired proteasomal function in motor neurons has been observed in ALS [45]. The decrease in proteasome subunits was observed during disease progression in the spinal cord of these transgenic mice, as well [46]. Another observation was the locomotive alteration accompanied by progressive motor neuron loss detected in the conditional knockout of the murine

proteasome subunit Rpt3 in a motor neuron-specific manner [29]. Proteasome inhibition, using lactacystin, induced selective motor neuron death in organo-typic slice cultures [47]. Importantly, genetic evidence has also revealed an implication of defective UPS in ALS pathogenesis. For example ubiquilin 2, whose mutated form causes X-linked ALS/frontotemporal dementia (FTD), physically associates with ubiquitin ligases E3 and the proteasome to mediate protein degradation [48]. Furthermore, mutated valosin-containing protein (VCP) has been discerned in ALS [49]. One of the suggested functions of VCP is to maintain the solubility or to reverse the aggregation of insoluble, misfolded proteins prior to their proteasomal degradation [50]. The best characterized function of VCP is to participate in the endoplasmic reticulum-associated protein degradation (ERAD), a system linked to UPS [51].

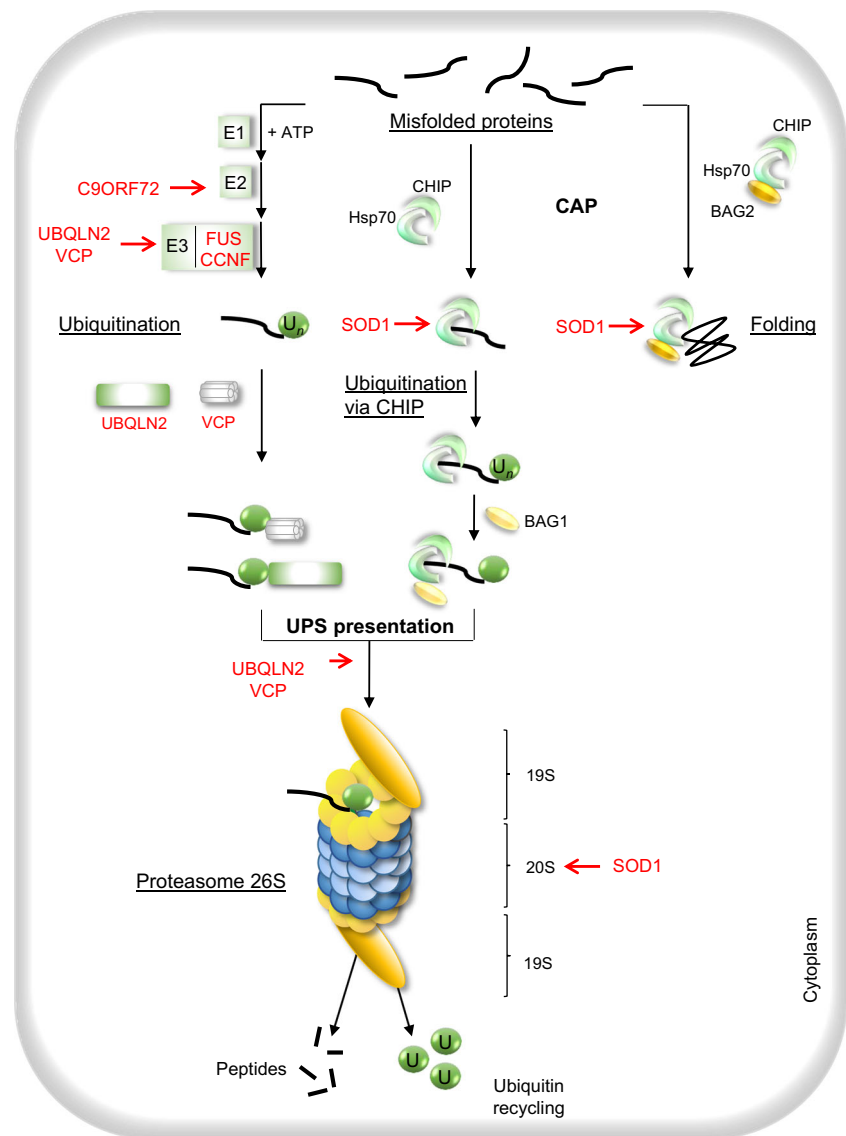


**Fig. 1** Trend of publications in PubMed (MEDLINE) for research with the words: “ALS ubiquitin,” “ALS proteasome,” and “ALS autophagy”

## ERAD and ALS

Proteins of the secretory pathway represent one third of the proteins synthesized in cells. These proteins enter the ER in order to be properly folded. If proteins remain unfolded or are misfolded in the ER lumen, they enter the endoplasmic reticulum-associated protein degradation (ERAD) pathway. ERAD consists of the translocation of these proteins from the ER to the cytosol to be ubiquitinated and, in turn, degraded by the proteasome machinery [52]. An accumulation of unfolded/misfolded proteins in the ER results in a stress response, which activates the unfolded protein response (UPR). The UPR stimulates the production of chaperones and components of the ERAD to handle this protein accumulation (Fig. 3). The

**Fig. 2** Schematic representation of mechanisms implicated in ubiquitin proteasome system (UPS) and links with ALS. Misfolded proteins are poly-ubiquitinated ( $U_n$ ) through the coordinated activity of an ubiquitin activating enzyme (E1), a conjugating enzyme (E2), and a ligase (E3). UBD (ubiquitin-binding domain) containing proteins (such as UBQLN2, VCP) bind ubiquitin-tagged substrates before degradation by the proteasome 26S. Chaperone-assisted proteasomal degradation (CAP) pathway contributes to improve the presentation of ubiquitinated proteins to the proteasome thanks to co-chaperone and an E3 ligase called CHIP. CHIP is able to bind Hsc70/Hsp70 which are associated with misfolded or aggregated proteins during protein quality control. Other proteins are implicated in CAP: BAG1 recruitment leads to proteasomal degradation and BAG2 binding leads to correct folding and stops degradation. In red, genes mutated in ALS and their implication in UPS: chromosome 9 open reading frame 72 (C9ORF72), ubiquitin 2 (UBQLN2), valosin-containing protein (VCP), fused in sarcoma (FUS), cyclin F (CCNF), superoxide dismutase 1 (SOD1)

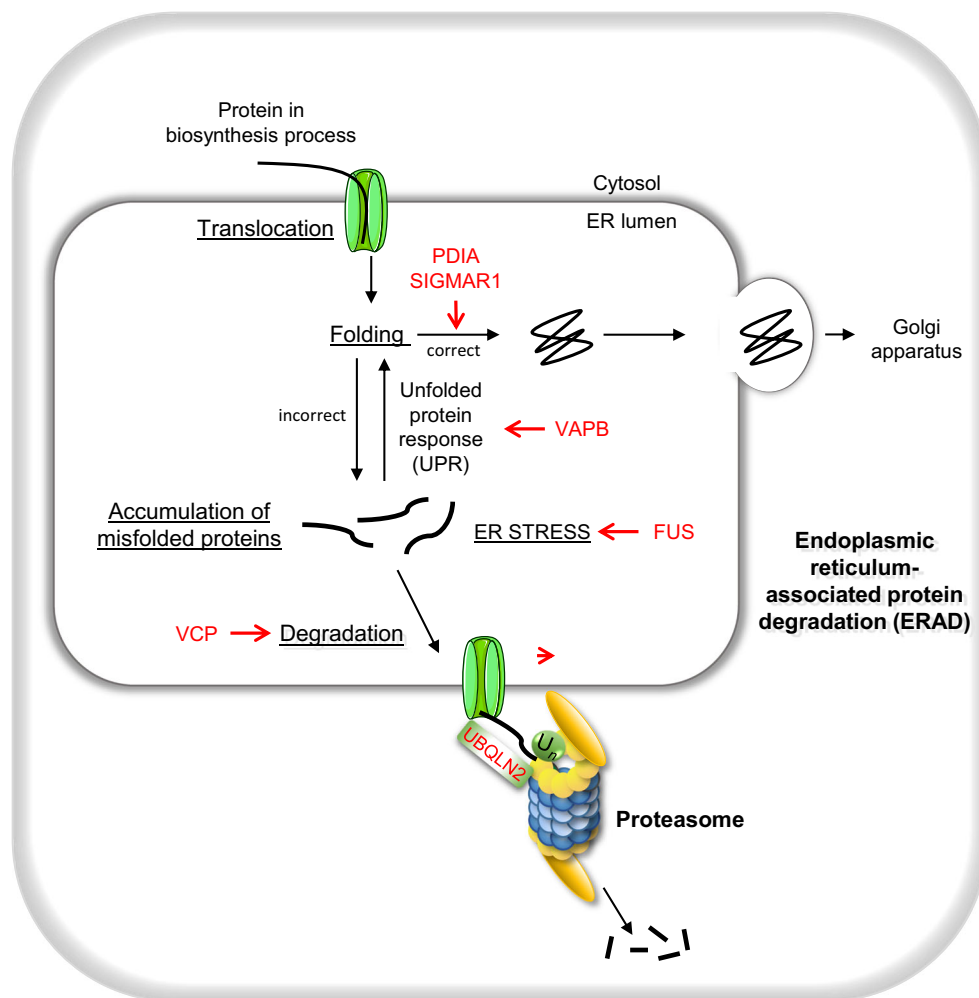


implication of the ER and, particularly, ER stress in ALS has been questionable for many years [53, 54]. A thorough review on this particular point has been previously published [55]. Causes of ER stress in motor neurons are several. Briefly, however, as protein folding is calcium-dependent, an imbalance of calcium levels in the ER may be at play [56]. Inhibition of ER-Golgi transport by mutant SOD1, TDP-43, or FUS can also result in ER stress [57]. The most studied causes of ER stress in ALS are genetic mutations leading to accumulations of proteins in the ER and consequently to a stress.

### SUMO Pathway

The UPS system has a tight relationship with the SUMO pathway. Like ubiquitin, SUMO proteins are expressed as precursors and, after being cleaved by SENPs (sentrin-specific

proteases), they enter the SUMO pathway. The main contribution of the SUMO pathway in proteostasis is its ability to cooperate with or balance the UPS system [58]. Indeed, these two systems work cooperatively [59]. Nevertheless, they can compete if a certain lysine residue on the target protein can be either ubiquitinated or SUMOylated. Proteins encoded by genes mutated in ALS patients, such as SOD1, VCP, and TDP-43, are targets of the SUMO pathway. SOD1 mutants co-localize in aggregates in cells with ubiquitin but also with SUMO proteins. Studies have reported that SUMO1 and SUMO3 increase aggregation of SOD1 mutants [60, 61]. Remarkably, we observed an inhibition of mutant SOD1 aggregation in motor neurons through the prevention of its SUMOylation on lysine 75 [62]. We have also reported that other proteins encoded by ALS-related genes, such as *OPTN* or *VAPB*, contain SUMO consensus sites and, thus, could be SUMOylated [63]. Finally, an



**Fig. 3** Schematic representation of endoplasmic reticulum-associated protein degradation (ERAD) and links with ALS. After synthesis, proteins are translocated in the inner face of the ER to be folded and undergo protein quality control. If the folding is correct, proteins are sent to the Golgi apparatus, where they will undergo post-translational modifications. If proteins are misfolded and accumulated, ER is subjected to stress. Unfolded protein response (UPR) up-regulates molecular chaperones involved in protein folding, allowing misfolded protein to be correctly folded. If not, this response encourages degradation of misfolded protein

additional observation relating the SUMO pathway to ALS is the fused in sarcoma protein (FUS), a SUMO1 E3 ligase encoded by an ALS causative gene [64].

### Chaperone-Assisted Proteasomal Degradation

Molecular chaperones are facilitators of protein folding and assembly. They also play a central role in protein degradation by facilitating the sorting of misfolded proteins to the proteasome or the lysosomal compartment. The chaperone-assisted proteasomal degradation (CAP) is one of these facilitating systems. CAP improves the access of ubiquitinated proteins to the 26S proteasome using a co-chaperone ubiquitin ligase called CHIP. CHIP binds Hsc70/Hsp70 and Hsp90, which are associated with misfolded or aggregated proteins during protein

quality control (Fig. 2). In ALS, CHIP promotes the proteasomal degradation of ALS-linked mutant SOD1 by ubiquitinating Hsp/Hsc70 [40]. CHIP-mediated CAP is also linked to the degradation of several proteins that aggregate in neurodegenerative diseases such as Alzheimer's and Parkinson's diseases [65]. Studies have revealed that Hsps are sequestered within SOD1-positive aggregates in ALS, reducing their capacity to take care of with misfolded proteins [66] (Fig. 2).

by UPS (ERAD). Because proteasomes are in the cytoplasm, ubiquitinated substrates are translocated back in cytosol through transmembrane proteins cooperating with protein such as UBQLN2 in order to present substrates to degradation. In red, genes mutated in ALS and their implication in ERAD: protein disulfide isomerase family A member (*PDIA*), sigma non-opioid intracellular receptor 1 (*SIGMAR1*), vesicle-associated membrane protein-associated protein B/C (*VAPB*), fused in sarcoma (*FUS*), valosin-containing protein (*VCP*), ubiquitin 2 (*UBQLN2*)

### Autophagy

Autophagy plays a significant role in neurodegenerative diseases. For instance, the inactivation of constitutive autophagy, in *Atg7* (autophagy-related 7)-deficient mice, results in the

formation of Ub-positive, cytoplasmic protein inclusions in neurons and drives behavioral defects and neurodegeneration [67]. Its implication in ALS is critical as demonstrated by the fact that several proteins encoded by genes mutated in FALS participate in autophagy processes. The term autophagy entails three protein-presentation mechanisms on lysosomal vesicles for degradation: macro-autophagy (including chaperone-assisted selective autophagy, CASA), chaperone-mediated autophagy (CMA), and micro-autophagy. Macro-autophagy comprises in the removal of aggregated proteins via the formation of a bilayer membrane autophagosome that encapsulates substrates in the cytosol, such as misfolded proteins, before fusing with the lysosome to allow degradation and recycling (Fig. 4). Chaperone-mediated autophagy (CMA) degrades cytosolic proteins containing the pentapeptide motif, KFERQ, recognized by Hsp70 and sent to lysosomes (Fig. 4).

P62/sequestosome1 is an autophagy receptor that binds ubiquitinated proteins and recruits them to the autophagosome [68]. Several observations directly link P62/sequestosome1 to ALS. For example, the depletion in C9ORF72 protein, which is one of the possible pathogenic mechanisms in C9ORF72-linked ALS, causes the accumulation of p62/sequestosome1-positive inclusions in cell lines and primary neurons. Notably, C9ORF72 controls the Rab1a-dependent trafficking of the ULK1 autophagy initiation complex [69]. Another example is that mutant SOD1 can be recognized by p62/sequestosome1 in an ubiquitin-independent manner and targeted to for autophagy [70].

Moreover, optineurin is another important autophagy receptor presenting ubiquitinated substrates to autophagic processes [71]. Target components of autophagy containing p62/sequestosome1 or optineurin, called cargo or substrates, are then associated with a bilayer membrane vacuole, called phagophore, through the interaction with autophagic receptor with light chain 3 II (LC3-II). Then, the phagophore extends its membrane to engulf cargo. This autophagosome can merge with the lysosome to create an autolysosome, where cargo degradation occurs. These formations of vesicles, from phagophores to autolysosomes, are regulated by several proteins, one of which is TANK-binding kinase 1, a cargo recruiter in autophagy [31]. Recently, mutations in its gene *TBK1* were found in ALS [72].

Autophagy and ubiquitin proteasome system and have been viewed as two separate pathways with no or little interplay. Recent works indicate that this is not the case. Crosstalk exists from the UPS to the autophagy and from the autophagy to the UPS [73]. For example, bortezomib, a proteasome inhibitor, induces autophagy [74]. Autophagic inhibition can also impair proteasomal function, resulting in protein accumulation and aggregation [75]. Cooperation seems also very important between UPS and autophagy. These two systems act together for protein degradation in cells, with the UPS mainly implicated in degradation of soluble proteins, and autophagy

in degradation of insoluble and/or aggregated proteins. For example, CHIP protein is directly implicated in the choice between UPS and autophagy. Indeed, as previously mentioned, CHIP acts in CAP, leading to proteasomal degradation of misfolded proteins. But it can also mediate autophagic degradation of misfolded and/or aggregated proteins [76].

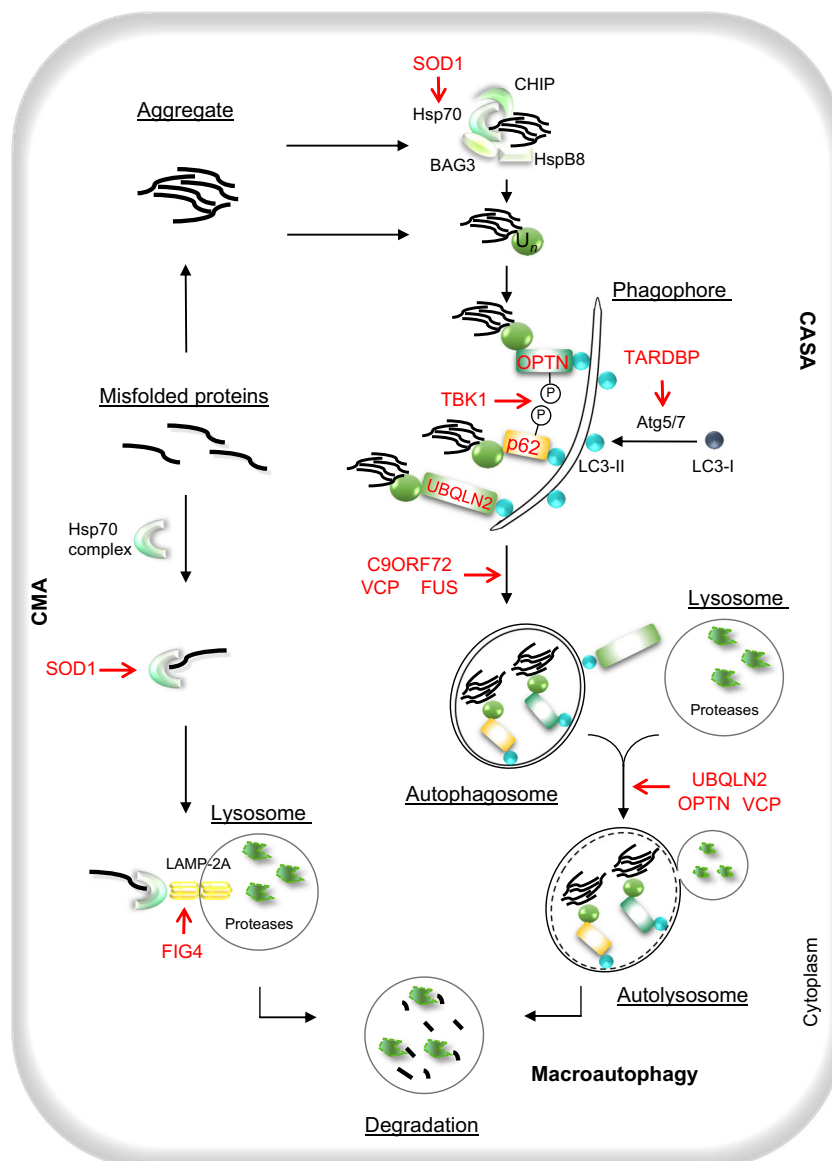
## Causative Genes in ALS and Protein Degradation Pathways

Significant advances have been made in the genetic footprint of ALS since the discovery of mutations in the *SOD1* gene in ALS patients in 1993 [26, 27]. To date, more than 30 genes have been demonstrated to be involved in familial ALS. They are listed in the Amyotrophic Lateral Sclerosis Online Genetics Database website (<http://alsod.iop.kcl.ac.uk/>). These genes have mostly been identified through exome screening analysis, candidate gene strategies and linkage approaches have also rendered important results. For instance, a hexanucleotide repeat expansion (HRE) in the first exon of *C9ORF72* was discovered in ALS patients thanks to linkage analyses followed by sequencing [77, 78]. These discoveries indicated the involvement of several molecular mechanisms in the disease, including those regulating proteins degradation in cells.

### *C9ORF72*

The pathogenic hexanucleotide repeat expansion (HRE) (GGGGCC)<sub>n</sub> found in *C9ORF72* is the most common genetic cause of ALS [77, 78]. It is present in up to 50% of FALS cases and around 10% of SALS cases [79]. This mutation in *C9ORF72* is associated with neuronal inclusions [80, 81]. The function of *C9ORF72* protein is still unclear. Conditional knockout of *C9ORF72* in mice does not result in motor neuron degeneration [82]. Bioinformatics studies have indicated the presence of a DENN domain (differentially expressed in normal and neoplastic cells) in *C9ORF72* protein. This particular domain has been ascertained in more than 20 proteins, whose function is often to act as guanine nucleotide exchange factors (GEFs) for Rab GTPases or Rag GTPases [83, 84]. The consequences of the HRE mutations could be multiple. Three mechanisms have been proposed: loss of function caused by a decreased expression of the protein, production of toxic GGGGCC repeat-containing RNAs, and accumulation of dipeptide repeat proteins (DPR) produced by non-canonical translation (repeat-associated non-ATG translation, RAN) [2, 85, 86].

Ubiquitin-positive inclusions are present in post-mortem brains and in skeletal muscles of *C9ORF72* ALS patients, suggesting an implication of the ubiquitin pathway in the pathogenesis [87]. Transcriptional studies in *C9ORF72*



**Fig. 4** Schematic representation of autophagy and links with ALS. Chaperone-mediated autophagy (CMA) pathway is dedicated to misfolded proteins containing a specific motif: KFERQ (sequence present in 30% of cytoplasmic proteins). These misfolded proteins are recognized by Hsc70/co-chaperones complex and delivered to the lysosome through lysosomal membrane-associated protein 2A (LAMP-2A), a CMA adaptor. Concerning macro-autophagy, poly-Ub chains on cytoplasmic proteins triggers autophagy receptor recruitment (such as OPTN, p62/sequestosome1, UBQLN2) thanks to their ubiquitin-binding domain. Cargos are associated with a double membrane vacuole (phagophore) through the interaction of autophagic receptor with light chain 3 II (LC3-II). LC3-II proteins are activated by cleavage and lipidation of LC3 form by autophagy-related proteins 5 and 7 (Atg5/7) before incorporation in the phagophore membrane. The phagophore extend his

membrane to engulf cargos. Next, the autophagosome can merge with lysosomes to create an autolysosome where protein degradation occurs. Chaperone-assisted selective autophagy (CASA) is a particular macroautophagy pathway: Hsc/Hsp70 and HspB8 chaperones with the association of BAG3, a co-chaperone, improves the recognition of cargos and attracts components of the autophagy machinery. BAG 3 facilitates binding with p62, an autophagic receptor. In red, genes mutated in ALS and their implication in autophagy: superoxide dismutase 1 (*SOD1*), optineurin (*OPTN*), TANK-binding kinase 1 (*TBK1*), TAR DNA-binding protein (*TARDBP*), ubiquilin 2 (*UBQLN2*), p62/sequestosome1 (*SQSTM1*), chromosome 9 open reading frame 72 (*C9ORF72*), valosin-containing protein (*VCP*), fused in sarcoma (*FUS*), FIG4 phosphoinositide 5-phosphatase (*FIG4*)

expansion carriers have displayed significant dysregulation of ubiquitination-related genes such as E2 genes *UBE21*, *UBE2Q1*, *UBE2E1*, and *UBE2N* [88] (Fig. 2). The two gain-of function hypotheses, i.e., the production of toxic hexanucleotide repeat-containing RNAs and toxic dipeptide

repeat proteins (DPR), could impair the ubiquitin proteasome pathway. Concerning the DPR hypothesis, a recent in vitro study on the effects of proline/arginine dipeptide repeats (20 repeats) in rat primary spinal cord cultures demonstrated a reduction in flux through both the proteasomal and autophagic

degradation pathways [89]. A recent study challenges the impact of DPR in neurodegeneration since there is not much DPR aggregation in motor neurons from C9ORF72 mutated ALS patient's spinal cord [90]. DPR accumulation may be not responsible for motor neuron loss; nonetheless, potential contribution of DPR in the disease could go through other mechanisms than aggregation. In vitro studies related an UPS impairment and evidence of ER stress in primary neurons expressing DPR poly-GA [91].

Neuronal, cytoplasmic inclusions are also positive for p62 protein (sequestosome 1, SQSTM1) in post-mortem brain tissues from C9ORF72 ALS patients [92]. Levels of p62 and LC3, another protein directly involved in autophagy, are increased in mice lacking C9ORF72 [93]. Abnormalities in autophagy pathways have been found in patient-derived neurons [94]. This might be a consequence of a defect in autophagosome formation, because C9ORF72 controls the Rabla-dependant trafficking of the ULK1 autophagy initiation complex. Cells expressing endogenous tagged C9ORF72, using a CRISPR-Cas9 approach, revealed its localization on the surface of lysosomes when cells were starved of amino acids [17]. Surprisingly, recent studies have indicated the implication of C9ORF72 in a protein complex containing SMCR8 (another DENN domain-containing protein) and WDR41 [18]. These proteins have also been found on lysosomes [19]. This complex regulates the autophagy-lysosome pathway (Fig. 4). Altogether, current knowledge supports a defect of both ubiquitin proteasome pathway and autophagic pathway in ALS pathogenesis linked to *C9ORF72* mutation.

## SOD1

The superoxide dismutase 1 (*SOD1*) gene is altered in 20% of FALS cases and in 2–7% of SALS cases (<http://alsod.iop.kcl.ac.uk>). More than 180 mutations have been described. The enzyme encoded by *SOD1* is part of the defense mechanisms against oxidative stress. SOD1 inactivates superoxide radicals by generating dioxygen and hydrogen peroxide [95]. Several transgenic murine lines for *SOD1* studies have been generated—knockout mice deficient in *SOD1* and mice bearing a human transgene of FALS-associated mutant SOD1 [96, 97]. These mice, which exhibit a reduced life span, together with in vitro experiments, support a gain-of-function mechanism for ALS-linked SOD1 mutations.

SOD1/ubiquitin-positive aggregates are found in ALS patients carrying a mutation in the *SOD1* gene [26, 98]. These protein aggregates can be observed both in animal and in cellular models expressing mutants SOD1 [99, 100]. Several mechanisms have proposed to explain the cause of toxicity by mutant SOD1 proteins, but the main hypothesis states that mutations induce structural destabilization, prompting the improper folding of SOD1 followed by its aggregation [101, 102]. The accumulation of mutant, unfolded/misfolded SOD1

proteins in the ER triggers a stress signal that activates the UPR. For example, mutants SOD1 interact with Derlin-1, a component of the ERAD machinery, and trigger ER stress [103]. This ER stress has been observed in human-transgenic animals and cellular models expressing mutants SOD1 [104]. SOD1 mutations can also impaired proteasome activity. Indeed the level of the 20S proteasome was reduced in lumbar spinal motor neurons relative to the surrounding neuropil in the spinal cord of transgenic mice expressing the familial ALS superoxide dismutase 1 (SOD1) G93A mutant. Several strategies have been employed to prevent or diminish the formation of SOD1 positive aggregates. We recently presented that inhibition of SOD1 SUMOylation inhibits mutant SOD1 aggregation in vitro [62]. In neurons, the E3 ubiquitin ligase dorfin ubiquitinates mutant SOD1 proteins and targets them for proteasomal degradation [105]. Interestingly, human Dorfin overexpression in SOD1<sup>G93A</sup> mice decreases the amount of mutant SOD1 protein in the spinal cord and improves neurological phenotypes [106]. Another mechanism for mutant SOD1 protein toxicity could be a direct effect on proteasomal activity. Double transgenic mice expressing a fluorescent reporter substrate of the proteasome (UbG76V-GFP) and SOD1<sup>G93A</sup> revealed a lowered expression of proteasomal subunits, followed by a build-up of the reporter substrate [46].

Autophagic processes also appear to play an important role in toxicity generated by mutants SOD1. Parkin, an E3 ubiquitin ligase related to Parkinson's disease, allows the polyubiquitination of mutants SOD1, promoting their degradation by the autophagy-lysosome system [107]. The clearance of aggregates, mutated SOD1 by macro-autophagy is mediated by Hsp70 and its co-chaperone BAG3 [108]. Not long ago, the E3 ligase Mahogunin ring finger 1 (MGRN1) was shown to contribute to the clearance of toxic mutant SOD1 aggregates likely through autophagy [109]. SQSTM1 and ALS2, two ALS-linked factors, have displayed additive protective roles against mutant SOD1-mediated toxicity through modulating proteostasis perhaps by way of autophagic processes [110]. The reason behind the selective degeneration of motor neurons in SOD1-linked ALS is still not understood. One proposed idea argues that these neurons are less able to correctly degrade unfolded/misfolded proteins. It is intriguing to note that comparisons between the NSC-34 motor neuronal cell line and the the C2C12 muscle cell line revealed that the efficiency of activation of the autophagic system in the context of SOD1 mutants expression is reduced in NSC-34 cells. All these data support an implication of both the UPS and autophagy in SOD1-associated ALS.

## TARDBP

The *TARDBP* (trans-activation element DNA-binding protein) gene is mutated in 5% of FALS cases and 1% in SALS cases [111, 112]. More than 50 different mutations have been



identified. A majority of these mutations are located in the 3' region encoding a glycine-rich domain in its product, TDP-43. TDP-43 is for the most part expressed in the nucleus. It participates in RNA metabolism in many ways—transcriptional regulation, splicing, mRNA stabilization (including its own transcript), and miRNA processing. TDP-43 also regulates axonal transport and neuronal plasticity [113]. In ALS, TDP-43 is often observed in the cytoplasm, which corresponds to its mislocalization. Post-mortem studies on ALS patients associated with studies on animal and cellular models expressing mutant human TDP-43 support the role of two mechanisms in TDP-43's pathogenesis [114]: A cytoplasmic buildup of hyper-phosphorylated TDP-43 and a clearance of nuclear TDP-43.

The observations of TDP-43-positive aggregates in post-mortem studies of ALS patients preceded the discovery of mutations in its gene *TARDBP* [9]. These aggregates were also ubiquitin-positive. Notably, TDP-43-positive aggregates are present even in the absence of mutations in the *TARDBP* gene. Mutations in other ALS-related genes, such as *C9ORF72* or *FUS* for example are associated with cytoplasmic aggregates containing TDP-43 [115, 116]. A strong connection between TDP-43 and UPS has been proposed. Cells treated with proteasome inhibitors demonstrated an accumulation of ubiquitinated and insoluble TDP-43 particles [117]. Several enzymes participate in TDP-43 ubiquitination, such as UBE2E3 and UBPY [118]. TDP-43 aggregates consist of the adaptor proteins sequestosome 1 and ubiquilin 2, two proteins associated with UPS and autophagy [119]. The precise role of UPS in TDP-43 proteostasis still remains to be determined. Research efforts in cell lines showed that the UPS primarily acts on the degradation of soluble TDP-43 proteins, whereas aggregated TDP-43 requires autophagic clearance [120].

Several observations support an important role for autophagy in TDP-43-linked pathogenesis. RNAi knockdown of TDP-43 in Neuro2A cells shows downregulation of autophagy-related protein 7 (ATG7) at both the mRNA and protein level [121]. ATG7 is an integral constituent of the autophagy machinery (Fig. 4). Several chemical compounds that stimulate autophagy improved TDP-43 clearance and enhanced survival of primary murine neurons and human stem cell-derived neurons harboring mutant TDP-43 [122]. TDP-43 could also influence or interfere with autophagy by an effect on stress granules (SGs). TDP-43 mutants perturb SG dynamics, engendering their persistence in the cytoplasm and a possible toxic gain-of-function [123]. SG breakdown is dependent on selective autophagy [22]. In fact, administration of rapamycin, an inducer of autophagy, promotes SG clearance. It is now clear that TDP-43 is a major factor in motor neuron death in ALS by directly or indirectly disturbing the dynamics of several cellular machineries, such as the UPS, the breakdown of stress granules, and autophagy. Further studies on the

mechanisms of TDP-43 aggregation and its localization in the nucleus versus the cytoplasm in motor neurons are necessary. Remarkably, a recent study showed that a physiological oligomerization of TDP-43 through its N-terminal domain antagonizes its pathological aggregation [124].

## FUS

The implication of the *FUS* gene (fused in sarcoma) mutations in ALS was recognized in 2009 [23, 125]. Mutant *FUS* is observed in 4% of FALS patients and 1% of SALS patients [23, 112, 125]. More than 79 mutations have been described at present, predominantly in the 3' region encoding an arginine/glycine-rich region and a NLS domain (nuclear localization signal). *FUS*, which is essentially localized in the nucleus, regulates RNA processing, splicing, and mRNA trafficking [126]. *FUS* can also bind DNA, taking part in genomic integrity. Furthermore, *FUS* possesses SUMO E3 ligase activity [64] (Fig. 2). In contrast to *Tdp-43*, the homozygous knockout of *Fus* is lethal in embryos [127]. *FUS*'s connection to ALS pathogenesis could be related to both loss and gain-of-function mechanisms [128].

Pathogenic mutations of *FUS* are associated with aggregates immune-reactive for *FUS* within the cytoplasm of neurons and glial cells [129]. These aggregates and the mislocalization of *FUS* appear essential in the processes of degeneration in ALS. In *Drosophila*, the ectopic expression of human *FUS* mutant influences motor neuron degeneration. Strikingly, if mutations are introduced into the RNA-binding region of *FUS*, aggregation and degeneration are abridged, suggesting that the RNA-binding activity of *FUS* is critical for aggregate formation [130]. Several studies have exhibited a co-localization of *FUS* and ubiquitin in some aggregates, but not all [24, 131]. This suggests that ubiquitination of *FUS* could be a late event in the formation of aggregates and that the UPS may not be crucial in *FUS*-associated neurodegeneration [132].

A recent work on transgenic mice overexpressing *FUS* without a NLS domain showed neuronal loss in the motor cortex and the presence of ubiquitin/p62-positive aggregates. RNA-seq analysis revealed specific transcriptome alterations, such as genes regulating endoplasmic reticulum stress [133]. Studies also showed an implication of *FUS* in SGs dynamics. ALS-associated mutants of *FUS* increased the lifetime of SGs in the cytoplasm [134]. These mutants also disrupt the autophagy mechanisms by inhibiting the formation of autophagosomes [135] (Fig. 4). Overexpression of Rab1, a protein involved in autophagosome formation, restores the autophagy defects induced by mutant *FUS*. Despite these recent efforts, the role of autophagy in degeneration associated with mutant *FUS* remains enigmatic. Nevertheless, many findings advocate that abnormalities in RNA metabolism encompass the cause of neurodegeneration in *FUS*-linked ALS.

Altered RNA metabolism promotes the expression of atypical RNAs and proteins that form aggregates, disrupting the ubiquitin pathway and autophagy.

### OPTN

Mutations in the *OPTN* gene coding for optineurin were first linked to glaucoma [136]. The mutation in *OPTN* was then described in ALS [137]. Mutations were found in approximately 1% (2/161) of FALS and 3.5% (4/113) of SALS in an Italian cohort [138]. In a Japanese cohort, 3.8% of FALS patients and 0.29% of SALS patients carried *OPTN* mutations [28]. Optineurin plays a role in the maintenance of Golgi apparatus and vesicular and membrane trafficking. It contains a ubiquitin-binding domain (UBD) and a LC3-interacting region (LIR), indicating functions in both the UPS and autophagy.

Optineurin-positive aggregates are present in FALS and less frequently in SALS. These inclusions are also composed of ubiquitin and other proteins involved in ALS, such as TDP-43 and FUS [131, 137]. A recent work showed that optineurin is mainly degraded by the UPS [139]. The E3 ubiquitin ligase Hdr1 might be a key protein in this pathway because its overexpression enhances the degradation of optineurin. This study also suggested that the formation of optineurin-containing aggregates is dependent on the UPS. Autophagy seems to play a less significant role in this particular function. However, optineurin has paramount functions in autophagy in physiological conditions. It is a ubiquitin-binding receptor that interacts, through its UBD, amino acid 474–479, in a complex with TBK1 (TANK-binding kinase 1), another protein whose gene is mutated in ALS [140]. TBK1 phosphorylates optineurin, enhancing its interaction (amino acid region 412–520 containing UBD) with LC3 [71] (Fig. 4). Optineurin also regulates autophagy through its interaction with myosin VI, which promotes the fusion of autophagosomes with lysosomes [141, 142] (Fig. 4). Genetic analyses of ALS patients pinpoint a mutation within the UBD (p.E478G) which provokes alteration in LC3 level and turnover thereupon decreased autophagy and displayed weakened interaction with myosin VI, engendering autophagosomes' poor maturation [143]. Conjointly, this mutant and another ALS-related mutation (p.Q398X) disrupt myosin VI-mediated autophagosome-lysosome fusion and intracellular trafficking pathways and induce ER stress [144]. Recent studies support several major roles for optineurin in neuroprotection: regulation of autophagy, participation in inflammatory signaling, blockade of necroptosis, and regulation of apoptosis [145]. A recent study reports that *OPTN* regulates apoptosis via linear ubiquitin binding [146]. Additional research will consequently be necessary to precisely describe which functions of optineurin are particularly involved in neurodegenerative processes in ALS.

### SQSTM1/p62

Mutations in *SQSTM1* have been described in 1 to 3% of patients with FALS [25, 147]. *SQSTM1* encodes the protein p62/SQSTM1 (sequestosome 1) which regulates various biological processes such as transcription, degradation by UPS and autophagy, and apoptosis.

Aggregates harboring p62 have been identified in patients with ALS and ALS/FTD [148]. p62 is a ubiquitin-binding protein and, thus, may be involved in the regulation of the UPS [149]. p62 is better known for its role in autophagy. Indeed, as optineurin, p62 is an autophagy receptor that interacts with LC3, a key protein in autophagy [150] (Fig. 4). ALS-associated mutations located in the LC3-interacting region of p62 have been characterized in ALS [151]. Inhibition of p62 expression utilizing RNA interference has been seen to lead to autophagy defects. In vivo studies on a zebrafish knockdown model on a *sqstm1* ortholog have showed locomotive impairment and have supported an association between ALS and mutated *SQSTM1* through a loss-of-function mechanism [152]. Yet, gain-of-function mechanism is also fathomable, because p62 is related to the UPS and the proteostatic and redox balance. p62 participates in one of the mechanisms controlling redox stress, the Keap1-Nrf2 pathway that activates the expression of antioxidant enzymes [153, 154]. In fact, a recent study proposed that the E3 ligase TRIM21 ubiquitinates p62 and that the binding of p62 to ubiquitin plays an essential role in p62-regulated redox homeostasis [155].

### VCP

Mutations in the *VCP* gene (valosin-containing protein, p97) were initially observed in inclusion body myopathy associated with Paget Disease of bone and frontotemporal dementia [156]. *VCP*'s pathology is characterized by aggregates containing ubiquitin and TDP-43. Neuroanatomic results have revealed the presence of *VCP* in ubiquitin-staining inclusions in several other neurodegenerative diseases, including ALS [157]. A mutation in *VCP* has been also identified in familial case of ALS with dominant inheritance [49]. Today, mutations in the *VCP* gene are found in approximately 2% of FALS case studies [49]. This gene codes for a protein from the AAA+ ATPase family involved in protein homeostasis and protein structure remodeling [30]. Loss of *VCP* in mouse models is lethal prior to implantation, indicating a crucial role for this protein [158].

*VCP* is involved in many cellular functions, including regulation of transcription and cell proliferation. Its participation in ALS pathogenesis may be related to its role in mitochondrial energy production [159]. But *VCP* is best recognized for its role in proteostasis, particularly for the UPS. It binds to ubiquitinated proteins through its N-terminal domain [160] (Fig. 2). This function overlaps with the ERAD mechanism,

which allows the release of aberrant proteins from the ER to be degraded by the proteasome [161] (Fig. 3). VCP has links with ER stress. The mutation in the domain of VCP that encodes a hexameric ATPase crucial for protein degradation during ER stress. Also, ER stress caused by protein aggregation activates the SUMOylation of VCP, a post-translational modification, generating its assembly into a hexameric form and its capacity to participate in the ERAD response [162]. The SUMOylation of VCP at its N-terminal domain is reduced by ALS-causing mutations. Another link between VCP and the UPS is its interaction with PI31 protein (*PMSF1*), a regulator of proteasome complex activity [163]. It is very likely that there is a strong relationship between defects in the UPS caused by mutated VCP and motor neuron degeneration in ALS, but defects in autophagy may also be at play. Indeed, depletion of VCP in cell cultures disrupts autophagosome maturation [164, 165] (Fig. 4). Also, a recent study showed that VCP regulates the clearance of lysosomes by autophagy [166] (Fig. 4). In motor neurons, many endogenous factors, such as protein aggregates and reactive oxygen species (ROS), induce lysosomal damage. A loss of this particular function of VCP in autophagy could, as a result, influence ALS pathogenesis, by way of defects in protein degradation by the UPS.

### UBQLN2

The *UBQLN2* gene is currently the only X-linked gene involved in ALS. Its mutation results in a dominant inheritance pattern [48]. *UBQLN2* encodes for the ubiquilin 2 protein containing an N-terminal ubiquitin-like domain (UBL) and a C-terminal ubiquitin-associated domain (UBA) [167]. The UBA domain binds poly-ubiquitinated proteins, while the UBL domain binds the cap of the 26S proteasome. Thus, ubiquilin 2 delivers poly-ubiquitinated proteins to the proteasome for degradation (Fig. 2). It is also involved in autophagy, cell signaling, cell cycle progression, and cytoskeletal association. [20].

Histopathological analyses on familial and sporadic ALS cases have showed aggregates with ubiquilin 2, regardless of *UBQLN2* mutation. These aggregates are sometimes also positive for TDP-43, FUS, p62, and ubiquitin [21, 48]. A recent study showed that ubiquilin 2 associates with the chaperone HSP70 to clear protein aggregates via the proteasome, and that ALS-related mutants ubiquilin 2 are defective in chaperone binding [168]. Moreover, ubiquilin 2 appears to be involved in the ERAD response [169] (Fig. 3). Concerning autophagy, ubiquilin 2 interacts with LC3, suggesting that it participates in delivering cargo to autophagosomes [170, 171] (Fig. 4). This could mean that mutant *UBQLN2* fails to deliver cargo to the proteasome for degradation [172] (Fig. 4). Altogether, these findings indicate that ubiquilin 2 has a significant function in regulating protein homeostasis by

mediating protein degradation by UPS and autophagy and thus acts as a neuroprotective protein. A deficit in functional ubiquilin 2 would therefore be a risky situation for motor neurons. Current efforts in rodent models, such as transgenic rats, have advocated that an excess of ubiquilin 2 instills a toxic gain-of-function in motor neurons [173]. In contrast, another study showed that mice expressing ALS/FTD-linked *UBQLN2* mutants demonstrate cognitive deficits and develop motor neuron disease, but mice overexpressing WT *UBQLN2* were mostly devoid of clinical and pathological signs of ALS [174]. In conclusion, the relation between *UBQLN2* mutations and defects in proteostasis appears to be paramount in ALS, but the precise mechanisms have not been unequivocally identified. A possible solution could result from studies done directly on patients' cells. For example, a recent work on lymphoblasts from ALS patients carrying mutations in *UBQLN2* reported increased LC3-II and p62 levels, supporting a deregulated proteasome and lysosome in ALS [175]. Further investigations on these cells could further shed extensive information on this matter.

### VAPB

VAMP/synaptobrevin-associated protein (*VAPB*) gene is mutated in rare cases of ALS. The first mutation was discovered in a Brazilian family in 2004 [32]. This study, together with others, indicated that mutations in *VAPB* are associated with the following phenotypes with dominant, autosomal inheritance: classic, adult form of ALS, atypical form with postural tremor, and adult late-onset proximal SMA [32]. *VAPB* participates in the UPR [176] (Fig. 3). However, mechanisms by which *VAPB* mutations cause motor neuron degeneration are still unclear.

The most studied mutation, P56S, causes aggregation of *VAPB* in transfected cells and animal models [177, 178]. Overexpression of wild-type or mutant *VAPB* in primary motor neuronal cultures leads to cytosolic aggregates and ER stress [179]. Chen and collaborators also performed functional studies on neuronal cells expressing mutant *VAPB* (*VAPB*<sup>T46I</sup>). They observed intracellular protein aggregates containing ubiquitin ultimately resulting in cell death [180]. Co-transfection experiments have suggested that mutant *VAPB* inhibits the ability of wild-type *VAPB* from mediating the unfolded protein response. These data support the argument that mutations in *VAPB* in ALS stimulate ER stress and produce defects in the UPS, contributing to weakened protein homeostasis and, consequently, to motor neuronal loss. Interestingly, cellular models expressing moderate levels of mutant *VAPB* contain cytoplasmic aggregates that are not only cytosolic but are also associated with the ER. In these models, *VAPB* mutants cause dramatic ER restructuring [181, 182].

## TBK1

*TBK1* (TANK1-binding kinase 1) has recently emerged as a new gene related to ALS, ALS-FTD, and FTD. Causal mutations could be responsible for 0.4 to 4% of ALS cases [72, 183, 184]. This protein is involved in two pathological mechanisms in ALS: inflammation and autophagy [185, 186]. TBK1 participates in the autophagosomes engulfment of poly-Ub targets by phosphorylating autophagic receptors, such as OPTN and p62/SQSTM1 [71, 187, 188] (Fig. 4). Phosphorylation aids to increase the affinity for LC3 [189].

Neuropathological studies of FTD patient carrying mutations in *TBK1* display moderate to high amount of TDP-43 and p62 neuronal, cytoplasmic inclusions in agreement with a TDP-43 proteinopathy [72, 190]. These observations support the hypothesis that a loss of TBK1 function induces flaws in protein clearance in neurons. The relationship between TBK1 and the autophagy adapters OPTN and p62 also enforces the notion that a defect in mitophagy leading to neurodegeneration [191, 192]. It is worthwhile to note that ALS/FTD-linked mutations in *TBK1* result in a reduction in the amount of TBK1 and/or in modifications in its C-terminal region capable of binding OPTN [72]. TBK1 is also implicated in the innate immune response by regulating the production of IFN $\alpha$  and  $\beta$  [193]. These various roles of TBK1 in the central nervous system suggest a pathogenic mechanism. TBK1 mutations probably generate malfunctions in protein clearance and mitochondrial turnover, which would lead to neuronal damage triggering innate responses by surrounding neurons.

## FIG4

Mutations have also been discovered in the *FIG4* gene in ALS [194]. However, its role remains debatable with respect to ALS [195]. A recent finding, nonetheless, suggests a role specifically in ALS with longer disease duration and upper motor neuron predominance [196]. *FIG4* is a member of the SAC domain-containing protein family with a phosphoinositide 5-phosphatase activity. *FIG4* regulates the cellular level of PI(3,5)P2 maintaining endomembrane homeostasis and endosomal trafficking. PI(3,5)P2 is recognized by lysosomes and merges to gain access to the lysosomal machinery. Inactivation of *FIG4* in spontaneous mutant mice reveals neurodegeneration and large vacuoles containing lysosome receptor LAMP-2A, suggesting perturbations in the endosome-lysosome network (Fig. 4). Autophagic substrates (p62, Ub, LAMP-2A, and LC3-II) are upregulated and accumulate in neurons in the spinal cord of these mutant mice.

## SIGMAR1

The *SIGMAR1* gene encodes for the transmembrane chaperone called sigma non-opioid intracellular receptor 1 (Sig-R1)

that localizes predominantly on the mitochondrion-associated ER membrane [197]. Sig-R1 not only facilitates the proper folding of newly synthesized proteins, but also prevents the accumulation of misfolded proteins by consigning them to the ERAD [198] (Fig. 3). Sig-R1 has also a role in autophagy through its participation in autophagosome maturation [199]. It is also involved in the ER stress response, Ca<sup>2+</sup> metabolism, and chaperone activity, all implicated in neurodegeneration [200]. In 2011, a missense mutation in *SIGMAR1* leading to an amino acid change in the transmembrane domain of Sig-R1 was associated with a juvenile ALS incidences [201]. This mutation creates an abnormal subcellular distribution and modification of Sig-R1 provoking modifications in the ER structure, formation of ER-derived autophagic vacuoles, and the induction of ER stress [202]. A contemporary study on an ALS-linked mutant, E102Q of *SIGMAR1*, proposed a synergistic mechanism of both a gain in toxic function and a loss of function involving altered ER activity, a lack of in protein homeostasis, and a dysregulation of RNA-binding proteins [203].

## CCNF

Another current study by whole exome sequencing of an ALS-FTD family identified the *CCNF* gene as a new ALS-generating gene [204]. *CCNF* encodes the ubiquitously expressed cyclin F protein member of cyclin protein family that acts in cell cycle, mainly by activating cyclin-dependent kinases (CDK) enzymes. Compared to others cyclins, cyclin F is the only one that cannot bind CDK enzymes. Actually, it is part of a Skp1-Cull1-F-box (SCF) E3 ubiquitin ligase complex enabling ubiquitination of target proteins for proteasome-directed degradation [205, 206] (Fig. 2). Later studies identified other mutations in *CCNF* in FALS and SALS cases from various countries. Functional studies on some of these mutant proteins in NSC-34 and Neuro-2A cell lines showed perturbations in the UPS and increased ubiquitination of TDP-43.

## PDIA1 and PDIA3

*PDIA1* (*P4HB*) and *PDIA3* (*ERp57*) are genes encoding the proteins disulfide-isomerases 1 and 3, respectively [207]. These proteins are mostly present in the ER where proteins are synthesized. They catalyze the rearrangement of disulfide bond between cysteine residues in proteins (Fig. 3). This confers to PDIA proteins their role as chaperones in protein folding. In 2015, a novel study described variants in these two *PDIA* genes in ALS patients [208]. Functional studies in zebrafish models and in cell lines showed that these ALS-related *PDIA* mutations cause defects in the function of PDIA1/3 proteins leading to the disruption of motor neuron connectivity and the impairment neuritogenesis [209]. This

observation supports ER proteostasis imbalance as a risk factor for ALS.

## Proteostasis and Therapeutic Strategies in ALS

Recent genetic discoveries in ALS will have reasons for clinical management [27]. We can also hope that these discoveries lead more rapidly to therapeutics against ALS. Evidently, the mechanisms of proteostasis are attractive targets. Many compounds targeting various elements of the UPS and autophagy pathways are currently being tested, either as single agents or in combination, in various pathologies including cancer and neurodegenerative diseases, such as ALS. It is possible to remove pathogenic proteins by stimulating the expression or function of chaperones engaged in the UPS or in autophagy [210]. Indeed, the specificity of certain heat-proteins to misfolded proteins can be stimulated for degradation by autophagy. For example, the enhanced expression of Hsp22 increases clearance of mutant SOD1 [211]. Directly targeting autophagy in neurodegenerative diseases with aggregates may also be of interest, as shown by several studies employing small molecules such as valproate, rapamycin, or lithium, which are autophagic inducers [212–214]. However, they do not have the same impact depending on the disease. Rapamycin, an inhibitor of mTOR, ameliorates clearance of pathogenic aggregates in Parkinson's and Alzheimer's mouse models but not in a SOD1<sup>G93A</sup> model of ALS. The mood stabilizer lithium succeeds in promoting the degradation of SOD1<sup>G93A</sup> in cytoplasmic inclusions and extends survival and delays the onset of ALS [214]. Targeting heat shock proteins seems also interesting, since these proteins are participants in the unfolded protein response, proteasomal degradation and autophagy [215]. Several studies highlight the neuroprotective effects of arimoclomol, a hydroxylamine derivative that co-induces heat shock proteins expression. Still under investigation, this compound extends motor neuron lifespan, diminishes muscular dysfunction, and protects motor neurons in a mouse model of ALS [216, 217]. In addition, these small molecules can have interesting effects on cells by acting on other pathways, leading to synergistic effects. The development of new monoclonal antibodies could also create a very interesting future for ALS treatment, as in other neurodegenerative diseases [218, 219]. Injected by the intracerebroventricular route, a monoclonal antibody against mutant SOD1 was able to reduce its level in the spinal cord and to prolong the lifespan of transgenic SOD1<sup>G93A</sup> mice [220]. Another captivating strategy to reduce the formation of misfolded proteins aggregates is, of course, to reduce the synthesis of the mutated protein. Several antisense strategies are currently being examined. For example, an ISIS antisense technology targeting at the genetic level the production of SOD1 protein

and its mutant form is presently being tested in ALS patients (ClinicalTrials.gov ID:NCT02623699). The mouse model SOD1 G93A is the most commonly used animal model to test potential therapeutic agents in ALS. Several preclinical studies using these mice reported interesting results as previously described. However, success in human clinical trials following preclinical-studies using these mice was rare, suggesting for example that this animal model is not relevant for all preclinical studies. Indeed, this model could be of interest for only familial forms of ALS with mutation in SOD1 gene (5% of FALS), but not for the majority of ALS patients [221]. Nevertheless, in conclusion, blocking aggregation, modifying aggregate formation dynamics, modifying the location of aggregates in cells, and promoting disaggregation all seem to be promising research for therapeutic strategies in combatting ALS.

## Conclusion

The observation of aggregates of misfolded proteins in ALS has argued for more than 20 years in the implication of impaired protein degradation pathways in the disease. In parallel with these histological observations, mutations have been discovered in *SOD1*, *TARDBP*, and *FUS* genes. Interestingly, proteins encoded by these genes turn out to be also present in aggregates. Thirty other genes associated with familial ALS have been linked to ALS to date [222]. Many of them are directly involved in proteostasis and particularly in the protein degradation pathways UPS and autophagy. The UPS plays a major role in the degradation of a plethora of cellular proteins, especially short-lived proteins. Autophagy also plays an essential role in degrading misfolded or abnormally long-lived proteins. The proper activity of these pathways is essential to remove misfolded proteins that are naturally produced in cells. These pathways are not independent; several connections exist such as their links with ER stress. These two mechanisms, UPS and autophagy, become less efficient with age. In ALS, genetic mutations and environmental factors such as oxidative stress and excitotoxicity promote the production of misfolded proteins by acting directly on mechanisms of proteostasis [223]. The UPS and/or autophagy dysfunction induces vulnerability that ultimately lead to cell death. Thus, the misfolding of proteins and certainly their aggregation are directly involved in ALS pathogenesis. One has yet to understand the relationships between these steps. Numerous studies argue to investigate more closely the intracellular localization of these aggregates and their formation instead of their size [224]. For these experiments, it will be essential to develop innovative cellular and animal models with chronic expression of mutant proteins, for example, and not simply their overexpression. These studies can be performed ideally beginning directly from cells of the patient, such as lymphocytes if the gene is

expressed there. The studies can also be done using differentiated motor neurons or glial cells from induced pluripotent stem cells derived from fibroblasts of patients. The CRISPR/CAS 9 system could also be employed in order to create models carrying ALS mutations that would better mirror the conditions of the patients. These new models will certainly aid in better understanding the mechanisms of aggregation, the precise location of these aggregates in cells, and in testing novel therapeutic strategies in the upcoming years.

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