VALOSIN CONTAINING PROTEIN IN AMYOTROPHIC LATERAL SCLEROSIS: NEW INSIGHT IN PATHOLOGICAL MECHANISMS

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INTRODUCTION

NEURODEGENERATIVE DISEASES AND PROTEINOPATHIES

Neurodegenerative diseases (NDs) are characterized by the progressive degeneration of neurons in different region of the nervous system. NDs are a very heterogeneous set of disorders that can be differentiated by the age onset, the brain region effected, the clinical outcome, and the progression of the pathology. Although NDs are very heterogeneous for their clinical features and eziopathology, they share several cellular mechanisms, as, for example, misfolded protein accumulation, formation of protofibrils (Hardy, J. 1997), alteration of degradation mechanisms (Crippa *et al.*, 2010), oxidative stress (Palacino *et al.*, 2004), mitochondrial damage (Sipione & Cattaneo, 2001), failure of axonal transport and loss of synapsis (Li *et al.*, 2001).

Many mechanisms have been identified that concur to neuron death in NDs (Bossy-Wetzel *et al.*, 2004). Nevertheless, most NDs, like Alzheimer's disease (AD), Parkinson's disease (PD), tauopathies, Amyotrophic Lateral Sclerosis (ALS), Frontotemporal Dementia (FTD), are characterized by accumulation of specific protein in nervous tissue. For this common feature some NDs are also known as proteinopathies (Bayer, 2013) (Figure 1).

Proteinopathies are characterized by the aggregation of a single protein or two different proteins that misfold and expose hydrophobic amino acid side chains that are normally confined inside the protein in its native state. This increases the hydrophobic interactions that leads to protein self-association and formation of oligomers, capable to elongate and to bind other intracellular proteins forming insoluble aggregates; then, these aggregates may become insoluble and precipitate inside the cells. The insoluble aggregates can be amorphous or can be organized in fibrils (Chiti & Dobson, 2006). They can be found in cells altering cellular homeostasis or can be released and deposit in the extracellular environment effecting the organ integrity (Almeida & Saraiva, 2012; Zraika *et al.*, 2010).

The origins of misfolded proteins are multiple: proteins may misfold in a stressed environment where proteins synthesis is enhanced (Chiti *et al.*, 2001) or their clearance is reduced (Chiti *et al.*, 2001); there can be a misfunctioning of the folding mechanisms as well as a failure of the protein quality control mechanisms (McNaught *et al.*, 2002; Waelter *et al.*, 2001). In addition, mutations in genes that express unstable proteins which cannot properly fold, may also occur. All of these situations may occur in combination, summing up their deleterious effects in cells.

At first, it was thought that proteinopathies were triggered by the aggregation of normal physiological proteins that became pathologically active after their misfolding (Brundin *et al.*, 2010; Carrell & Lomas, 1997). Afterwards, data showed that in the brain tissue of affected

patients, were present aggregates of proteins truncated and/or post-translationally modified. It has been demonstrated that these protein modifications enhance aggregation and increase protein-toxicity (Barrow & Zagorski, 1991; Dong *et al.*, 2003; Masters *et al.*, 1985).

Insoluble protein aggregates lead to cell toxicity and death in many ways. In first place, misfolded proteins lose their functionality. Moreover, misfolded proteins or disorganized aggregates bind to cellular proteins preventing their functionality (Radford & Dobson, 1999). They can also interact with many cellular molecules and oligomers, leading to a multiplicity of mechanisms of toxicity. In fact, it has been demonstrated that misfolded proteins can interact with cell membrane phospholipid bilayer and receptors (Hirakura & Kagan, 2001; Kourie & Shorthouse, 2000; Lin *et al.*, 2001) and membranes of organelles like mitochondria, ER and Golgi, causing release of Ca^{2+} and oxidative stress, consequently leading to apoptotic or necrotic cellular death (Ross, 2002).

Cells prevent the accumulation of misfolded proteins or the formation of unstable toxic aggregates using specific mechanisms, such as the unfolded protein response (UPR) in the ER or the heat shock protein (HSP) response in the cytosol. These mechanisms, supported by the degradation pathways, are known as the protein quality control (PQC) system (Rubinsztein, 2002; Tofaris *et al.*, 2001).

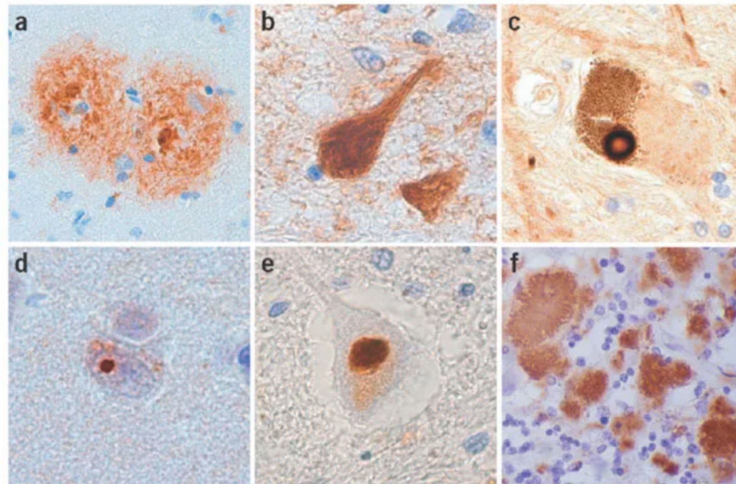


Figure 1 Protein aggregates in neurodegenerative disease: a.Alzheimer disease; b.Frantotempral Dementia; c.Parkinson disease; d.Huntington disease; e.ALS; f.CJD. (Forman et al., 2004)

AMYOTROPHIC LATERAL SCLEROSIS

Amyotrophic Lateral Sclerosis (ALS) is a ND characterized by a remarkable component of alteration in proteostasis. In fact, for these features, is also considered as a proteinopathy.

ASL has been described for the first time by Jean-Martin Charcot in 1869 and is currently diagnosed to 3-4 individuals over 100'000 people. Commonly the disease onset is in adulthood, with an age range around 55 years, and the patient dies after 3-5 years after the diagnosis, however it might strike earlier and the survival can be of only 6 month or, in fewer cases, it might extend up to 25 years. ALS is characterized by the progressive loss of both upper and lower motor neurons (MNs). These neurons are responsible of the control of skeletal muscles; because of that, the progressive death of MNs leads to weakness and atrophy of muscles causing loss of the voluntary muscular movement (Al-Chalabi & Hardiman, 2013).

ALS can be classified in familiar forms (fALS) and sporadic form (sALS). About 10% of the ALS cases are forms of fALS, where mutations of genes are inherited in families (at least two people in the same family). The inherited mutations are almost always a dominant trait and have frequently a high penetrance. The remaining 90% of the cases are a sALS form. All ALS cases are characterized by the presence of ubiquitin (Ub) positive inclusions (Peters *et al.*, 2015). Moreover, besides the presence of inclusions positive to misfolded proteins encoded by mutated genes, around 97% of ALS cases, inclusions are positive to wild type proteins such as TDP-43 (Bosco *et al.*, 2010; Neumann *et al.*, 2006). Furthermore, another evidence of proteotoxicity in ALS is the presence of signs of impairment in the PQC system. Finally, many genes mutated associated to ALS encode proteins involved in PQC system as chaperones and ubiquitin proteasome system (UPS) or autophagic regulators.

CLINICAL SIGNS

The clinical signs of the disease can subdivide ALS in different subsets according to which MNs are firstly and mainly affected. About 70% of the patients present a limb-onset. When spinal neurons or lower MNs are affected first, patients develop weakness, flaccidity and atrophy of limbs. Conversely, if firstly corticospinal MNs are affected and the involvement of spinal MNs is limited (known as primary lateral sclerosis) patients show hyperreflexia, spasticity, increased limb tone and little muscle atrophy. Moreover, about 25% of the patients present a bulbar-onset. The loss of brainstem MNs (known as bulbar ALS) causes tongue atrophy that leads to thickness of speech and difficulty in swallowing. The degeneration of cortical fronto-bulbar MNs (known

as pseudobulbar-palsy) leads to a slow and highly dysfunctional speech and swallowing but without any tongue atrophy. Finally, the remaining 5% of patients present trunk onset with respiratory involvement (Vucic *et al.*, 2007). Most of ALS cases evolve in the other areas of the brain tissue and end with death due to respiratory failure. The heterogeneity of medical signs and symptoms, leads to a difficult diagnosis that is worsened by the overlapping of clinical features with other adult-onset NDs, like FTD (Kiernan *et al.*, 2011; Nass *et al.*, 2012; Phukan *et al.*, 2007).

RISK FACTORS

The causes of ALS are attributable to genetic alterations and the environment. In some sALS patients are found genetic variants present in fALS forms. However, there are no clear differences between fALS cases and sALS in clinical symptoms and sings. This leads to think that there could be common molecular mechanisms that cause the disease (Hanspal *et al.*, 2017).

ENVIRONMENTAL FACTORS

Through years, many environmental factors, that contribute to ALS onset, have been analyzed. Studies show that long exposure to physical and chemical substances such as heavy metals, pesticides, electromagnetic fields may concur to the onset of the disease. Data show that high body mass index (BMI), physical activity or head traumas can also increase risk of ALS onset.

The exposure to heavy metals, like lead (the most studied), were demonstrated to lead to a high risk of ALS. However, in the last decade the knowledge of the risk brought people to decrease or even nullify the exposure to lead or other heavy metals. This temporarily eliminated the exposure to these metals as a cause of ALS onset (Trojsi *et al.*, 2013; Wang *et al.*, 2017).

Pesticides include rodenticide, insecticides and herbicides. Studies show that the exposure to pesticides induces oxidative stress, mitochondrial dysfunction, α-synuclein storage, that lead to neuronal loss. It has been demonstrated that a high exposure to pesticide concurs to ALS onset as well as other NDs. Exposure to pesticide risk is strictly correlated to genetic factors. In fact, it has been demonstrated that mutations in a specific gene (PON1) increase pesticide induced damage. PON1 gene expresses an enzyme that hydrolyzes organophosphate pesticides so its misfunctioning worsens pesticide exposure effects (Bozzoni *et al.*, 2016; Slowik *et al.*, 2006).

Long exposure to electromagnetic fields might also be related to ALS-onset. Some studies have associated the increasing risk of motor neuron disease to occupations related to electricity. In

particular, works with a high exposure to low frequency electromagnetic fields and electric-shocks are more subjected to ALS risk (Johansen, 2000; Mitchell, 2000; Park *et al.*, 2005) .
Nowicka and colleagues show that all of these risk factors alter cellular homeostasis by inducing oxidative stress (Nowicka *et al.*, 2019). In particular, they suggest that these risk factors decrease cell capability of eliminating excessive reactive oxygen species (ROS) leading to alteration and damage of cell organelles that, if not reverted, leads to cell death. In fact, high exposure to heavy metals leads to cell depletion of antioxidant molecules and enzymes (Ercal et al,. 2001). Also pesticides increase oxidative stress. Studies demonstrated that pesticides alter the total amount of thiol molecules decreasing the antioxidant capacity, and they activate cell membrane lipid peroxidation (Mostafalou & Abdollahi, 2018). Finally, in vitro studies showed that exposure to low frequency electromagnetic waves resulted in generation of a larger quantity of cellular ROS. This was confirmed in vivo where it was also demonstrated that the exposure to low frequency electromagnetic fields disables antioxidant properties within cells (Martínez-Sámano *et al.*, 2012).
Even if, environmental factors have an important role in ALS onset, in most of the cases the simple exposure to environmental factors cannot explain by themselves the onset of the disease. In these cases, environmental factors are strictly correlated to genetic factors.

GENETIC FACTORS

Most of the fALS cases and some sALS are correlated to mutation of genes that lead to motor neuron degeneration. It is difficult to define the genetic contribute to ALS onset, as not all mutations have a full penetrance. Some genes have dominant inheritance with a full penetrance, some only with a partial penetrance, others are recessive mutations, and finally, some mutations can even be X-linked (Zufiría *et al.*, 2016). Moreover, mutations frequency is population-specific. In 1993, the first mutation correlated to ALS was found, which was a mutation in superoxide dismutase 1 (SOD1) gene. Up to now, sequencing and advanced molecular biological technologies have permitted to find up to 50 mutated genes which can concur to the onset of ALS. The genes found involved in ALS-onset can be classified by the mechanisms that the proteins they express are involved in. Some mutated genes express proteins involved in oxidative stress like SOD1, others involved in RNA metabolisms like TAR-DNA binding protein 43 (TDP-43) and Fused in Sarcoma (FUS), others implicated in vesicle trafficking like Optineurin (OPTN), Alsin (ALSIN), VAMP Associated Protein B (VAPB), others regulate or are implicated in degradation systems like Valosin Containing Protein (VCP), Sequestosome 1 (SQSTM1), Ubiquilin 2 (UBQLN2),

others cooperate with actin polarization like Profilin 1 (PFN1), and finally there are some mutations, which occur in intronic gene sequences resulting in expression of proteins that are normally not express, like *C9ORF72* gene.

The most recurrent mutated genes in both European and Asian ALS-patients are *SOD1*, *TDP-43*, *FUS* and *C9ORF72* (Figure 2). These mutations are present in familiar and sporadic ALS.

SOD1

Rosen et al. discovered in 1993 mutations of *SOD1* gene correlated to fALS cases. In particular, they found 11 missense mutations in 13 different fALS families. Since then, more than 180 different mutations have been discovered (Rosen *et al.*, 1993). These mutations are mainly single point mutations, but there are also deletions, insertions and truncations spread through all the 5 exons of *SOD1* gene (Yamashita & Ando, 2015).

SOD1 gene is localized on chromosome 21q22.11 and it expresses SOD1 monomeric protein with a mass of 16kDa and composed by 153 highly conserved amino acids (Rosen *et al.*, 1993); (Doucette *et al.*, 2004). SOD1 is active as a homodimer metalloprotein that binds a Cu^{2+} and a Zn^{2+} ions. It is localized mainly in the cytoplasm, but is also distributed in the nucleus, mitochondria and lysosomes (Getzoff *et al.*, 1989; Zelko *et al.*, 2002). SOD1 main function is to protect from oxidative stress by dismutating the free superoxide radicals ($O_2^{\bullet -}$) into oxygen (O_2) and releasing the less reactive hydrogen peroxide (H_2O_2) (McCord & Fridovich, 1969).

Mutations in *SOD1* are correlated to fALS and to some sALS-patients. These mutations are causative of nearly 15% of European-fALS and up to 30% in Asian-fALS cases. While they are correlated to only 1.2% of European-sALS and 1.5% in Asian-sALS cases (Zou *et al.*, 2017). ALS caused by any *SOD1* mutations is known as ALS1. ALS1 can have many different phenotypes depending on the variants that are present and if they are homo o heterozygous. In fact, ASL1-cases can differ for disease duration and severity. For example A4V and G93A mutations are correlated to rapid disease progression and shorter survival, while homozygous D90A has a slowly progressive paresis that starts in the legs and has some atypical features like bladder disturbance (Andersen *et al.*, 1996; Juneja *et al.*, 1997; Yamashita & Ando, 2015). Whereas, heterozygous D90A is associated with various ALS forms including bulbar, upper limb or lower limb onset with a faster progression (Li & Wu, 2016). The most common and studied mutations are D90A, A4V and G93A.

D90A has an alanine in place of the aspartic acid 90. Patients carrying this mutation can have different clinical symptoms and disease course depending on whether is homo or heterozygous

as it was first described. Of the three, it is the most common mutation in Europe. In fact, it is correlated to 50% of all ALS cases in Sweden and Finland. This SOD1-mutant has normally a recessive inheritance, but in fewer cases, it can be also inherited as a dominant mutation.
A4V has alanine 4 changed to a valine. It is the most common SOD1 mutation correlated to ALS that is found in North America (nearly 50%). Biochemically, this mutation leads to the formation of aggregates in the nucleus and in cytoplasm.
G93A mutation has an alanine in place of glycine 93. It is the rarest of the three, but is the most researched. In fact, G93A was the first mutation correlated to ALS that was studied in a transgenic mouse which had the phenotype of a motor neuron syndrome. G93A is correlated to a disease with a rapid progression and a short survival (Andersen, 2006; Yamashita & Ando, 2015). Biochemically, the mutation is correlated to the formation of cytoplasmatic aggregates and mis-localization of the protein that loses the capacity to be retained in the nucleus (Sau *et al.*, 2007). Mutations alter SOD1 conformation, destabilizing the dimer and decreasing the enzyme activity up to 80% in same cases (Deng *et al.*, 1993; Rosen *et al.*, 1993). However, it has been demonstrated that the correlation between SOD1-mutants and ALS is not due to a loss of functioning of the enzyme, but probably to a toxic gain of function (Cleveland et al., 1995). This is confirmed by the fact that SOD1-knockout mouse model does not develop an ALS phenotype (Siwek, 1996). Conversely, many pathological mechanisms have been proposed, but the main toxic pathway is still not fully known. Firstly, aberrant SOD1 leads to anomalous chemical reaction as tyrosine nitration, peroxidation and reverse catalysis (Pasinelli & Brown, 2006). Moreover, SOD1 altered conformation increases protein-protein interaction and triggers the formation of protein aggregates. These results alter cellular homeostasis increasing oxidative stress, DNA damage, mitochondrial dysfunction, disturbance in axonal transport and alteration of the PQC system in particular, decreasing the functionality of the UPS (Boillée *et al.*, 2006; Ikenaka *et al.*, 2012; Sau *et al.*, 2007).
Increase in oxidative stress could be due both to a decrease in SOD1-mutant functioning correlated to insufficient degradation of ROS or to an over-functioning of SOD1-mutant with an increase in hydrogen peroxide radical levels (Allen *et al.*, 2003). Both can lead to peroxidation of fatty acids with alteration in cells membrane. Moreover, increased ROS levels lead to activation of signaling pathways and alteration in protein structure and functionality. Nervous tissue is particularly sensible to oxidative stress. In fact, in nervous tissue, energy is produced by catabolic mechanisms dependent from O_2 levels; moreover, there are many unsaturated fatty acids that

are sensible to ROS; finally, there is a very low concentration of glutathione, an important antioxidant molecule (Wang *et al.*, 2009).

Oxidative stress and formation of aggregates concur to ER stress. Endoplasmic reticulum stress activates UPR which, if the stress persists, activates apoptotic neuron death (Malhotra & Kaufman, 2007). The increase in DNA damage is due to mislocalization of SOD1 in cytoplasm aggregates that causes a reduction of soluble SOD1 in the nucleus. The loss of soluble SOD1 in then nucleus nullify its protective role from ROS and it consequently increases DNA damage (Brasil *et al.*, 2018; Inoue *et al.*, 2010; Sau *et al.*, 2007). Aggregates formation also damage organelles like mitochondria or proteasome. Mitochondrial damage results in reduction of adenosine triphosphate (ATP) synthesis and increased production of oxidative stressors. The decrease of ATP levels blocks Na^+/K^+ pumps leading to a slow depolarization with hyperexcitability, and blocks of Ca^{2+} pumps with an increased intracellular Ca^{2+} level that activates apoptotic pathways leading to cell death (Miquel *et al.*, 2012; Szelechowski *et al.*, 2018). Moreover, mitochondrial damage is associated to an increased production of oxidative stressors. Several data demonstrate that the increase in ROS further enhance alteration in mitochondrial morphology and inhibition of ATP production (Bernard *et al.*, 2007). Mitochondrial damage is strictly correlated to alteration in axonal transport. Axonal damage results in alteration of organelles transport and in accumulation of neurofilaments that can be found in the brain tissue of affected patients (Hirano *et al.*, 1984).

The progression of the disease correlated to SOD1-mutant and the spreading from lower MNs to upper or vice versa could be explained by the fact that SOD1-mutants can spread from cell to cell. In fact, it has been demonstrated in vitro and in vivo that SOD1-mutants upregulate the release of extracellular vesicles that contain the mutant protein. Moreover, it was observed that neurons can intake extracellular vesicles that carry SOD1-mutant (Grad *et al.*, 2014; Münch & Bertolotti, 2011).

TDP-43

TAR DNA binding protein 43 (TDP-43) is a DNA/RNA binding protein that belongs to a heterogeneous nuclear ribonucleoprotein (hnRNP) family. It was firstly identified in 1995 as a binding and repressor protein of pyrimidine-rich DNA motifs in a long terminal repeat called TAR of HIV1 virus (Ou *et al.*, 1995). Afterword, it was discovered that TDP-43 mainly binds RNA with a highly conserved RNA recognition motif (RRM). In 2006, TDP-43 was for the first time associated to ALS and FTD as it was discovered to be the main component of insoluble inclusions present in

the brain tissue of affected patients (Arai *et al.*, 2006; Neumann *et al.*, 2006). From 2008, dominant mutations in TDP-43 gene (*TARDBP*) were correlated to ALS providing evidence that aberrant TDP-43 could be causative of neurodegeneration. To date, nearly 50 mutations in *TARDBP* are correlated to ALS; in particular they are correlated to 4.2% of European fALS cases and 1.5% of Asian fALS cases and only 0.8% of European sALS cases and 0.2% of Asian sALS cases (Figure 2). However, TDP-43 in its wild type form, is highly relevant in ALS pathology as it is a key component of the insoluble and ubiquitinated inclusions of nearly 97% of ALS cases.

TDP-43 is a ubiquitous protein localized mainly in the nucleus, but it can be also found in cytoplasm. TDP-43 can shuttle from a compartment to the other thanks to a nuclear export signal (NES) and a nuclear localization signal (NLS) that are present in the protein structure.

TDP-43 has a key role in RNA metabolism. In fact, it is involved in exon splicing, gene transcription, mRNA stability, mRNA biosynthesis, mRNA transport, mRNA degradation and non-coding RNA regulation. Moreover, data show that TDP-43 concurs in stress granules (SGs) formation.

TDP-43 can bind RNA through two RRM present in a central domain which is linked to a N-terminal domain and a C-terminal domain. The N-terminal domain (NTD) function is to bind another N-terminal domain forming a homodimer. It is debated if TDP-43-NTD dimerization is a physiological or a pathological mechanism. Data suggest that dimerization is necessary for TDP-43 physiological functions, but other suggest that is involved with TDP-43 aggregation. In fact, Tsoi and colleagues showed that NTDs dimerization is reversible, but it enhances the propensity of the C-terminal region to aggregate (Tsoi *et al.*, 2017). While Jiang and colleagues showed that NTD dimerization enhances TDP-43 role in pre-mRNA splicing, improves its solubility and protects from the formation of cytoplasmic inclusions (Jiang *et al.*, 2017).

The two RNA recognition motifs (RRM1, RRM2), attached to one another by 15 aa linker, recognize with high specificity short TG/UG-rich sequences of DNA/RNA (Kuo *et al.*, 2014). The RRMs contribute in different ways in TDP-43 functioning. They make possible specific mRNA recognition and regulation, among which they can also recognize its own mRNA with a resulting autoregulation mechanism that controls TDP-43 own total cellular concentration (Ayala *et al.*, 2011). Moreover, by binding RNA or single stranded DNA (ssDNA), RRMs concur in regulating the TDP-43 solubility and helping preventing its aggregation (Huang *et al.*, 2013; Sun & Chakrabartty, 2017).

The C-terminal domain is a glycine-rich segment enriched with uncharged polar amino acids. This disordered region resembles highly aggregating prion-like domain (King *et al.*, 2012; Liebman & Chernoff, 2012). C-terminal functions are mostly unknown. Recently, it has been shown that C-terminal domain is involved in stress granule formation. In fact, it concurs in the formation of dynamic protein droplets where it has mild transient interactions that seem crucial for the formation of SGs (Conicella *et al.*, 2016; Mcdonald *et al.*, 2011).

TARDBP mutations associated to ALS are mainly localized in exon 6 which encodes TDP-43 C-terminal region. Mutations modify TDP-43 functioning and stability. In fact, they increase TDP-43 propensity to aggregate, enhance its cytoplasmic mis-localization, alter protein stability and resistance to proteases, and modify TDP-43 interactions with other proteins (Buratti, 2015; Lattante *et al.*, 2013; Pesiridis *et al.*, 2009). Some mutations are present in both sporadic and familiar forms of ALS. Some are also correlated to other diseases like FTD. However, the overall percentage of mutations correlated to ALS, as first shown, is quite low. Conversely, a very high percentage of ALS-cases are correlated to inclusions positive to TDP-43 wt. In fact, all cases of ALS, except from ALS cases correlated to SOD1 and FUS mutants, present insoluble species positive to TDP-43. TDP-43, present in these insoluble species, can be found ubiquitinated, phosphorylated and truncated (Arai *et al.*, 2006; M. Hasegawa *et al.*, 2008; Inukai *et al.*, 2008; Neumann *et al.*, 2006, 2009). TDP-43 has different potential phosphorylation sites: 41 serine, 15 threonine and 8 tyrosine residues. Kinases as CK1, CK2 and GSK3 have been identified to phosphorylate TDP-43 in various phosphorylation sites like Ser-409/Ser-410, that is considered a signature of ALS pathology (Neumann *et al.*, 2006, 2009). Data show that in neuronal cells, phosphorylation enhances mis-localization and aggregation (Barmada & Finkbeiner, 2010; Liachko *et al.*, 2010; Nonaka *et al.*, 2009; Takashi Nonaka *et al.*, 2016). Data also show that TDP-43 is found ubiquitinated. In particular, the ubiquitin ligase, Parkin, ubiquitinates TDP-43 with both K-63 and K-48 polyubiquitin chains. Ubiquitination concurs in formation of aggregates that could be then addressed to different degradation systems (Hebron *et al.*, 2013; Scotter *et al.*, 2014). TDP-43 can be found in inclusions as full-length or as truncated forms. The truncated forms are C-terminal fragments of 25 or 35 kDa (TDP-25, TDP-35) generated by proteolytic cleavages of caspase and or alternatively calpain proteases (Tsuji *et al.*, 2012; Xiao, Sanelli, *et al.*, 2015). Data demonstrate that chronic oxidative stress or ER stress can increase caspase activity generating TDP-43 C-terminal fragments (Meyerowitz *et al.*, 2011; Suzuki *et al.*, 2011). In these studies, TDP-35 is well detected while TDP-25 is found in low levels. On the contrary, in inclusions

in brain tissue of effected patients, TDP-35 is rarely observed while TDP-25 is always present, for this reason is considered a pathological signature of the ALS and FTD brain (Hasegawa *et al.*, 2008; Neumann *et al.*, 2006, 2009). Other studies show that C-terminal fragments could be a result of an alternative translation of *TARDBP* that is pathologically upregulated in ALS. Data show that could be either the translation of an alternative transcript, or the in-frame translation that starts from a downstream initiation codon (Nishimoto *et al.*, 2010; Xiao *et al.*, 2015). In any case, C-terminal fragments are found phosphorylated mainly in inclusions of the brain cortex, whereas in the spinal-cord there is a predominant deposition of phosphorylated full-length TDP-43 (Neumann *et al.*, 2009).

TDP-25 lacks of the NLS as a result of proteolytical cleavage (Lee *et al.*, 2011; Winton *et al.*, 2008). The lack of NLS added to the typical prion like structure of the TDP-43 C-terminal domain confers to TDP-25 a high propensity to aggregate and to form cytoplasmatic inclusions. The high capacity to aggregate of C-terminal truncated forms are considered a possible pathological mechanism that triggers aggregation and mis-localization of full-length TDP-43 (Shimonaka *et al.*, 2016).

TDP-43 mis-localization and aggregation results in triggering different pathogenic pathways. In first place, TDP-43 mis-localization leads to a loss of its nuclear functions. Nuclear loss of function results in differential splicing and/or expression of TDP-43 targets (Colombrita *et al.*, 2015; Highley *et al.*, 2014; Klim *et al.*, 2019). In support that the loss of TDP-43 nuclear functions can be a pathway of the disease, the homozygous TDP-43 null mice are not viable (Kraemer *et al.*, 2010; Sephton *et al.*, 2010) and inducible TDP-43 knockout in adult mice is lethal (Chiang *et al.*, 2010). However, the main pathogenic mechanisms seem to be attributable to TDP-43 gain of neuronal toxicity function in the cytoplasm. In fact, overexpression of either wild type or mutant-TDP-43 in animals results in a neurodegenerative phenotype (Ash *et al.*, 2010; Kabashi *et al.*, 2010; Liachko *et al.*, 2010; Stallings *et al.*, 2010; Wils *et al.*, 2010).

Increased concentration of TDP-43 alters endocytosis pathways. In fact, it was observed in ALS patients and in yeast that, in abnormal abundance of TDP-43, it localizes with endocytosis-associated proteins. Moreover, Liu and colleagues demonstrate that TDP-43 inhibits endocytosis (Liu *et al.*, 2017). On the other hand, endocytosis seems to be involved in TDP-43 turnover, so alteration in this pathway increases TDP-43 aggregation and toxicity (Leibiger *et al.*, 2018; Liu *et al.*, 2017). Independently from endocytosis, also other degradation pathways are involved in TDP-43 toxicity. In fact, TDP-43 can also be degraded by UPS, when it is in its soluble form, and autophagic pathway, when it is in its aggregated and insoluble form (Crippa *et al.*, 2016; Scotter

et al., 2014; Zhang *et al.*, 2010). Alteration in these systems, that can be due to mutation in genes that encode protein involved in the systems, leads to an increase in insoluble TDP-43 aggregates (Budini *et al.*, 2017; Filimonenko *et al.*, 2007; Osaka *et al.*, 2016). Whereas the increased activity of the systems can decrease the levels of the insoluble TDP-43 (Barmada *et al.*, 2014; Crippa, Cicardi, *et al.*, 2016). In parallel, autophagic pathway can be altered by TDP-43 mutations or mis-localization as proteins involved in autophagy regulation have mRNAs that are targets of TDP-43 (Bose *et al.*, 2011) .

The abnormal levels of TDP-43 wild type and mutants alter mitochondria structure, functioning and transport. Wang and colleagues demonstrate in primary MNs that TDP-43 toxicity results in mitochondrial length alteration and in impaired mitochondrial movement. Alteration in mitochondria dynamics was also detected in mouse and flies models (Altanbyek *et al.*, 2016). Mitochondria alteration leads to oxidative stress and increases levels of metal ions like zinc, manganese and copper. Moreover, TDP-43 aggregates directly increase oxidative stress and damage and cause increased accumulation of the anti-oxidant response (Duan *et al.*, 2010; Tian *et al.*, 2017). Finally, TDP-43 mutations are also correlated to alteration of SGs assembly and release. In particular recent data show that in primary motor neurons, TDP-43 is recruited to SGs and cells expressing TDP-43-mutant have less and smaller SGs (Gordon et al., 2019).

In the same way as SOD1-mutants, TDP-43 mutants and/or aggregates spread from cell to cell with a prion-like mechanism of self-templating propagation (Bräuer *et al.*, 2018; Smethurst *et al.*, 2016). There are various different mechanisms that assist TDP-43 aggregate spreading. There are data that show that TDP-43 aggregates can propagate via cerebrospinal fluid (CSF) (Ding *et al.*, 2015). Others demonstrate that aggregates are transported along neuroanatomical pathways thanks to axonal transport (Fallini *et al.*, 2012; Kassubek *et al.*, 2014). Finally, studies show that they can be released locally and spread from cell to cell (Iguchi *et al.*, 2016; Smethurst *et al.*, 2016). Recently, several studies have identified the secretion of exosome as a new pathway of transmission of TDP-43 aggregates (Feiler *et al.*, 2015; Iguchi *et al.*, 2016). It has been demonstrated in neuronal cell line that exosomes contain TDP-43 overexpressed and that inhibition of exosomes-release increases TDP-43 aggregates. To support the importance of exosomes secretion, in sALS-patient exosomes were found higher levels of TDP-43 (Feiler et al. 2015).

C9ORF72

In 2011 *C9ORF72* gene, localized on locus 9p21 of chromosome 9, was correlated to ALS and FTD (DeJesus-Hernandez *et al.*, 2011; Renton *et al.*, 2011). Mutations in *C9ORF72* gene are found mainly in European population, in fact they are associated to nearly 34% of European-fALS and more than 5% of European-sALS cases. It is also present in lower levels in Asian population, with 2.3% in fALS cases and 0.3% in sALS cases (Zou *et al.*, 2017).

C9ORF72 is transcribed in two "long" variants and one "short" variant. The long variants (variant 2 and 3) encode for full length proteins of 481 aa, while the "short" variant (variant 1) encodes for short isoform of 222 aa. Isoform 2 is the most expressed in central nervous system (Rizzu *et al.*, 2016). C9ORF72 protein function is still to be well clarified. Recent data show that C9ORF72 protein is involved in endosomal trafficking and autophagy regulation. In fact, bioinformatic analysis show that C9ORF72 protein has a high homology with DENN protein family, activators of RAB GTPase and regulators of membrane trafficking (Levine *et al.*, 2013). Moreover, C9ORF72 forms a complex that interacts with different RAB proteins regulating various steps of autophagy (Amick *et al.*, 2016; Farg *et al.*, 2014; Sullivan *et al.*, 2016). In support to C9ORF72 protein involvement in autophagy regulation, the knockdown of C9ORF72 in primary neurons and in iPSC–derived neurons from patients, leads to the accumulation of some autophagy substrates like p62, a marker of the autophagic flux (Aoki *et al.*, 2017; Webster *et al.*, 2016).

Mutations of *C9ORF72* correlated to ALS and FTD are hexanucleotide repeat expansions (G_4C_2) of a non-coding region. In particular, the expansion in variants 1 and 3 is located in an intron between two alternatively spliced exons, while for variant 2 is located in the promoter region. In physiological condition the GGGGCC expansion is less then 11 repeats, whereas in pathological condition, it could reach hundreds or even thousands repeats (Beck *et al.*, 2013; DeJesus-Hernandez *et al.*, 2011; Dobson-Stone *et al.*, 2013; Ishiura *et al.*, 2012). The length of the repeat seems to be correlated to survival and age onset: the longer is the expansion the worst is survival and the earlier is age onset (Van Blitterswijk *et al.*, 2013; Gijselinck *et al.*, 2016).

In first place, it was supposed that expansion in *C9ORF72* gene led to a loss of function, as in patients were found lower levels of C9ORF72 mRNA and protein (Waite *et al.*, 2014; Xiao, MacNair, *et al.*, 2015). However, the knockout mouse does not show motor-neuron degeneration or ALS phenotype (Koppers *et al.*, 2015). Therefore, C9ORF72 loss of function is not sufficient but it has to be associated to a toxic gain of function. Toxicity can be caused by the transcription of

sense and antisense RNA with GGGGCC or CCCCGG repeats or it can be due to the ATG independent translation (RAN-translation) of dipeptide repeated proteins.

The repeat-containing RNA forms particular secondary structures as i-motifs, hairpins, and G-quadruplexes. These structures can interact and sequester RNA-binding proteins forming nuclear RNA foci (Fratta *et al.*, 2012; Rahimov & Kunkel, 2013). The sequestration of RBP prevents their functioning, altering proper metabolism of target mRNAs (Fratta *et al.*, 2012). RNA foci can be found in neurons but also in microglia, astrocytes and oligodendrocytes.

Dipeptide repeat (DPR) proteins are translated in an ATG independent mechanism called RAN translation. They can be translated on both sense GGGGCC and anti-sense CCCCGG mRNA: in particular, poly-GA and poly-GR are uniquely translated from the sense RNA, poly-PA and poly-PR are uniquely translated from the antisense RNA, and poly-GP is translated in both directions. DPRs have a highly propensity to aggregate forming inclusions. Inclusions of different DPRs can co-occur in the same neuron, but normally there is a higher predominance of inclusions positive to sense-strand RNA-encoded DPRs (Mori *et al.*, 2013). DPR inclusions are found mainly in the brain and in lower levels in the spinal cord (Gomez-Deza *et al.*, 2015). They are localized in the cytoplasm but also in the nucleus of neurons. To date no DPR inclusions are found in microglia, astrocyte or oligodendrocyte (Mori *et al.*, 2013). Studies show that all DPRs clearance, except for poly-PR, occurs mainly through the autophagic pathway. In fact, the upregulation of genes involved in the autophagic machinery as Heat Shock Protein B8 (HSPB8) enhances the degradation of DPRs (Cristofani *et al.*, 2018).

DPR proteins and mRNAs presence activate various pathological pathways as: DNA damage, nucleolar disfunction, altered nucleo-cytoplasmatic translocation, dysregulated formation and clearance of SGs, translation inhibition and block of the UPS.

DNA damage is mediated mainly by poly-GR, that alters mitochondria functions causing oxidative stress which in turn leads to DNA damage (Choi *et al.*, 2019; Lopez-Gonzalez *et al.*, 2019). Alteration in nucleolus leads to cell death as nucleolus activity is fundamental for ribosomal-RNA metabolism. The accumulation of DPRs in this compartment leads to altered ribosomal-RNA biogenesis by interfering with rRNA splicing and maturation (Kwon *et al.*, 2014). Moreover, RNA-foci sequester nucleolin, one of the principle components of the nucleolus leading to abnormal nucleolar morphology and volume (Haeusler *et al.*, 2014; Mizielinska *et al.*, 2017; Wen *et al.*, 2014).

Nucleo-cytoplasmatic translocation is essential for the functioning of various proteins. DPRs alter these mechanisms mainly in different ways. DPRs bind and sequester in RNA foci proteins involved in regulating interaction between cargo-proteins and receptors (Zou *et al.*, 2017). Moreover, DPRs bind nucleopore proteins causing a reducing in trafficking (Shi *et al.*, 2017).
SGs dynamics are altered by the presence of DPRs. In fact, DPR proteins interact with prion-like domains of RBPs altering capacity of forming SGs (Boeynaems *et al.*, 2017). Moreover, data show that the overexpression of DPRs lead to a decrease of cytoplasmatic larger size P-bodies and an increase in the formation of SGs (Wen *et al.*, 2014).
Finally, studies show that the overexpression of DPRs alter proteostasis by blocking translation and by preventing proper UPS activity. In fact, DPRs can either bind mRNAs blocking their interaction with translation machinery or they can bind and sequester initiation and elongation factors or even ribosomal subunits (Green *et al.*, 2017; Kanekura *et al.*, 2016). Data show that DPRs also block UPS by directly binding and sequestering proteasome 26 subunits (Guo *et al.*, 2018).

FUS

Fused in sarcoma (FUS) is a DNA/RNA binding protein that belongs to the FET family. In 1993, *FUS* gene was discovered as an oncogene in malignant human mixoid liposarcoma (Crozat *et al.*, 1993). In 2009, the first mutations of *FUS* where identified and correlated to ALS patients (Kwiatkowski *et al.*, 2009; Vance *et al.*, 2009). *FUS* mutations are correlated mainly to fALS. In particular mutations in *FUS* are present in 2.8% of European fALS cases and 6.4% of Asian-fALS cases. Mutations are also correlated to a small percentage of sALS cases in both European and Asian patients (Zou *et al.*, 2017). *FUS* gene is collocated in chromosome 16 and it encodes a ubiquitous protein of 526 amino acid. FUS protein mainly localizes in the nucleus but in lower levels it can be found distributed in cytoplasm. FUS shuttling from one compartment to the other is possible thanks to NES and NLS present in its structure. FUS structure also presents a RRM and a zinc finger motif that permit binding with RNA and DNA. Moreover, FUS structure presents a prion-like domain in its N-terminal domain that increases its aggregating propensity (Morohoshi *et al.*, 1998).
FUS is involved in different physiological functions: it is involved in different point of RNA metabolism; it regulates DNA transcription; it concurs in DNA damage response. FUS RNA-targets are different from TDP-43 targets (Colombrita *et al.*, 2015; Lagier-Tourenne *et al.*, 2012). It regulates targets transcription by binding to ssDNA motifs in the promoter region of certain

genes and accumulates near the transcription start site (TSS), where it binds and recruits RNA polymerase II (Tan & Manley, 2012). Moreover, FUS is implicated in RNA splicing, maturation and translocation in cytoplasm (Alliegro & Alliegro, 1996; Fujii *et al.*, 2005). Finally, FUS is also involved in RNA degradation by regulating the synthesis of miRNAs in Drasha complex (Gregory *et al.*, 2004). Many studies demonstrated that FUS also has an important role in DNA damage repair. In fact, FUS is one of the first protein recruited in DNA damage site, where it is involved in the activation of ATM/γH2AX signaling pathways and it binds histone deacetylase 1 (HADC1) (Wang *et al.*, 2013). FUS promotes the interaction between a single-stranded oligonucleotide and a homologous superhelical DNA to form a D-loop, an essential step in DNA double-strand break repair (Baechtold *et al.*, 1999).

To date, more then 50 mutations in FUS gene have been correlated to ALS. Most of mutations are missense, but there are also rare insertions, deletions, splicing, and nonsense mutations (Lattante et al. 2013). Most of mutations are found in the NLS leading to nucleus depletion (Niu *et al.*, 2012; Vance *et al.*, 2013). Other mutations are in the prion-like domain increasing the aggregation propensity. The increase of aggregation tendency is enhanced also by the increase of FUS cytoplasmatic levels due to its mis-localization (Nomura *et al.*, 2014). FUS-ALS is characterized by the presence of inclusions positive to FUS and negative to TDP-43. It is still debated if FUS aggregates are caused or concur to increase FUS mis-localization.

FUS pathological mechanisms are due to FUS loss of function, caused by its mis-localization, and FUS gain of function. FUS loss of function contribute to ALS disease is still debated. In fact, the FUS knockout mouse model does not present a neurodegenerative phenotype, conversely a Drosophila knockdown model presents neurodegeneration and locomotive defects (Kino *et al.*, 2015; Sasayama *et al.*, 2012). Nevertheless, there are many evidence of FUS toxic gain of function. In fact, a FUS mouse model overexpressing FUS wild type has a neurodegenerative phenotype characterized by FUS positive cytoplasmatic insoluble inclusions (Mitchell et al. 2013).

VCP

Many other genes mutated are correlated to a small fraction of ALS cases. One of these genes is *VCP,* that encodes for Valosin Containing Protein (VCP), an AAA$^+$ (ATPases Associated with diverse cellular Activities) protein. VCP was associated to ALS in 2010 (Johnson *et al.*, 2010). Different mutations in VCP were found to be associated to about 2% of fALS cases. VCP is involved in many pathways of the PQC system like control of misfolded protein and their degradation, and autophagy regulation. VCP is also involved in DNA damage regulation, damaged organelles

degradation, membrane fusion and NF-κB activation (Franz *et al.*, 2016; Hemion *et al.*, 2014; Meyer, 2005; Neal *et al.*, 2017; Schweitzer *et al.*, 2016). VCP function is regulated by its ATPase activity and its interaction with various co-factors and adaptors, which concur to its localization in different cellular compartments, mediate its binding with various targets and cooperate with its activity. VCP mutants are shown to lose some of VCP functionality. An extensive description of VCP will be provided below in the Valosin Containing Protein chapter.

PATHOGENIC MECHANISMS

ALS main pathogenic molecular mechanism is not known, yet probably ALS is caused by an interplay between different pathogenic cellular pathways that are not mutually exclusive. Moreover, it is still not known if there is a relation between them or if they are caused by an upstream alteration. ALS pathogenic mechanisms have been previously outlined in the description of the main ALS mutations. However, ALS possible causative mechanisms will be described to be better defined.

OXIDATIVE STRESS

Oxidative stress is caused by an imbalance between anti-oxidant cell capacity and the production of ROS. Oxidative stress was firstly studied in relation to SOD1-mutants discovery, even if after it was discovered that SOD1 toxicity is due to a gain of function rather than a loss of function (Reaume *et al.*, 1996; Rosen *et al.*, 1993). Nevertheless, oxidative stress is considered a pathological mechanism of ALS as there are various signs that show the presence of an oxidative imbalance. Oxidative stress results in accumulation of ROS with alteration in proteins, DNA and RNA species. Data on CSF and on serum analysis from ALS patients show an increase of damaged products induced by ROS (Vance *et al.*, 1998; Lyras *et al.*, 1996; Mitsumoto *et al.*, 2008).

The presence of ROS leads to oxidative post-translational modifications of proteins, altering their solubility and folding, and triggering the formation of insoluble aggregates (Barber & Shaw, 2010; Beckman *et al.*, 2001; Bonafede & Mariotti, 2017; Grune *et al.*, 2004). Moreover, ROS alters RNA species. In fact, data show that oxidation of mRNA is already present in MNs and spinal cord oligodendrocytes in pre-symptomatic SOD1 mice (Chang *et al.*, 2008).

Another signal of oxidative stress in ALS patients is the very low glutathione level detected in the brain tissue compared to controls (Iguchi *et al.*, 2012).

MITOCHONDRIAL DYSFUNCTION

Alterations in mitochondrial functions have been described in the spinal cord and the skeletal muscle of ALS patients (Hirano *et al.*, 1984; Wiedemann *et al.*, 1998, 2002). In particular, it was reported reduced levels and increased mutations of mitochondrial DNA, and alteration in respiratory chain complexes activity. Moreover, studies show altered calcium homeostasis in SOD1-G93A mice caused by altered mitochondrial calcium buffering capacity (Petri *et al.*, 2006). The exact mechanism of mitochondrial disfunction is unclear. However, in SOD1-ALS models it has been proposed that misfolded SOD1 could aggregate and prevent mitochondrial protein import by blocking TOM/TIM protein import machines (Liu *et al.*, 2004; Wong *et al.*, 1995).

IMPAIRMENT OF AXONAL TRANSPORT

MNs are polarized cells with a very long axon. Therefore, proteins and organelles have to be transported further then other cell types. This increases the importance and the critical role of axonal transport in MNs. Data on ALS patients show neurofilament accumulation and alteration in organelle transport that are signs of impaired axonal transport (Breuer *et al.*, 1987; Hirano *et al.*, 1984; Julien, 1997; Julien *et al.*, 1998). Mutant-SOD1 mouse models also present neurofilament accumulation along with impaired anterograde transport (Zhang et al. 1997). In these models the cause of impairment of axonal transport could be either: the increase of inflammatory and excitotoxic mediators, or the overload of kinesin-associated protein caused by misfolded SOD1 that impede the transport of the physiological cargos (De Vos *et al.*, 2008). Moreover, Nicolas and colleagues found mutations in *KIF5A* gene associated to ALS (Nicolas *et al.*, 2018). *KIF5A* encodes for a kinesin that is part of a complex involved in axonal transport of organelles like mitochondria and granules composed of RNA and RNA binding proteins. Nicolas and colleagues speculate that *KIF5A* mutations cause disease by disrupting axonal transport as KIF5A has a central role in axonal transport.

EXCITOTOXICITY

Excitotoxicity is due to an excessive activation of glutamate receptors, that it may be the result of the failure of glutamate clearance in the synaptic boutons, an increase in glutamate release, or the increase in postsynaptic sensitivity to glutamate. Excitotoxicity results in a massive Ca^{2+} influx that activates different cellular pathways leading to activation of proteolytic enzymes, increase in ROS levels, alteration in mitochondrial functions and energy imbalance (Arundine & Tymianski, 2003).

Evidences of the presence of excitotoxicity in ALS patients are various. The most important proofs are the strong increase in glutamate levels present in ALS patients CSF and the positive effects that Riluzole treatment has (Ludolph & Jesse, 2009; Perry *et al.*, 1990; Shaw *et al.*, 1995).
One of the hypothesis of the possible cause of glutamate increase could be the decreased levels of the excitatory amino acid transporters (EAAT2) reported in ALS patients (Foran & Trotti, 2009). Physiologically EAAT2 reduces glutamate concentration at synapsis level after its excitatory action. The reduced levels of EAAT2 is probably correlated to an aberrant EAAT2 mRNA (Guo *et al.*, 2003; Lin *et al.*, 1998). Another hypothesis on the increase of glutamate levels can be found in a higher rate of glutamate release. Milanese and colleagues showed that mutant-SOD1 mouse models present higher levels of Ca^{2+} in spinal cord nerve terminals. This is correlated to activation of a kinase, calmodulin, which in turn activates synapsin I phosphorylation and subsequently glutamate release (Milanese *et al.*, 2011).

PROTEIN AGGREGATION

As mentioned above, one of the main characteristics of ALS is the presence of insoluble inclusions (Al-Chalabi *et al.*, 2012). Inclusions originate by the misfolding of protein that expose hydrophobic residues promoting interactions with other misfolded proteins. Protein improper interaction with other proteins leads to formations of oligomers which then sequester other cellular proteins eventually forming aggregates (Rowinska-Zyrek *et al.*, 2015; Soto & Estrada, 2008). The contribute of aggregates to the development of the disease is still very debated. Some studies claim that aggregates are beneficial for cell as they protect from the presence of oligomers or misfolded proteins, that are the toxic species (Ciechanover & Kwon, 2015; Guo *et al.*, 2011). Conversely, other studies show that disorganized aggregates lead to cell toxicity in many ways: by binding to proteins that lose their functionality (Radford & Dobson, 1999); by interacting with components of cell membrane (Hirakura & Kagan, 2001; Kourie & Shorthouse, 2000; Lin *et al.*, 2001) or of organelles membranes; or by causing the release of Ca^{2+} and oxidative stress (Ross, 2002).
Inclusions can be positive to mutated misfolded proteins or to wt proteins. As described above most of ALS patient inclusions are positive to wt TDP-43, in particular in all SOD1-negative familial ALS patients (Mackenzie, 2007; Neumann *et al.*, 2006).
The presence of these insoluble aggregates can also be a consequence of alteration of the PQC system. The misfunctioning of the system can be due to different reasons: overwhelming of the degradative pathways; the interaction with misfolded proteins that leads to sequestration of

proteins involved in the system or damage of organelles like lysosomes or proteasome; or the mutations of genes that encode for proteins involved in the system as VCP, ubiquilin and p62.
The alteration of degradative pathways as UPS and autophagy can cause and also be caused by the increase of aggregates concentration. In fact, data show in ALS a decrease in autophagy and UPS functionality due to different reasons as: interaction with aggregates and mutations in genes that encode for proteins involved in the system. An extensive description of the UPS and autophagic pathway involvement with ALS will be provided below in the Protein Quality Control chapter.

ENDOPLASMIC RETICULUM STRESS

Accumulations of misfolded proteins activates Endoplasmic Reticulum Stress Response (ERAD) and the Unfolded Protein Response (UPR) that are initially protective but if they are prolonged they can trigger apoptosis (Kaufman, 2002). In sALS patients and in mutant-SOD1 models UPR markers are found upregulated showing an activation of the pathway (Atkin *et al.*, 2008; Saxena *et al.*, 2009). The contribute of the ER stress seems non to be a primary pathogenic mechanism, but a consequence of misfolded proteins or the ER-Golgi transport disruption (Chen & Madura, 2005).

ABNORMAL RNA PROCESSING

Alteration of physiological RNA dynamics have been discovered in ALS since the association to ALS of mutations in genes that encode for RNA binding proteins as TDP-43 and FUS. As previously described, mutations in these genes lead to a mis-localization and aggregation of the RNA binding proteins. This is correlated to a loss of function and an altered RNA metabolism.

NEUROINFLAMMATION

Neuroinflammation is a pathological mechanism in many NDs. The main effectors of neuroinflammation are microglia and astrocytes, in fact they generally coordinate the immune response after neurons injury. In ALS, it has been reported an increased activation of microglia and astrocytes analysing spinal cord tissue and CSF of effected patients. Moreover, it has been detected an increased infiltration in nervous system of T cells and an increase in the levels of proinflammatory mediators (Henkel *et al.*, 2004; Kuhle *et al.*, 2009; Sta *et al.*, 2011; Troost *et al.*, 1990; Zhao *et al.*, 2013). Analysis in mutant-SOD1 mice also revealed an increase of inflammatory-related molecules levels in particular in the late stages of the disease (Ferraiuolo

et al., 2007; Lincecum *et al.*, 2010). In fact, during disease progression, SOD1 is released by affected neurons triggering the switch of M2 microglial cells (neuroprotective and anti-inflammatory) into M1 microglial cells (pro-inflammatory and neurotoxic cells that secerns ROS and cytokines) (Almer *et al.*, 1999; Appel *et al.*, 2011; Y. Zhang *et al.*, 2009). Moreover, data show that astrocytes decrease EAAT2 levels leading to an increase in the concentration of glutamate with a resulting excitotoxicity (Howland *et al.*, 2002).

THERAPIES

To date there is no cure that stops the progression of ALS. All therapeutic approaches that are used at the present aim to extend survival by decreasing progression rate and to improve clinical features making the disease more bearable. The only two drugs that are approved by Food and Drug Administration (FDA) are Riluzole, in 1995 and Edaravone (RadicavaTM) only recently. Riluzole slows down the progression of the disease but with a modest efficacy, in fact, it increases survival of approximately 3 months and only in 9% of the cases it increases the probability of surviving one year (Dharmadasa & Kiernan, 2018). Riluzole inhibits glutamate release reducing the excitotoxicity in neurons, but its specific mechanism of action is still unknown (Miller *et al.*, 2012). Edaravone is a strong antioxidant drug that eliminates lipid peroxides and hydroxyl radicals, but its mechanism of action is also still uncertain (Ikeda & Iwasaki, 2015). Its use, thought, is limited to patients in early disease stages (within 2 years from the onset) with a forced vital capacity of >80%, which is around 7% of ALS cases (Kiernan, 2018).

As pharmacological approach can still only partially decrease progression and not cure the disease, gene therapy and stem cells therapy have started to be considered and studied (Bonafede & Mariotti, 2017). To date, these two strategies are studied in animal models and in some clinical trials.

Gene therapy approach is the use of antisense oligonucleotides (ASOs) that leads to RNA interference. Studies in mouse model of ALS-SOD1 show that the use of ASO therapy leads to a significant delay of disease progression. Phase I clinical studies are testing ASO on patients to reduce the expression of mutated SOD1. The limit of this strategy is patient compliance as the infusion of ASO should be continuous and administration is intrathecal (Smith *et al.,* 2006). Stem cells therapy consist in the use of Neural Stem Cells (NSCs) that are self-renewing and multipotent with the ability to promote the formation of novel neuronal cell. In fact, NSCs have neurotrophic and anti-inflammatory capacities by producing and secreting immunomodulatory molecules that

regulate cell migration, cell growth, and cell differentiation (Mazzini *et al.*, 2016). To date some studies on animal models have been carried out with promising results. (Faravelli *et al.*, 2014).

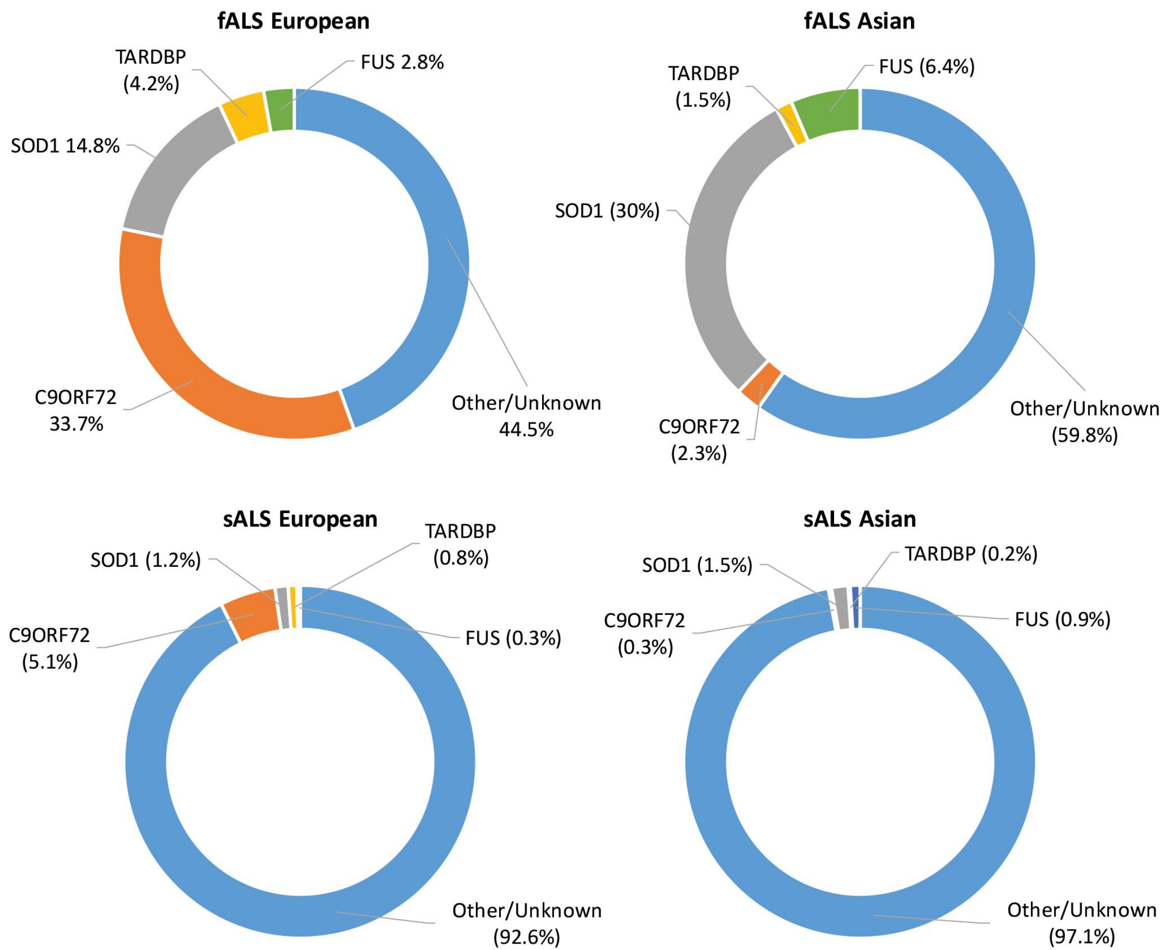


Figure 2 Proportion of the four most commonly mutated genes in Asian and in European ALS populations (Mejzini et al., 2019)

Locus	Gene	Protein	Protein function	Mutations	Proportion of ALS		Date of discovery
					Familial	Sporadic	
21q22.1	*SOD1*	Cu–Zn superoxide dismutase	Superoxide dismutase	>150	20%	2%	1993 (ref. 2)
2p13	*DCTN1*	Dynactin subunit 1	Component of dynein motor complex	10	1%	<1%	2003 (ref. 52)
14q11	*ANG*	Angiogenin	Ribonuclease	>10	<1%	<1%	2006 (ref. 141)
q36	*TARDBP*	TDP-43	RNA-binding protein	>40	5%	<1%	2008 (refs 67 and 142)
16p11.2	*FUS*	FUS	RNA-binding protein	>40	5%	<1%	2009 (refs 68 and 69)
9p13.3	*VCP*	Transitional endoplasmic reticulum ATPase	Ubiquitin segregase	5	1–2%	<1%	2010 (ref. 44)
10p15-p14	*OPTN*	Optineurin	Autophagy adaptor	1	4%	<1%	2010 (ref. 42)
9p21-22	*C9orf72*	C9orf72	Possible guanine nucleotide exchange factor	Intronic GGGGCC repeat	25%	10%	2011 (refs 8 and 77)
Xp11.23-Xp13.1	*UBQLN2*	Ubiquilin 2	Autophagy adaptor	5	<1%	<1%	2011 (ref. 40)
5q35	*SQSTM1*	Sequestosome 1	Autophagy adaptor	10	<1%	?	2011 (refs 41 and 143)
17p13.2	*PFN1*	Profilin-1	Actin-binding protein	5	<1%	<1%	2012 (ref. 144)
12q13.1	*HNRNPA1*	hnRNP A1	RNA-binding protein	3	<1%	<1%	2013 (refs 70 and 71)
5q31.2	*MATR3*	Matrin 3	RNA-binding protein	4	<1%	<1%	2014 (ref. 76)
2q36.1	*TUBA4A*	Tubulin α-4A chain	Microtubule subunit	7	<1%	<1%	2014 (ref. 145)
22q11.23	*CHCHD10*	Coiled-coil-helix-coiled-coil-helix domain-containing protein 10	Mitochondrial protein of unknown function	2	<1%	<1%	2014 (ref. 146)
12q14.1	*TBK1*	Serine/threonine-protein kinase TBK1	Regulates autophagy and inflammation	10	?	?	2015 (ref. 147)

Figure 3 most common mutations in ALS (Taylor et al., 2016)

Figure 4 ALS pathogenic mechanisms (Mancuso & Navarro, 2015)

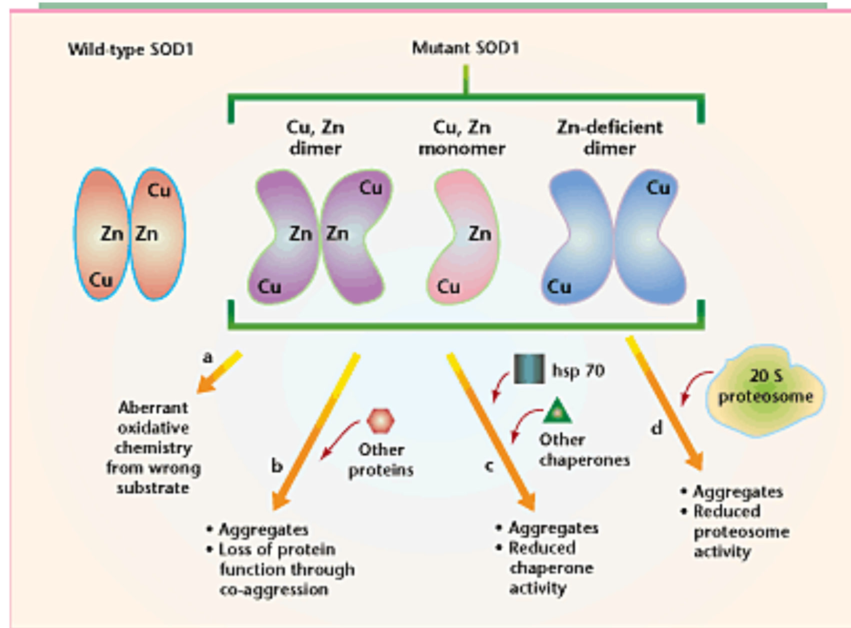


Figure 5 mutant-SOD1 pathogenic mechanisms (Cleveland & Liu, 2000)

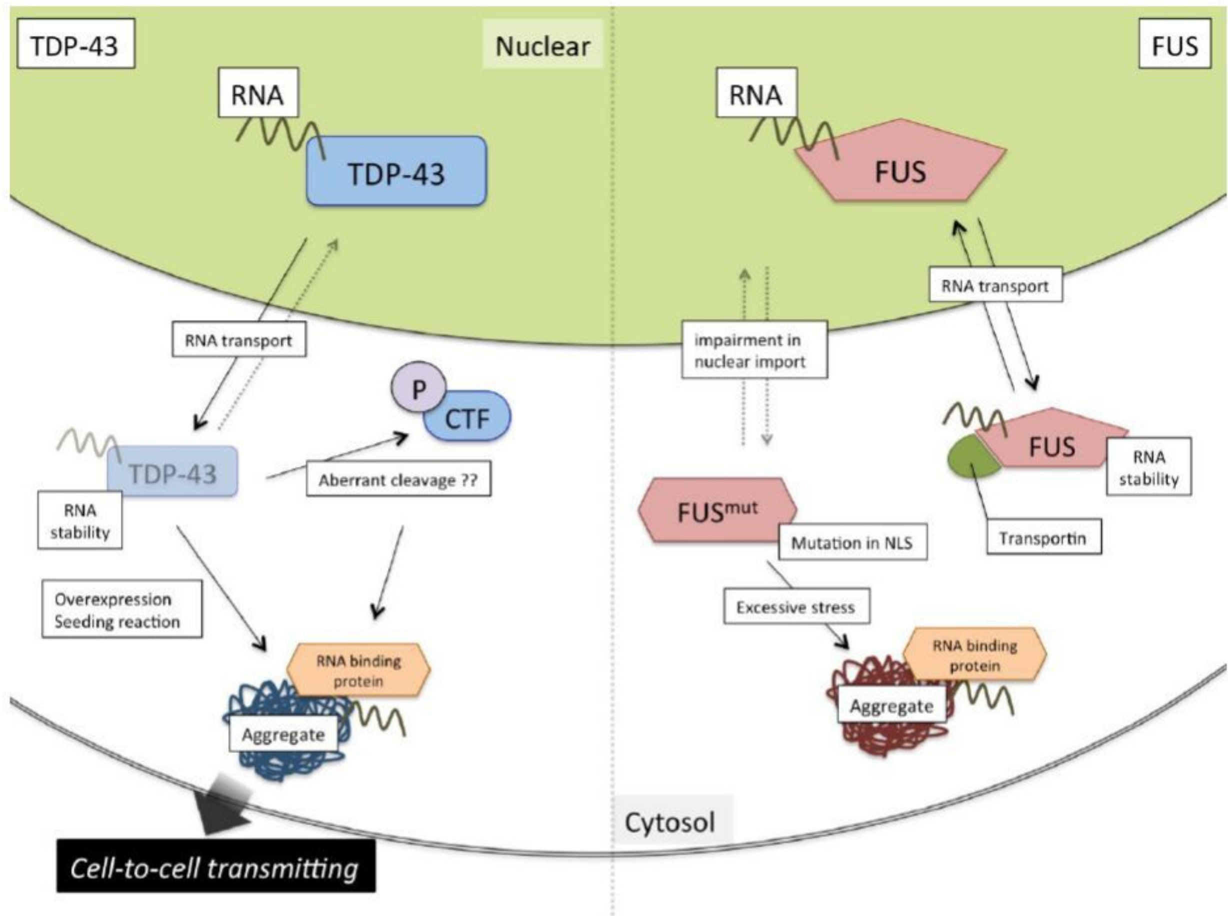


Figure 6 TDP-43 and FUS pathogenic mechanisms (Tsubota et al., 2016)

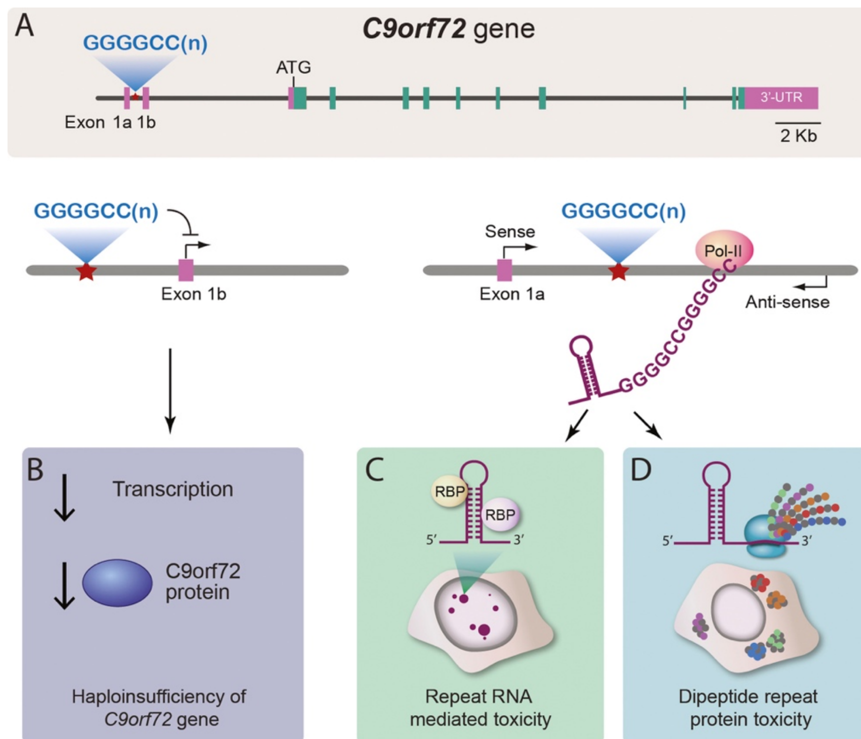


Figure 7 C9ORF72 pathogenic mechanisms (Gitler & Tsuiji,2016)

VALOSIN CONTAINING PROTEIN

Valosin Containing Protein (VCP) or p97 is an AAA^+ protein. It is a highly conserved protein as homologs are found in all species: it is called Cdc48 in yeast, archaea and *Caenorhabditis elegans* and TER94 in drosophila. In 1982, Cdc48 was identified for the first time in *S. cerevisiae*. In *S. cerevisiae,* Cdc48 function as a regulator of cell cycle in particular it is an inhibitor of cell cycle in G2-M transition stage (Moir *et al.*, 1982). In 1987 was identified VCP, the mammalian homolog (Koller & Brownstein, 1987). It was thought to be the precursor of a small peptide valosin that turned out to be a purification artifact *(Gill et al.,* 1989).

VCP belongs to type II AAA^+ ATPase family. Mammalian AAA^+ proteins are involved in degradation pathways that serve in different processes as in the remodeling of protein-DNA complexes, of protein-protein complexes and of protein aggregates. They use ATP hydrolysis energy to generate mechanical forces to act on their substrates. AAA^+ proteins structure is generally a homoexamer where monomers bind together forming a ring structure. They are classified according to the number of the ATPase domain present in the structure. Type I AAA^+ ATPase has only one ATPase domain, while type II ATPase has two ATPase domains in tandem.

VCP is highly ubiquitously expressed protein. It is mainly diffused in the cytoplasm, but a fraction localizes on different organelles membranes like mitochondria, ER, Golgi and endosomes (Acharya *et al.*, 1995; Latterich *et al.*, 1995; Rabouille *et al.*, 1995; Ramanathan & Ye, 2012; Xu *et al.*, 2011). Another smaller fraction can be found in the nucleus, where it cooperates in DNA remodeling and damage repair (Madeo *et al.*, 1998). The localization in different intracellular compartments is possibly through the binding between VCP and a large number of specific adaptors.

In each compartment VCP is involved in different pathways. It has a key role in PQC system by segregating proteins from membranes, from chromatin, from protein complexes or from protein aggregates and by addressing them to UPS. Moreover, VCP is involved in the degradation of organelles like mitochondria and lysosome through the autophagic pathway. Furthermore, it cooperates in ribosome quality control (RQC), in the clearance of SGs, in endosomal trafficking, in Golgi and nuclear envelope reassembly and in the activation of NF-kB. Finally, VCP is implicated in DNA replication, transcription and repair. The involvement of VCP in so many and various pathways is possible thanks to the mediation of different co-factors, that cooperate with VCP functioning, and adaptors that recruit VCP in different cellular compartment.

Altered expression or mutations of *VCP* gene, collocated on chromosome 9p13.3-12, are correlated to pathological phenotypes. VCP upregulation has been detected in some types of cancer, for which VCP inhibitors have been developed. In fact, in cancer cells characterized by high VCP mRNA levels, the VCP protein could be a possible target for effective therapies (Anderson *et al.*, 2015). Instead, mutations and misfunctioning of VCP are correlated to degenerative diseases among which the most recurrent are Inclusion Body Myopathy associated with Paget's disease of the bone and Frontotemporal Dementia (IBMPFD) and ALS.

STRUCTURE

VCP structure resembles the structure of type II AAA$^+$ ATPase. As shown in Figure 8, VCP functional structure is a homoexamer formed by monomers with two ATPase domain each, that work in a strictly coordinated manner. Every monomer is composed by a N-terminal domain, two ATPase domain (D1 and D2) and an unstructured C-terminal tail. The N-terminal domain role is to bind co-factors and adaptors coordinating VCP functioning. ATPase domain D1 binds to N-terminal and the D2 domains through flexible linkers. D1 main role is to assembly the hexamer using ATP hydrolysis. However, studies that use D2 inhibitors, show that D1 also contribute in a small percentage (~30%) to VCP functioning (Anderson *et al.*, 2015; Chou *et al.*, 2014; Wang *et al.*, 2004). Whereas, D2 is the driving force of VCP, in fact its ATP hydrolysis is the main force of VCP activity. D2 can be found bound to a molecule of ADP, ATP or in some cases it can be in a no binding state (Apo state) (Huyton *et al.*, 2003). Finally, C-terminal binds to a small set of co-factors and adaptors mediating VCP activity, but its main role is to participate with D2 ATP hydrolysis by interacting with the other monomers present in the hexamer (Hänzelmann & Schindelin, 2016; Niwa *et al.*, 2012).

Six VCP monomers bind to form a hexamer, which is the VCP functioning structure. The VCP hexamer arrange itself in a mushroom-like shape where the ATPase domains form two rings stack on top of each other. One ring is formed by D1 domains (D1 ring) and the other by D2 domains (D2 ring). In D1 ring there is a restriction site formed by six histidine residues, also known as Hys-gate. N-D1 ring is larger than D2 ring as it has the N-terminal domains laterally attached (Banerjee *et al.*, 2016; Brunger & DeLaBarre, 2003; Davies *et al.*, 2008; Huyton *et al.*, 2003; Peters *et al.*, 1990; Schuller *et al.*, 2016; X. Zhang *et al.*, 2000). The D2 ring has two pores: a smaller pore (loop1) and a larger pore (loop2) that contains positively and negatively charged residues.

To permit its activity, VCP is subjected to conformational changes regulated by a ATP hydrolysis cycle (Beuron *et al.*, 2006; DeLaBarre & Brunger, 2005; Rouiller *et al.*, 2002; Tang *et al.*, 2010). ATP/ADP binding and hydrolysis produce the energy that enhances conformational changes. The mechanical force generated by these conformational changes influence in turn substrate molecules stability and function. The conformational changes are mainly in the N-D1 ring: the N-terminal domains are co-planar to D1 ATPase domains when D1 domains bind ADP (down-conformation), whereas N-terminal domains change into 'up' conformation when D1 domains change from a Apo state to a ATP-bound state binding ATP (Banerjee *et al.*, 2016). D1 bond to nucleotides is highly regulated: in each hexamer there are always at least three ADP bound to D1 ring. These chemical bonds prevent ATP binding which has a higher affinity for D1 in Apo state (Brunger & DeLaBarre, 2003; Tang *et al.*, 2010; Tang & Xia, 2013). In D2 ring ATP hydrolysis cycle leads to other conformational changes with the purpose of regulating D2 pore opening: loop1 and loop2 modify their conformations resulting in D2 more open pore with flexible loops for substrate interactions. It is still debated if the opening of D2 pore is due to ATP binding or to its hydrolysis (Banerjee *et al.*, 2016; Brunger & DeLaBarre, 2003).

CO-FACTORS AND ADAPTORS

VCP is implicated in a large number of pathways and cellular processes despite having the ability of performing only one mechanism: the hydrolysis of ATP. This is only possible thanks to VCP binding with many different co-factors and adaptors. Co-factors are normally enzymes that cooperate with VCP activity by modifying VCP substrates with N-glycan or Ub-conjugates. In fact, co-factors as well as adaptors, may harbor different structural motif that can be involved in substrate processing. They can also have substrate recognition motif like Ub-binding domains which permit the specific binding to different Ub-chains, in particular K48-, K63-linked chain.

Co-factors and adaptors can be classified in two groups depending on what part of VCP they interact with. Nearly all co-factors and adaptors bind to VCP N-terminal domain, but a smaller set bind to the C-terminal tail. The motifs that recognize and bind the N-terminal domain are: ubiquitin regulatory X (UBX), UBX-like (UBX-L), VCP interacting motif (VIM), VCP binding motif (VBM) or SHP (binding segment 1) motif (Boeddrich *et al.*, 2006; Bruderer *et al.*, 2004; Schuberth & Buchberger, 2008). Most of these motifs target the same binding site present on N-terminal domain, for these reasons the binding to VCP is retained mutually exclusive (Jentsch & Rumpf,

2007; Meyer *et al.*, 2000). However, there are some co-factors/adaptors that can bind simultaneously, collaborating in their activity like UFD1-NPL4 complex (Isaacson *et al.,* 2007).

UBX motif is a globular domain of 80 aa that is structurally homologous to ubiquitin. To date 12 proteins are known to have UBX motif. Of these 12 proteins 5 also present an additional ubiquitin-associated (UBA) domain that recognizes and binds polyubiquitylated substrates (Meyer and Weihl 2014). They all have different cellular role, for example UBXD8, FAF1, SAKS1 are involved in ERAD pathway (LaLonde & Bretscher, 2011; Madsen *et al.*, 2011) UBXD7 cooperates in the repair of damaged protein (Huang *et al.,* 2016); and p47 contributes in membrane fusion (Kondo *et al.*, 1997). VIM and VBM have a single alfa-helix structure with a linear sequence where the arginines are essential for the interaction with VCP. VIM sequence is RX_5AAX_2R, while VBM is a highly polarizing linear sequence motif (RRRRXXYY). VIM is found in different proteins as gp78, VIMP and SVIP (Ballar *et al.*, 2006, 2007; Ye *et al.*, 2004). VBM is found in ataxin-3, UFD2 and HRD1 (Boeddrich *et al.*, 2006). The SHP motif is a short module with a high concentration of hydrophobic residues. It has been identified in proteins like UFD1-NPL4 (Meyer *et al.*, 2000) and Derlin-1 (Greenblatt *et al.*, 2010; Lilley & Ploegh, 2004; Ye *et al.*, 2004), but also in protein containing UBX motif like p47 (Kondo *et al.*, 1997). These proteins probably use a bipartite mechanism to form a complex with VCP by binding in different position of VCP structure.

C-terminal tail motif is PNGase/UBA (PUB) domain that binds the sequence D_3LYG. The main proteins that harbor PUB domain are: a PNGase, that functions as an enzyme for the removal of N-glycan from misfolded glycoproteins present on the ER (Blom *et al.*, 2004); and Phospholipase A2 Activating Protein (PLAA), that is implicated in a large number of pathways like the processing of misfolded mitochondria outer-membrane proteins (Wu *et al.*, 2016), endosomal trafficking (Ren *et al.*, 2008), ribophagy (Ossareh-Nazari *et al.*, 2010) and lysophagy (Papadopoulos *et al.*, 2017).

Generally, VCP hexamer binds to co-factors/adaptors in a sub-stoichiometric manner (Hänzelmann & Schindelin, 2016). This can be caused by steric hindrance that prevents a 6:6 binding and can also enable VCP to interact concurrently with other different co-factors/adaptors inducing a specific cellular response.

FUNCTION

VCP by binding different co-factors and adaptors and by forming very dynamic complexes, is involved in several pathways of the PQC system, thus is an essential component of the cellular proteostasis (Meyer *et al.*, 2012). In general, VCP activity can be summarized in: the energy from ATP hydrolysis is used to segregate polypeptides from protein complexes, from protein aggregates or from cellular structures such as membranes or chromatin. After polypeptides are released, they are degraded through the UPS or transported into the nucleus to promote gene expression in response to specific stimulating cues.

ENDOPLASMIC RETICULUM-ASSOCIATED DEGRADATION

Endoplasmic reticulum-associated degradation (ERAD) is a cellular process used to control proteins formation in the ER. It can be divided in three types, depending on which type of protein is involved. It is called ERAD-L for luminal proteins, ERAD-M for membrane proteins and ERAD-C for cytosolic domain of membrane proteins (Christianson & Ye, 2014; Xudong Wu & Rapoport, 2018). Proteins that are unproperly folded, are retro-translocated to the cytoplasm, where they are ubiquitinated and degraded through the UPS. VCP promotes proteins retro-translocation using the energy ATP hydrolysis (Stein *et al.*, 2014; Ye, 2006).

Proteins are retro-translocated by a complex composed by membrane proteins HRD1, HRD3, and other accessory proteins like DERLIN1 and HERP that all together form the protein-conducting channel (Baldridge & Rapoport, 2016; Schoebel *et al.*, 2017). The complex UFD1-NPL4 recruits VCP to the ER where there are ubiquitinated substrates (Ye *et al.*, 2004). Also UBX2 recruits VCP to the ER membrane and facilitates the interaction between VCP and HRD1 which is a membrane-anchored ubiquitin ligase that ubiquitinates substrates. Once HRD1 has polyubiquitinated substrates, VCP binds to gp78, an E3 ligase that mediates the substrates degradation through the UPS (Ballar *et al.*, 2006; Gauss *et al.*, 2006; Kikkert *et al.*, 2004).

ORGANELLE DEGRADATION

VCP is also involved in the turnover of mitochondrial membrane proteins. In fact, VCP coordinates the removal of mis-folded proteins from the mitochondria outer membrane (Hemion *et al.,* 2014). Moreover, by the removal of proteins on the outer membrane of the mitochondria, VCP participates to damaged mitochondria degradation via the autophagic pathway, also known as mitophagy. (Tanaka *et al.*, 2010). The complex UFD1-NPL4 recruits VCP to the surface of

mitochondrial membrane. In mitophagy, the damaged mitochondria stabilizes PIKN1 exposure on outer membrane by inhibiting its degradation. PINK1 regulates the ubiquitination of substrates and recruits various E3 ubiquitin ligases like Parkin, that amplifies ubiquitination of proteins present on the damaged organelle. Some of these proteins, like mitofusin, have K48-Ub chains. These proteins are substrate of VCP and are eliminated via UPS. The degradation of these proteins is necessary for mitochondria degradation (Tanaka *et al.*, 2010).

Furthermore, VCP in similar mechanism cooperates to the degradation of damaged lysosomes, known as lysophagy. VCP forms a complex with YOD1, UBXD1 and PLAA. Together they selectively remove K48-linked Ub-conjugates from damaged lysosomes promoting their degradation (Papadopoulos *et al.*, 2017). An extensive description of lysosome degradation will be provided below in the Protein Quality Control-Lysosome chapter.

RIBOSOME-ASSOCIATED DEGRADATION

VCP cooperates in the removal of aberrant nascent proteins translated from a defective mRNA. When there is an altered mRNA translation the ribosome stalling occurs. The defective mRNA is decomposed and the aberrant polypeptide needs to be degraded. Various factors promote the release of the subunits of the stalled ribosome, allowing the recruitment of a ribosome-associated ubiquitin ligase (listerin 1) to polyubiquitinate the aberrant nascent polypeptide. Then the polyubiquinated substrate and the ribosome factor Rqc1 recruit VCP bound to the complex UFD1-NPL4 that promotes defective polypeptide degradation through the UPS (Brandman *et al.*, 2012; Defenouillère *et al.*, 2013; Verma *et al.*, 2013).

REGULATION OF AUTOPHAGY

Data show that VCP is also implicated in another degradation pathway: autophagy, that targets degradation of misfolded proteins, protein aggregates or damaged organelles through lysosomes. VCP contribute in these processes is controversial. In fact, some studies demonstrate that VCP is a positive regulator of autophagic flux, whereas others demonstrate that VCP inhibits autophagic degradation. In support to a positive contribute of VCP in autophagy studies on *S. cerevisiae* show that a Cdc48 co-factor named SHP1P binds the autophagic regulator ATG8 to promote autophagy. However it is not known if the human analogue has the same function (Krick *et al.*, 2010). Conversely, a study demonstrated that VCP inhibition leads to an increase in the clearance of autophagic substrates rather than a decrease, suggesting an inhibitory role of VCP (Anderson *et al.*, 2015).

CHAPERONE ACTIVITY

Various studies demonstrate that VCP acts in a chaperone like way by segregating misfolded proteins from cytoplasmatic aggregates and addresses them to the proteasome for degradation, or by simply preventing protein aggregation (Gallagher *et al.*, 2014; Neal *et al.*, 2017; Nishikori *et al.*, 2008; Yamanaka *et al.*, 2004). In fact, by studying VCP effects on heat-denatured firefly luciferase, it was demonstrated that VCP wild type is involved in the re-folding and the consequently reactivation of luciferase. Moreover, it was shown that VCP ATPase activity is essential for this function. Indeed, mutants with a decrease ATPase activity fail to reactivate luciferase (Kobayashi *et al.,* 2007).

VCP was shown to co-localize with preformed insoluble aggregates until they completely disappear (Kobayashi *et al.*, 2007). VCP un-aggregating role suggests that its activity could be critical for degradation of aggregation-prone proteins (Gallagher *et al.*, 2014). In support to this, recently Ghosh and colleagues show that VCP functions as a disaggregase chaperone by disassembling polyglutamine-expanded Huntigntin-exon1 aggregates (Ghosh *et al.*, 2018). However, it was also shown that VCP enhances both aggregate formation and clearance. In fact, if there is a high concentration of soluble aggregate-prone proteins, VCP catalyzes protein aggregation. While, VCP catalyzes aggregates degradation when there is a low concentration of soluble aggregate-prone proteins as in presence of pre-formed aggregates (Kitami *et al.*, 2006; Kobayashi *et al.*, 2007).

CHROMATIN-ASSOCIATED DEGRADATION

One of VCP central function is the removal of proteins from chromatin to allow the access of repair factors in sites of DNA damage, or to facilitate helicase and polymerase activity. There have been identified many nuclear substrates that are removed by VCP activity. The degradation of these proteins is needed to disassemble complexes or to facilitate the binding of other proteins (Polo & Jackson, 2011; Schwertman *et al.*, 2016).

DNA double-strand-break is repaired thanks to many complexes that are regulated by phosphorylation and ubiquitination. In particular, ubiquitination has an important role in the assembly and disassembly of protein complexes. In these processes VCP has a key role *(Franz et al.,* 2016). For example, one of VCP substrates is the complex Ku70/Ku80 which binds the open ends of DNA double-strand break to enhance the reparation via a non-homologous end joining (van den Boom *et al.*, 2016; Taccioli *et al.*, 1994). RNF8 ubiquitinates with K48 chains

Ku70/Ku80 complex, once it accomplished the task, K48-Ub chains recruit VCP that removes the complex from the DNA. To coordinate VCP activity there have been identified different co-factors: either FAF1 or UFD1-NPL4 complex. In addition, VCP removes proteins involved in DNA repair, to allow the functioning of other proteins that bind downstream. An example of this is VCP involvement in the recruitment of downstream DNA damage response proteins like BRCA1, 53BP1 and RAD5. VCP bound to NPL4 co-factor, is recruited to DNA double-strand break by RNF8-generated K48-Ub chain and removes K48-Ub conjugates. The removal of these substrates permits the proper binding of BRCA1, 53BP1 and RAD5 (Meerang *et al.*, 2011). Other VCP substrates are: RNA polymerase Pol II complex (Verma *et al.*, 2013), transcriptional repressor α2 (Wilcox & Laney, 2009) mitosis regulator Aurora B kinase (Ramadan *et al.*, 2007; Sasagawa *et al.*, 2012) and certain DNA polymerases (Davis *et al.*, 2012; Mosbech *et al.*, 2012).

NF-κB ACTIVATION

NF-κB is a transcription factor that enhances the expression of cytokines, immunoreceptors and other components of the immune system (Pahl, 1999). NF-κB activation is regulated by membrane receptors like Toll-like receptors or interleukin-1 receptor. The activation of these receptors triggers downstream phosphorylation and K63-ubiquitination of proteins which leads to NF-κB activation and translocation from cytoplasm to the nucleus (Chen, 2012). When NF-κB is in an inactive state it is found in the cytoplasm bounded to IκBα. To be activated NF-κB has to release IκBα which is degraded (Dai *et al.*, 1998; Henkel *et al.*, 1993). Firstly, both NF-κB and IκBα are phosphorylated, then IκBα is ubiquitinated by CRL1$^{\beta\text{-TrCP}}$ recruiting VCP *(Schweitzerand et al.*, 2016). VCP co-factors implicated are not well characterized but data show that co-factors p47 and FAF1 inhibits activation of NF-κB (Kinoshita *et al.*, 2006; Shibata *et al.*, 2012).

MEMBRANE FUSION

With a totally different mechanism from the other pathways, VCP is implicated in membrane fusion of most of the membranes present in cellular compartments such as Golgi, ER, nuclear membrane and lysosomes. In membrane formation processes VCP functions as a scaffold, and its ATPase activity does not seem essential.

Golgi membranes undergo to disassembly and re-assembly during cell cycle. In these cycles, ubiquitination cooperates in the regulation membrane dynamics (Meyer, 2005). The co-factors that are involved in Golgi membrane regulation are p47, the E3 ubiquitin ligase HACE1 and the DUB VCIP135 (Kondo *et al.*, 1997; Meyer, 2005; D. Tang *et al.*, 2011). HACE1 and VCIP135 interact

with SYN5, the t-SNARE receptors present on Golgi membrane. t-SNARE proteins are membrane receptors that bind v-SNARE proteins present on vesicles mediating their interaction and fusion. During early mitosis HACE1 ubiquitinates SYN5, preventing the interaction with BET1, its corresponding v-SNARE. VCP-p47 complex binds the ubiquitinated SYN5. Afterword, during late mitosis VCIP135 associates with VCP complex and deubiquitinates SYN5 permitting its interaction with BET1, membranes fusion and finally Golgi cisternae formation (Huang *et al.,* 2016).
The contribute of VCP in formation of ER and nuclear membrane is still not so clear. It is known that in nuclear membrane formation VCP co-factor UFD1 binds CHMP2A, a protein present in ESCRT-3 complex involved in the membrane transport and remodeling. The inhibition of UFD1 prevents CHMP2A localization to the nuclear envelope (Olmos *et al.*, 2015). In ER membrane formation VCP seems involved in a similar way then to Golgi membrane formation forming complexes with p47 and VCIP135 (Totsukawa *et al.*, 2011).

VCP DISEASE-ASSOCIATION

Alteration or mutation in *VCP* gene expression is correlated to various diseases. The upregulation of VCP expression is correlated to some type of cancers, while its mis-functioning due to mutations leads to degenerative diseases. The main pathologies, to which VCP is correlated, are IBMPFD and ALS. VCP is also correlated to PD, CMT and AD.
A *VCP* mutation was first associated to IBMPFD, a dominant disorder with a multisystem involvement and an adult onset (Watts *et al.*, 2004). IBMPFD was firstly described in 2000 when a new autosomal dominant disease was reported with a clinical myopathy that resembles limb girdle muscular dystrophy, in the majority of the cases was associated to Paget disease of bone and in fewer cases was also associated to FTD (Kimonis *et al.*, 2000). Patients can manifest myopathy with disabling muscle weakness that can lead to the involvement of cardiac and respiratory muscles (Nalbandian *et al.*, 2011). Moreover, they can have an involvement of bone tissue with bone pane and fracture due to excessive osteoblastic and osteoclastic activity (Farpour *et al.*, 2012). Finally, 30% of patients can have brain involvement with incapacity of learning and deficits in memory, problems in speaking, altered personality and social skills. This is caused by the degeneration of neurons in the frontal and anterior temporal lobes of the brain (Kimonis *et al.*, 2008; Neary *et al.*, 1998).
More than 40 missense mutations in *VCP* gene have been found in IBMPFD patients. Mutations involve amino acids in 29 different positions (Mehta *et al.*, 2013; Nalbandian *et al.*, 2011).

Mutations are mostly localized near the interface between the N-terminal and D1 domains of VCP. These mutants alter D1 affinity for ADP (Tang *et al.,* 2010b), leading to an increased ATPase activity of D2 domain and a loss in the coordinated movement of N-terminal domain (Halawani *et al.*, 2009; Schuetz & Kay, 2016; Tang *et al.*, 2010; Tang & Xia, 2013). Moreover, these mutants alter some of co-factors binding, decreasing its affinity or even preventing it (Bulfer *et al.*, 2016; Fernández-Sáiz & Buchberger, 2010). The most abundant mutations are missense mutations in Arginine-155 like R155C and R155H. Generally, patients having the same mutation might have completely different phenotypes. However, R155C mutation normally, is correlated to patients with a more severe phenotype, with an earlier onset and with a decrease survival compared to those with the R155H mutation. Moreover, VPC R155C patients generally have clinically a myopathy and Paget disease (Mehta *et al.,* 2013).

At cellular level, muscles fibers present vacuoles that contain ubiquitin and VCP (Watts *et al.*, 2004). While neurons present nuclear and cytoplasmatic inclusions positive to VCP and ubiquitin (Kimonis & Watts, 2005). Moreover, both muscles and neurons present TDP-43 inclusions. In fact, studies have demonstrated a correlation between VCP and TDP-43 cytoplasmatic redistribution, and TDP-43 cytotoxicity in presence of VCP mutants (Ritson *et al.*, 2010).

Mutants of VCP were correlated to ALS in 2010 by Johnson and colleagues. Using exome sequencing they identified, in an Italian family with autosomal dominant fALS, a point mutation in VCP: VCP R191Q (Johnson *et al.,* 2010). The subsequent screening on a cohort of ALS patients showed other mutations correlated to the disease: R155H, R159G and D592N. To date VCP is correlated to all most 2% of fALS cases. Almost 20 mutations in 12 different positions have been correlated to ALS. Although there is an overlapping between ALS and IBMPFD mutations, most of ALS mutants are located in the D2 domain and most of them are not in the interface between the N-terminal and D1 domains as it was for IBMPFD. At cellular level, Johnson and colleagues found inclusion positive to ubiquitin and/or deposition of TDP-43 positive aggregates (Johnson *et al.,* 2010).

Mutations in *VCP* gene do not prevent VCP total functionality, but impair a subset of its functions. In fact, VCP-knockout mice are not vital as there is an early embryonic lethality (Muller *et al.,* 2007), yet generally patients and mice with VCP mutations develop normally and have disease symptoms manifesting only late in life (Badadani *et al.*, 2010).

The persistence of inclusions in IBMPFD and VCP-ALS patients suggests an alteration in the PQC system, in particular in protein degradation pathways. Indeed, VCP mutants affect the

consolidation of aggregate-prone proteins like TDP-43, into insoluble aggregates. Moreover, in some cases mutations in *VCP* disrupt the degradation of ubiquitylated proteins through the autophagic pathway, leading to the accumulation of autophagosomes, a common pathologic feature (Ju *et al.*, 2009; Ju *et al.*, 2008; Tresse *et al.*, 2010). In support to this theory, it was reported that mutants like VCP R155C and VCP R191Q cause alterations in the maturation of autophagosome, defects of autophagosome-lysosome fusion and autolysosome formation (Ju *et al.*, 2009; Tresse *et al.*, 2010). Moreover, recently data showed that VCP mutants can alter autophagic functioning by preventing damaged lysosomes degradation. Alteration in lysophagy mechanism results in the accumulation of damage lysosomes that leads to a cellular severe stress condition. Moreover, it alters autophagic pathway by decreasing the pool of lysosome available for the process (Papadopoulos *et al.*, 2017).

Conversely, in some cases data show a different pathological mechanism. In fact, some IBMPFD-mutants like VCP P137L and VCP R93C were shown to stimulate both autophagosome and autolysosome formation (Bayraktar *et al.*, 2016). This suggests that cellular mechanisms leading to VCP-disease may be highly variable.

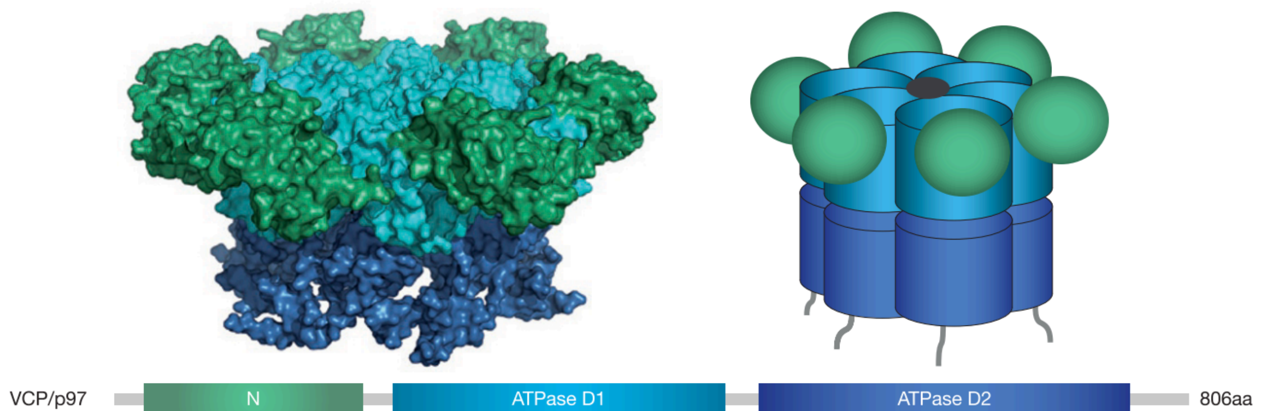


Figure 8 VCP structure (Meyer et al., 2012)

	Change in amino acid	Change in gene	Location in protein	Phenotype	References
I27	I27V	79A>G	N domain	IBM, FTD, PDB	Rohrer et al., 2011; Majounie et al., 2012; Weihl et al., 2015
R93	R93C	277C>T	N domain	IBM, PDB, FTD	Guyant-Maréchal et al., 2006; Hübbers et al., 2007
	R93H	278G>A	N domain	HSP	Neveling et al., 2013
R95	R95C	283C>T	N domain	IBM, ALS	Weihl et al., 2015
	R95H	284G>A	N domain	AD	Kaleem et al., 2007
	R95G	283C>G	N domain	IBM, PDB, FTD, ALS	Watts et al., 2004; Kimonis et al., 2008b
G97	G97E	290G>A	N domain	IBM, PDB, FTD	Gu et al., 2012; Jerath et al., 2015
I114	I114V	340A>G	N domain	ALS	Koppers et al., 2012
I126	I126F	376A>T	N domain	IBM, PDB, FTD	Matsubara et al., 2016
T127	T127A	379A>G	N domain	FTD, AD	Shi et al., 2016
P137	P137L	410C>T	N domain	IBM, PDB, FTD	Stojkovic et al., 2009; Palmio et al., 2011
I151	I151V	451A>G	N domain	IBM, ALS	DeJesus-Hernandez et al., 2011; Boland-Freitas et al., 2016
R155	R155S	463C>A	N domain	IBM, PDB, FTD	Stojkovic et al., 2009
	R155L	464G>T	N domain	IBM, PDB, FTD	Kumar et al., 2010
	R155H	464G>A	N domain	IBM, PDB, FTD, ALS	Watts et al., 2004; Hübbers et al., 2007; Kimonis et al., 2008a; Viassolo et al., 2008; Stojkovic et al., 2009; González-Pérez et al., 2012
	R155C	463C>T	N domain	IBM, PDB, FTD, ALS	Watts et al., 2004; Schröder et al., 2005; Guyant-Maréchal et al., 2006; Gidaro et al., 2008; González-Pérez et al., 2012
	R155P	464G>C	N domain	IBM, PDB, FTD	Watts et al., 2004
G156	G156C	466G>C	N domain	ALS	Segawa et al., 2015
	G156S	466G>A	N domain	IBM, PDB, FTD	Komatsu et al., 2013
G157	G157R	469G>C	N domain	IBM, PDB, FTD	Djamshidian et al., 2009
		469G>A	N domain	IBM, PDB, FTD	Stojkovic et al., 2009
M158	M158V	472A>G	N domain	PDB, ALS	Ayaki et al., 2014
R159	R159G	475C>G	N domain	ALS, FTD	Johnson et al., 2010
	R159C	475C>T	N domain	IBM, FTD, PD, ALS	Bersano et al., 2009; Chan et al., 2012; de Bot et al., 2012; González-Pérez et al., 2012
	R159H	476G>A	N domain	IBM, PDB, FTD, ALS	Haubenberger et al., 2005; Stojkovic et al., 2009; van der Zee et al., 2009; Koppers et al., 2012
E185	E185K	553C>T	N domain	CMT2Y	Gonzalez et al., 2014
R191	R191G	571C>G	N-D1 linker	IBM, ALS	González-Pérez et al., 2012
	R191Q	572G>A	N-D1 linker	IBM, PDB, FTD, ALS	Watts et al., 2004; Kimonis et al., 2008b; Stojkovic et al., 2009; Johnson et al., 2010; González-Pérez et al., 2012
L198	L198W	593T>G	N-D1 linker	IBM, PDB, FTD	Watts et al., 2007; Kumar et al., 2010
G202	G202W	604G>T	N-D1 linker	IBM, FTD	Figueroa-Bonaparte et al., 2016
I206	I206F	616A>T	N-D1 linker	IBM, PDB, FTD	Peyer et al., 2013
A232	A232E	695C>A	D1 domain	IBM, PDB	Watts et al., 2004; Kimonis et al., 2008b
T262	T262A	784A>G	D1 domain	IBM, PDB, FTD	Spina et al., 2008
K386	K386E	1158T>C	D1 domain	IBM	Lévesque et al., 2016
N387	N387H	1159A>C	D1 domain	IBM, FTD	Watts et al., 2007
	N387S	1160A>G	D1 domain	IBM, PDB, FTD	Liewluck et al., 2014
	N387T	1160A>C	D1 domain	ALS	Abramzon et al., 2012
N401	N401S	1202A>G	D1 domain	FTD, ALS	Shi et al., 2016
A439	A439S	1315G>T	D1 domain	IBM, PDB	Stojkovic et al., 2009
	A439P	1315G>C	D1 domain	IBM, PDB, FTD	Shi et al., 2012; Kamiyama et al., 2013
	A439G	1316C>G	D1 domain	IBM, FTD	Figueroa-Bonaparte et al., 2016
R487	R487H	1460G>A	D2 domain	FTD, ALS	Hirano et al., 2015
D592	D592N	1774G>A	D2 domain	ALS	Johnson et al., 2010
R662	R662C	1984C>T	D2 domain	ALS	Abramzon et al., 2012
N750	N750S	2249A>G	D2 domain	ALS	Kenna et al., 2013

IBM, inclusion body myopathy; PDB, Paget's disease of bone; FTD, frontotemporal dementia; PD, Parkinson disease; ALS, amyotrophic lateral sclerosis; CMT2Y, Charcot-Marie-Tooth disease.

Figure 9 VCP mutations (Tang & Xia, 2016)

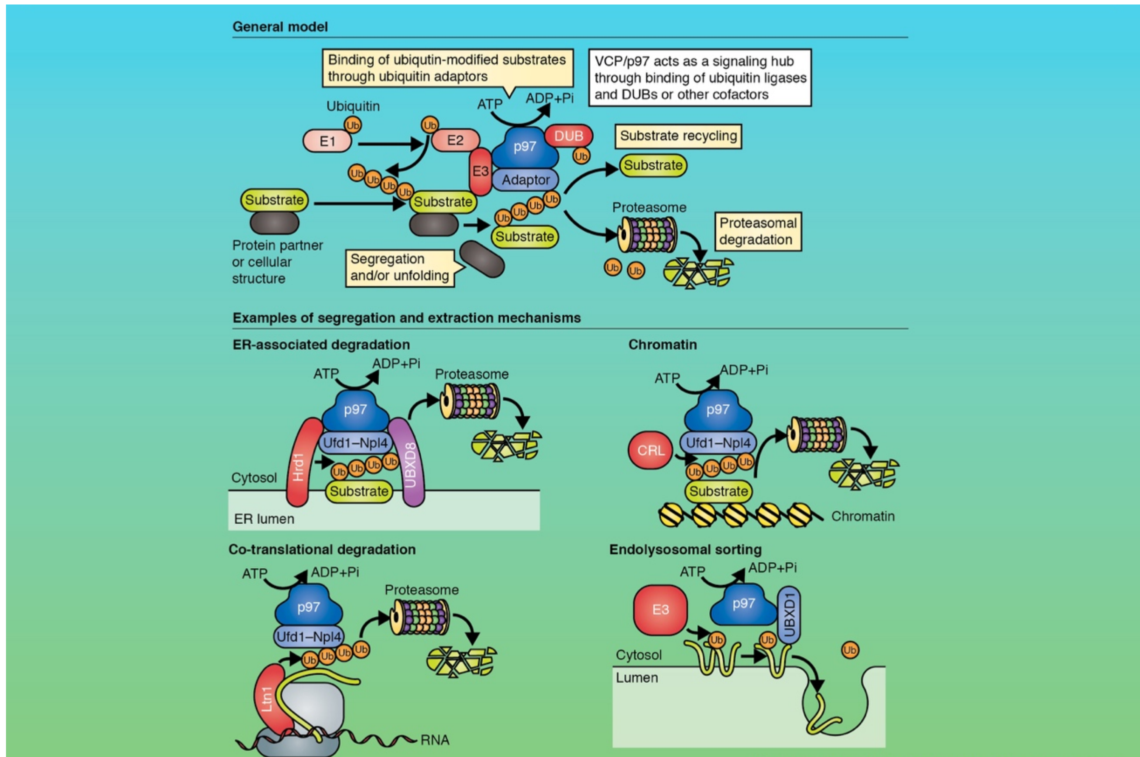


Figure 10 VCP functions (modified from Meyer & Weihl, 2014)

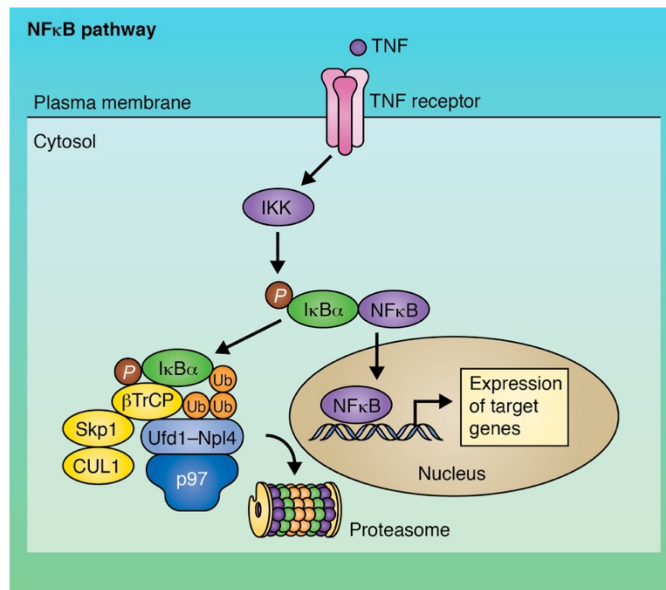


Figure 11 VCP functions (modified from Meyer & Weihl, 2014)

PROTEIN QUALITY CONTROL SYSTEM

The aggregation of misfolded proteins is one key element of ALS pathogenesis. In fact, most of the mutations in genes described before, lead to protein destabilization and to its incorrect folding, resulting in an increased tendency to aggregate. The formation of protein aggregates can then cause cytotoxicity by altering various cell mechanisms such as axonal transport, mitochondria processes and the degradative pathways. Moreover, aggregates can sequester other properly folded proteins preventing their functioning and threatening cell viability (Cozzolino *et al.*, 2008; Pasinelli & Brown, 2006; Seetharaman *et al.*, 2009). Neurons are much more sensitive than other cells to the accumulation of aggregates because they cannot "dilute" them through cell division as they are post-mitotic cells (Lee *et al.*, 2011). Therefore, cells to maintain their homeostasis and to protect themselves, use different mechanisms that prevent the formation of aggregates or eliminate them. The correct protein homeostasis is controlled by the chaperones and degradative systems.

Chaperones recognize and bind proteins that have not yet reached or have lost the correct conformation (unfolded, misfolded). By binding misfolded proteins, chaperones prevent proteins aggregation and enhance their refolding to proper conformation. If proteins are too much altered to reach their correct conformation, chaperones by forming complexes with co-chaperones, deliver them to the degradation pathways.

The main degradation pathways are: the UPS and the autophagic pathway.

CHAPERONE SYSTEM

Chaperones are proteins ubiquitously expressed that assist newly synthetized proteins to reach their correct conformation, they cooperate in the assembly of complexes, prevent protein misfolding and aggregation or, if it occurs, cooperate with protein unfolding and aggregate disassembly. In particular, they have a crucial role in preventing or in correcting protein denaturation caused by cellular stress. Cellular stresses include toxin exposure, heat shock or disease conditions, etc.. Generally, chaperone substrates have not specific post transcriptional signal recognitions but chaperones recognize proteins not properly folded that expose hydrophobic residues that are normally inside the protein structure. (Gidalevitz *et al.*, 2011; Hartl *et al.*, 2011; Saibil, 2013). To assist protein folding or refolding chaperones can either use ATP hydrolysis or they can just bind to substrates protecting them during their assembly (Mayer, 2010).

The major chaperone family is the family of the Heat Shock Proteins (HSPs). Initially, they were discovered as proteins that are upregulated after a heat shock (Schlesinger, 1990). HSPs expression is regulated by Heat Shock Factor 1 (HSF-1), a transcription factor normally found inactive in the cytosol as a monomer bound to HSP90, that becomes active after stress exposure migrating in the nucleus as a trimer to activate transcription of stress responsive genes (Clos *et al.*, 1990). HSPs are classified according to their molecular weight in: HSP40, HSP60, HSP70, HSP90, HSP100 and small HSP (sHSPs). Members of the HSP60, HSP70, HSP90 and HSP100 family have an ATP dependent activity while HSP40s and sHSPs do not use ATP energy to carry out their activity.

HSP70 is involved in various pathways by binding with different co-factors as HSP100, HSP40 also known as J proteins, and nucleotide exchange factors (NEF) that coordinates HSP70 ATP-binding. HSP70 functionality depends on its dynamic conformational cycle that is regulated by ATP binding, hydrolysis and release. HSP70 functions are: to bind and to maintain substrates that are in an unfolded state to prevent their aggregation or to permit organelle membrane translocation; to cooperate in the removal of Clathrin coat on endocytosis vesicles; to disassemble large aggregates (Rampelt *et al.*, 2012; Rothnie *et al.*, 2011; Sharma *et al.*, 2010).

HSP90, as well as HSP70, cooperates with various partners including HSP70. HSP90 also binds unfolded protein preventing their aggregation and cooperating with their proper folding. In particular, HSP90 activity occurs in the late stages of folding of substrates that are involved in cellular signalling, as hormone receptors, kinases and important oncogenic proteins. By binding to these specific substrates, HSP90 modulates their activity, their localisation and their degradation maintaining them in a un-folded conformation. HSP90 works as a dimer and is regulated by post-transitional modifications and co-chaperone binding, that modulates its activity and targets to its substrates (Johnson, 2012; Li *et al.*, 2012; Taipale *et al.*, 2010).

HSP60 or Chaperonins can be divided into two subgroups: group I is composed of the bacterial (GroEL, GroES), mitochondrial and chloroplast specific HSP60; and group II comprehends archaea and eukaryotic cytosolic proteins. HSP60 binds with few partners in fact, it forms a symmetrical, self-contained complex. HSP60 complex binds the substrate inside its structure enhancing and facilitating its correct folding (Goloubinoff *et al.*, 1989; Horwich & Fenton, 2009; Ostermann *et al.*, 1989).

HSP100 proteins function is to unfold protein or disaggregate by removing unfolded proteins from aggregates, and addressing them to degradation pathways. HSP100 proteins are members of the AAA$^+$ superfamily, that were previously described (Neuwald *et al.*, 1999).

sHSPs are characterized by a low mass (10-40kDa) and a particular α-crystallin domain of 90 aa. sHSPs role is crucial for the maintenance of proteostasis. In fact, they are their first chaperones that recognize misfolded proteins and bind them preventing aggregation in an ATP-independent manner (Ehrnsperger *et al.*, 1997) sHSPs can be found assembled in oligomeric forms, where their interaction with substrates are very low, or disassembled in smaller species, generally in dimers, that can easily interact with substrates. As dimers sHSPs bind substrates and they can either form assemblies smaller and easier to rescue than insoluble protein aggregates, or they can interact with other chaperonea and co-chaperones to enhance substrates degradation (Haslbeck & Vierling, 2015; Stengel *et al.*, 2010; Treweek *et al.*, 2015). To support chaperones activity there are different proteins families known as co-chaperones. There are several families of co-chaperones proteins, as the Bcl-2-associated athanogene (BAG) containing family that contains the BAG domain; the family containing the TPR motif, whose members are CHIP, HIP and HOP, and the co-chaperone protein HSP40.

UBIQUITIN PROTEASOME SYSTEM

The UPS is a cellular pathway responsible for removing short-lived or abnormal proteins. UPS is a selective proteolytic system that degrade misfolded or damaged proteins in small peptides. It is a selective degradation system, in fact proteins that are degraded, are recognized by the system only if they bind a polyubiquitin chain K48 (Ciechanover & Stanhill, 2014).

The proteasome is composed by an internal proteolytic subunit (20S) and two external regulatory subunits (19S) that harbour receptors to recognize substrates (Bedford *et al.*, 2011). The 20S subunit is composed of 4 rings stack one above the other, formed by 7 subunits each. The outer rings are called α while the two inner ones are called β ($\alpha_7\beta_7\beta_7\alpha_7$). Three of the seven β subunits have proteolytic activity and cleave hydrophobic, basic and acidic sites. The presence of multiple simultaneously active subunits leads to faster degradation of the substrate (Groll *et al.*, 1997). The 20S subunit can be found in three conformations: active state, inactive state and intermediate state. It is activated when it interacts with the 19S regulatory subunits (Unverdorben *et al.,* 2014). The two 19S subunits are located at the ends of 20S and are formed by nine subunits each. Their function is to recognize the ubiquitinated substrate, to

deubiquitinate it and to linearize proteins using ATPases energy. Once substrates are linearized, 19S introduce them into the core of the proteasome (Finley, 2009; Glickman *et al.*, 1998).

The proteasome substrates are misfolded proteins and fast-turnover proteins located in the cytoplasm, nucleus and ER. Proteins with incorrect conformation are recognized by chaperones and Ubiquitin-ligase (E3). These enzymes recognize the substrate that have altered structure as exposed hydrophobic residues or incorrect disulfide bonds. Once substrates are recognized, they are marked and then degraded (Clague & Urbé, 2010).

The UPS degradation mechanism is composed by various steps where about 500-1000 proteins cooperate. In the first step substrates are conjugated with an ubiquitin chain that is needed as recognition signal for the proteasome. Ubiquitin is regulatory protein of 76aa, it is activated in the active site of the enzyme E1 through the formation of a thioester bond between Gly76 in its C-terminal domain and a cysteine. Once the ubiquitin is activated it is transferred to enzyme E2 through another thioester bond. Then enzyme E3 ligase transfers ubiquitin and catalyzes its binding to a lysine residue of the substrate (Glickman & Ciechanover, 2002; Hershko, 2005). Once the substrate is bound to ubiquitin it can be addressed to different pathways. To be addressed to the proteasome, the protein must bind the ubiquitin in lysine 48. Furthermore, to be recognized by the 19S subunit, the chain must have at least 4 ubiquitins (Clague & Urbé, 2010; Thrower *et al.*, 2000).

The substrate is recognized through two receptors present on the 19S subunit: Rpn10 and Rpn13. To facilitate recognition there are shuttle proteins, such as Ubiquilin and p62, that have a UBL (Ubiquitin-like) domain that interacts with the proteasome, and a UBA (Ubiquitin-associated) domain that interacts with the polyubiquitin chain. Thanks to their structure, these proteins act as linkers between the proteasome and the target protein (Chen & Madura, 2005; Husnjak *et al.*, 2008).

After the interaction with the substrate, 19S subunit activates the enzymes that deubiquitinate the substrate: deubiquinating enzymes (DUBs). This step allows the protein to enter in the core of the 20S subunit and allows the ubiquitins to be recycled and used for other proteins degradation. Deubiquitination is an important phase that must take place in the right time since that if deubiquitinated proteins fail to enter the proteolytic site they are released again into the cell (Hanna & Finley, 2007). The deubiquitinated substrates then enter into the core of the proteasome passing through a very narrow opening (diameter of 13 A°) that mechanically linearize them. In the center of the 20S ring substrates are reduced to small peptides through the

β5, β2 and β1 subunits which have a chymotryptic, tryptic and post-acidic action (Hendil *et al.*, 1998; Tanahashi *et al.*, 2000).

UPS AND DISEASE

UPS malfunctioning leads to irreversible alteration of cell homeostasis resulting in the formation of aggregates which can lead to alteration of various vital cell processes and to subsequent cytotoxicity and cell death. The UPS malfunction may be due to problems in the ubiquitination phase, in the addressing of the substrate to the proteasome or in the activity of the proteasome itself (McKinnon & Tabrizi, 2014).

In neurodegenerative diseases, the alteration of UPS seems to be due to a decrease in proteolytic activity. In fact, the direct interaction between misfolded proteins and proteasome reduces or inhibits its activity. The alteration of UPS activity could also be due to toxic events associated with ALS such as ATP depletion or oxidative stress (McKinnon & Tabrizi, 2014).

In ALS, proteasome dysfunction plays an important role, in fact most inclusions contain ubiquitinated proteins. Studies on ALS proteasome dysfunction, have shown that the aggregation of mutated SOD1 leads to a significant decrease in the functioning of the proteasome (Crippa *et al.*, 2010; Sau *et al.*, 2007). In SOD1-ALS, proteasome defects are due to SOD1 aggregates that damages mitochondria causing ATP depletion and increase in the levels of free radicals (Ugarte et al. 2010). Some studies have also shown that SOD1 aggregates can inhibit the translocation of substrates to the proteasome preventing their degradation (Sau *et al.*, 2007). Studies conducted on TDP-43 associated ALS models show that inhibition of the proteasome leads to an increase in the levels of the C-terminal fragments of TDP-43 as it blocks their degradation. Indeed, proteasome inhibition increases toxicity of TDP-43 fragments as TDP-43 and its fragments are initially degraded by UPS. However, if during the transport to the proteasome TDP-43 and its fragments escape the control of ubiquitinating enzymes they are prone to aggregate. Once aggregated they can no longer be degraded by the proteasome but other degradative systems are needed (Andersen & Al-Chalabi, 2011).

The UPS dysfunction in ALS may also be caused by mutation of genes that encode for proteins involved in the system like UBQLN2 or SQSTM1, genes that encodes respectively for Ubiquilin-2 and p62 (Deng *et al.*, 2011; Fecto *et al.*, 2011). Mutations in UBQLN2 were associated to ALS in 2011. Mutated Ubiquilin-2 is shown to lead to impairment of protein degradation, with abnormal protein aggregation and neurodegeneration (Chang & Monteiro, 2015; Deng *et al.*, 2011).

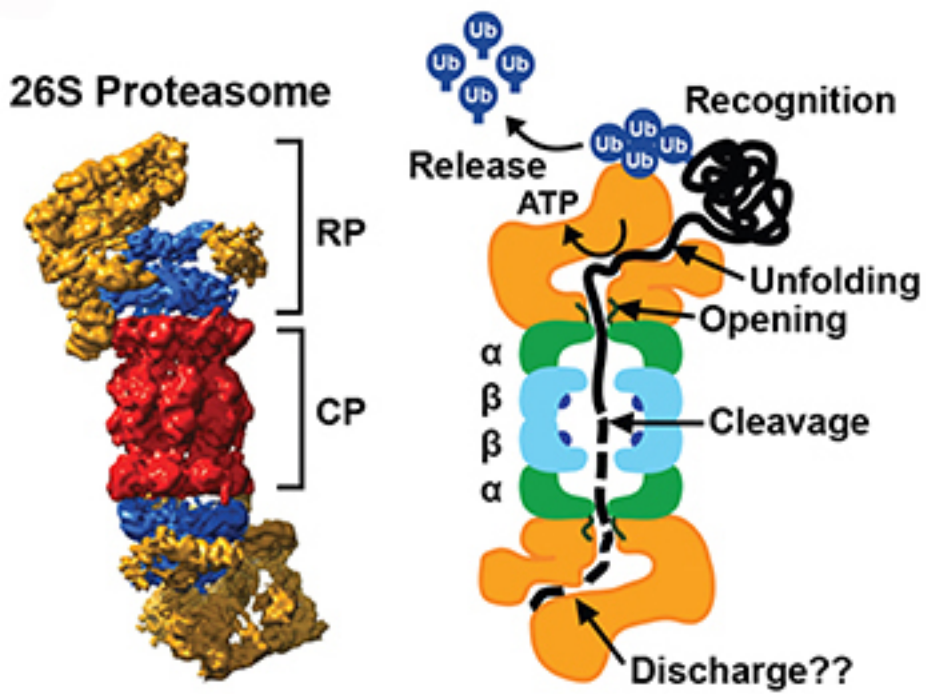


Figure 12 Proteasome structure (Marshall & Vierstra, 2019)

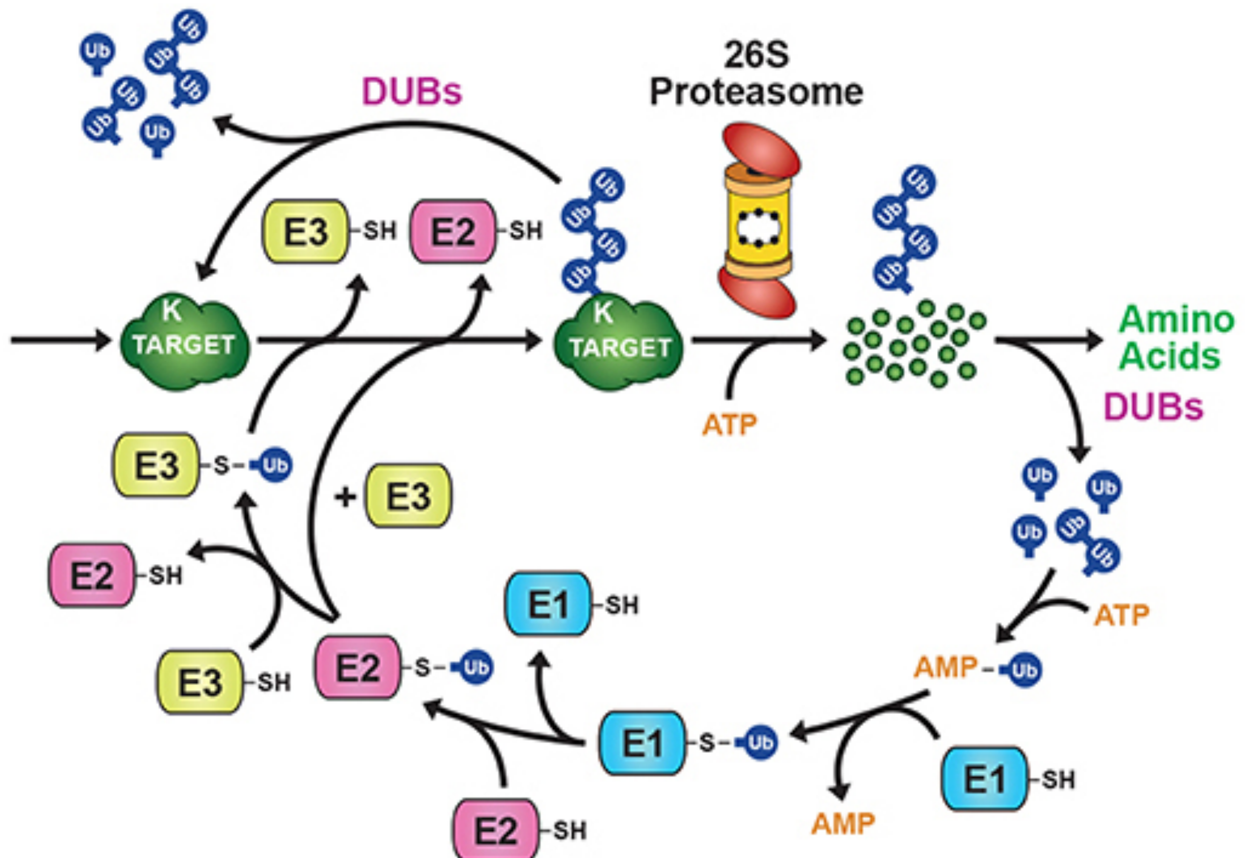


Figure 13 UPS mechanism (Marshall & Vierstra, 2019)

AUTOPHAGY

Autophagy is a catabolic process that eliminates large portions of cytoplasm, proteins with a long half-life and damaged organelles.

Autophagic degradation can be non-specific or targeted to ubiquitinated substrates such as protein aggregates, intracellular organelles and microorganisms (Kirkin *et al.*, 2009; Xie & Klionsky, 2007). These ubiquitinated complexes are recognized by receptors such as Microtubule-associated protein light chain 3 (LC3) and GABA (A) Receptor-Associated Protein (GABARAP) (Rogov *et al.*, 2014), via different mechanisms they are transported to lysosomes that degrade substrates by proteases and acid pH.

Initially, it was thought that the only role of autophagy was to enhance cell death by apoptosis (Ghavami *et al.*, 2014). However, recently autophagy has been shown to play an important role in maintaining cell homeostasis by degrading organelles and non-functional proteins (Mizushima & Klionsky, 2007). Indeed, several studies have shown that autophagy can be induced by denatured or aggregated proteins, damaged organelles, oxygen radicals, hypoxia or stress. (Kroemer *et al.,* 2010).

Autophagy can be subdivided in three different processes according to how the substrate reaches lysosome to be degraded. The three mechanisms are: chaperones-mediated autophagy (CMA), microautophagy and macroautophagy.

CHAPERONES-MEDIATED AUTOPHAGY

CMA degraded substrates that harbour in their structure a pentapeptide motif (LysPheGluArgGln; KFERQ) which is recognized and bound by a molecular chaperone called Heat Shock Cognate 70 (HSC70). Under physiological conditions this sequence is not exposed outside the protein, but due to protein misfolding or to mutations in its primary structure, it can be exposed outside and recognized (Kiffin *et al.*, 2004). The substrate-HSP70 complex is in turn recognized by Lysosome-associated membranes glycoprotein (LAMP2A), a protein present on the lysosome membrane. The interaction with LAMP2A leads, LAMP2A dimerization and to the internalization of the substrate and its degradation (Arias & Cuervo, 2011). This process can be activated to compensate the mis-functioning of macroautophagy.

MICROAUTOPHAGY

Microautophagy degradation is even more direct than CMA, in fact lysosomes themselves incorporate portions of the cytoplasm forming invaginations of the membrane (Glick *et al.,* 2010). Invagination of the membrane occurs where there is a very low content of transmembrane proteins and is regulated by dynamin-related GTPase, VPS1P (Uttenweiler *et al.,* 2005). First lysosome membrane bulges with a lateral segregation of lipids and a local exclusion of large transmembrane proteins (Uttenweiler *et al.,* 2005). Then the invagination extends inside lysosome lumen and forms tubular structure termed "autophagic tube" (Müller *et al.*, 2000). Thanks to enzymes, the invagination enlarges in a bubble-like structure enriched of lipids and without transmembrane protein (Sattler & Mayer, 2000; Uttenweiler *et al.*, 2007). Invagination is regulated by two Atg7-complexes. Moreover, in yeast the Vacuolar Transporter Chaperone (VTC) complex regulates protein redistribution and triggers membrane invagination through Calmodulin (Doelling *et al.*, 2002; Uttenweiler *et al.*, 2005). Finally, the vesicle formed separates from the membrane.

Microautophagy is generally non-selective, however in yeast are found different types of selective microautophagy. In selective microautophagy lysosomes sequester organelles with arm-like protrusions. The organelles, substrates of microautophagy, are: peroxisome (micropexophagy), non-essential parts of the nucleus (Piecemeal microautophagy of the nucleus, PMN) and mitochondria (micromitophagy) (Bellu *et al.*, 2001; Dawaliby & Mayer, 2010; Kiššova *et al.*, 2007).

MACROAUTOPHAGY

Macroautophagy (generally named autophagy) is a more complex mechanism. It consists in the degradation of substrate by lysosomes mediated by autophagosome that incorporates and transport the material. Macroautophagy can be divided into four main phases: *initiation*, where there is the recruitment of the initial membrane, called the phagophore; *elongation* of this membrane until it forms the autophagosome; *maturation*, when the autophagosome fuses with endosomes and finally lysosomes and *degradation* of the cytoplasmatic material.

During the *initiation* the internal cell membranes are reorganized to be used to incorporate the substrate. The cell membranes that are involved are still not clear but data show a contribute of ER, mitochondria, mitochondria-associated membranes (MAMs), Golgi, plasma membrane and recycled endosome (Frake *et al.*, 2015; Lamb *et al.*, 2013; Ravikumar *et al.*, 2010). *Elongation*

with autophagosome formation, is regulated by the assembly of two complexes: the protein-kinase autophagy complex (ULK1-Atg13-FIP200) and the lipid-kinase signalling complex (PI3KCIII complex: Vps34-Vps15-Atg14-Beclin-1). The reorganization of the membranes leads to the formation first of the phagophore and then of the autophagosome thanks to the recruitment of ATG proteins (AuTophaGy related).

The ULK1-Atg13-FIP200 complex is regulated by mTORC1 complex and by AMPK (Mizushima, 2010; Shang & Wang, 2011). mTORC1 is a complex containing mTOR, a serine/threonine protein kinase, that inhibits autophagy activation. mTORC1 actively inhibits ULK1-Atg13-FIP200 complex by phosphorylating ULK1 and Atg13 and directly binding ULK1 (Jia *et al.,* 2018). ULK1-Atg13-FIP200 complex activation is mediated by inhibition of mTORC1, by phosphorylation of AMPK and by autophosphorylation. AMPK phosphorylates ULK1 activating its functionality, and phosphorylates mTORC1 inhibiting it. ULK1 phosphorylates its self and both Atg13 and FIP200 activating the complex (Kim *et al.,* 2011). Once ULK1-Atg13-FIP200 complex is activated, it activates the PI3KCIII complex containing Beclin-1. This enzymatic cascade leads to the formation of the phagophore (Simonsen & Tooze, 2009). Substrate incorporation and autophagosome transformation occurs through two ubiquitin-dependent systems: Atg12-Atg5 conjugation system and microtubule-associated protein1A/1B - light chain 3 (LC3) conjugation system. The Atg12-Atg5 complex forms thanks to Atg7, an E1 enzyme, and Atg10, an E2 enzyme. Atg12-Atg5 complex enhances the formation of LC3-II. LC3-I is first clavated in its C-terminal end by Atg4, then it is conjugated to a phosphatidylethanolamine by Atg3 forming LC3-II. LC3-II enhances the fusion of the lipid double layer of the phagophore, thus forming the autophagosome. Once formed LC3-II remains anchored on the membrane (Hanada *et al.*, 2007). Before the formation of the autophagosome is complete, LC3-II interacts with autophagy receptors as p62, that mediate the internalization of the ubiquitinated substrates. In fact, p62 harbours UBA motif that when p62 is not activated, dimerizes by binding to another p62 on its UBA motif. Subsequent phosphorylation by ULK1 and TANK-binding kinase 1 (TBK1) destabilizes p62 dimer and increases its affinity for ubiquitin leading to p62 binding to ubiquitinated substrates. P62 also has a LC3-interacting region (LIR) that binds LC3 in different regions permitting the specific degradation of substrates (Ichimura *et al.*, 2008; Lim *et al.*, 2015; Pankiv *et al.*, 2007).

The *maturation* phase involves the transition from the autophagosome to the autolysosome through the fusion first with the endosome or amphysome and then with the lysosome (Longatti & Tooze, 2009). Firstly, the autophagosome formed must be transported to the cell body through

an antegrade transport which occurs thanks to the interaction with Dinactin and with Dinein (Eschbach & Dupuis, 2011). In neurons this transport, also known as axonal transport, is a critical passage. In fact, neuronal terminal parts, where the autophagosome are formed, are often very far from the cell body. The fusion of autophagosomes and lysosomes occurs thanks to the presence of various proteins including ESCRT (endosomal sorting complex required for transport), Rab and SNARE (N-ethylmaleimide-sensitive factor-activating protein receptor) (Ritz *et al.,* 2011).

The *degradation* phase takes place inside the autolysosome thanks to proteases, lipases and nucleases and thanks to the acid pH (pH 4.5-5) that activates the enzymes. The pH level is maintained by an ATPase proton pump (Saftig & Klumperman, 2009).

TRANSCRIPTIONAL REGULATION OF AUTOPHAGY

Besides post-translational modifications of proteins, autophagy is regulated also at transcriptional level. Different transcription factors are involved in promoting the expression of genes that are implicated in autophagosome formation, fusion of autophagosomes with lysosomes and lysosome biogenesis. Some members of the microphthalmia family of basic helix-loop-helix leucine-zipper transcription factors (MiT/TFE) are involved in these processes like: transcription factor EB (TFEB), TFE3 and MITF. MiT members recognize and bind palindromic CACGTG E-box and asymmetric TCATGTG M-box sequences present in the promoter of various genes, regulating them. MiT transcription factors bind these DNA regions as monomers, homodimer or heterodimer by interacting specifically with any other member of the MiT family. (Aksan & Goding, 1998; Hemesath *et al.*, 1994). TFEB and TFE3 regulate the expression of a similar set of genes. However, mice with different knockout-MiT transcription factors have different phenotypes proving that MiT members may have specific functions and a limited redundancy (Betschinger *et al.*, 2013; Ferron *et al.*, 2013; Steingrímsson *et al.*, 2002; Yagil *et al.*, 2012). TFEB and TFE3 regulates Coordinated Lysosomal Expression And Regulation (CLEAR) genes. CLEAR genes are involved in autophagosome biogenesis and are implicated in autolysosome formation (Sardiello *et al.*, 2009; Settembre *et al.*, 2011).

TFEB regulation depends on post-translational modifications, protein-protein interactions and spatial organization. In particular TFEB can be found in an inactive state: localized in cytoplasm, phosphorylated and bound to chaperone 14-3-3, or else it can be found in an active state: dephosphorylated and with a nucleus localization. Phosphorylation is the main regulator of TFEB activity. In particular, inactive TFEB is phosphorylated in Ser142 and in Ser211 (Martina, *et al.*,

2014; Roczniak-Ferguson *et al.*, 2012; Settembre *et al.*, 2012). Ser211 inhibits TFEB functioning by masking NLS motif present in TFEB structure and promoting binding with chaperone 14-3-3 (Roczniak-Ferguson *et al.*, 2012). TFEB phosphorylation and inactivation is regulated by mTORC1 complex. Activation of mTORC1 complex is promoted by v-ATPase that activate Rag (Ras-related GTP-binding) GTPases. Rag proteins recruit mTORC1 to lysosome membranes where GTPase Rheb activates the complex (Sancak *et al.*, 2010; Zoncu *et al.*, 2011). Rag GTPases also bind TFEB addressing it to lysosomes, where it is phosphorylated by mTORC1 (Martina & Puertollano, 2013). When starvation or lysosomal stress occurs, mTORC1 is inactivated and released from lysosomes (Sancak *et al.*, 2010) and in parallel cytoplasmatic Ca^{2+} increases activating phosphatases as Calcineurin/PPP3CB. In turn, Calcineurin dephosphorylates TFEB that translocate in the nucleus promoting gene transcription (Medina *et al.*, 2015).

Martina and colleagues showed that TFE3 is also regulated by Rag GTPase recruitment and mTORC1 phosphorylation. Moreover, TFE3 activation and nucleus translocation results in the expression of genes related to the autophagic pathway and lysosomal biogenesis (Martina *et al.*, 2014).

In same cell types TFE3 and TFEB role can seem redundant, however some biological functions may be unique and their expression levels are differentiated in some cell types (Martina *et al.*, 2014; Raben & Puertollano, 2016).

CHAPERONE-ASSISTED SELECTIVE AUTOPHAGY

Autophagy can be either non-specific or specific. In selective autophagy chaperone complexes recognize specific substrates and address them to autophagic degradation. A type of selective autophagy is named chaperone-assisted selective autophagy (CASA). In CASA pathway substrates are routed to autophagosomes by CASA complex. CASA complex is formed by HSPB8, a sHSP, BAG3, HSP70 and CHIP. HSPB8 recognizes and binds misfolded proteins and CHIP ubiquitinates them. BAG3 binds dynein that together with dynactin mediates substrates-complex transport along microtubules to the microtubule organizing centre (MTOC). At MTOC ubiquitinated substrates are bound by p62 proteins forming aggresomes that are then engulfed in autophagosomes (Arndt *et al.*, 2010; Massey *et al.*, 2006). Studies have tried to potentiate this mechanism in order to enhance degradation of misfolded proteins. To achieve this goal, members of the complex as HSPB8 or BAG3 were upregulated.

LYSOSOME

Lysosome is a double membrane organelle responsible of macromolecules, organelles and extracellular material degradation. Recent discoveries have found that lysosomes are involved in other cellular processes like metabolic signaling, regulation of genes, plasma membrane reparations, immunity and cell adhesion and migration (Ballabio & Bonifacino, 2020; Conus & Simon, 2008; Michelet *et al.*, 2018; Reddy *et al.*, 2001). Many proteins are present in lysosomal membrane and lumen to coordinate its functioning. Lysosome protein can be classified in soluble lysosomal proteins, present in the lumen and in the integral lysosomal membrane proteins (LMPs). Lumen proteins comprehend hydrolases, enzyme activators, protective factors and transport factors (Lübke *et al.,* 2009). To date there are at least 50 different hydrolases, that each targets different substrates degrading them. Besides from their key role in degradation, hydrolases are also involved processing of antigens cooperating with immune response, in degradation of extracellular matrix and in initiation of apoptosis (Conus & Simon, 2008). LMPs present in lysosomes are at least 20 and they also cooperate in many functions. The main LMPs are: v-ATPase that are responsible for the acidification of lysosomal lumen; Lysosome Associated Membrane Protein (LAMP), highly glycosylated proteins, that protect lysosome from hydrolyses and acid pH; ion channels that maintain ion homeostasis; transporters that permit the export of lysosomal degradation products; and finally SNARE proteins that coordinate lysosome fusion with vesicles or organelles (Eskelinen, 2006; Hasegawa *et al.*, 2015). Moreover, lysosomes through RAG-GTPases interact with various complexes cooperating in different signaling pathways as mTORC1/TFEB.

Another significant modality used by lysosomes to exert their activity is Ca^{2+} segregation and release. In fact, lysosomal Ca^{2+} release enhances various cellular processes as lysosomal re-formation, endosome-lysosome fusion, TFEB translocation to the nucleus, autolysosome formation and lysosomal exocytosis (Morgan *et al.*, 2011; Reddy *et al.*, 2001).

Several conditions as bacteria, photodamage, sterile damage and endocytosed neurotoxic aggregates lead to lysosome membrane permeabilization and rapture (Bussi *et al.*, 2018; Hung *et al.*, 2013; Thurston *et al.*, 2012). Lysosomal membrane permeabilization can eventually result in leakage of lumen cathepsins, that can trigger lysosome-dependent cell death (Aits & Jäättelä, 2013; Wang *et al.*, 2018). However, to prevent lysosome-dependent cell death, cell activates pathways, known as Endo-Lysosomal Damage Response (ELDR), that either repair or eliminate damaged lysosomes. Physiologically HSP70 prevents membrane permeabilization and maintains

balanced lipid composition by binding to an anionic phospholipid bis(monoacylglycero)phosphate (BMP) which is an essential co-factor for lysosomal sphingomyelin metabolism. When small damage occurs, lysosomal repair is activated (Kirkegaard *et al.*, 2010). ESCRT-I, ESCRT-II, and in particular ESCRT-III are recruited to the damaged lysosome. Lysosomal Ca^{2+} depletion triggers ESCRT-III recruitment through ALIX activation. Once ESCRT-III is localized on lysosomes membrane, it forms filamentous spirals on the surface of the lipid bilayer that could close membrane holes (Radulovic *et al.*, 2018; Skowyra *et al.*, 2018). When the ruptures is irreversible, damaged lysosomes can be eliminated through the autophagic pathway in a process called lysophagy (Maejima *et al.*, 2013; Papadopoulos *et al.*, 2017). Lysosomes activate their degradation via lysophagy in different ways. Firstly, ruptured lysosomes expose on their membrane a group of cytosolic lectins, known as galectins (GAL3, GAL8), to mark the damage. GAL8 directly recruits and binds NDP52 an autophagy receptor, that in turn recruits LC3 on the phagophore. GAL3 recruits and binds ULK1, that stimulates phagophore formation, and TRIM16. GAL3/TRIM16 complex enhances ubiquitination of LMPs with K48 and K63 polyubiquitin chains and recruits autophagic initiation factors to promote local phagophore formation (Chauhan *et al.*, 2016; Thurston *et al.*, 2012). Ubiquitinated K63 LMPs also recruit autophagy receptors. In parallel ubiquitinated K48 proteins are targeted by VCP to UPS degradation. VCP recruitment to lysosome membranes and functioning is mediated by its co-factors and adaptors: the deubiquitinating enzyme YOD1, UBXD1 and PLAA. UBXD1 has a UBA motif that permits ubiquitin chains recognition mediating substrates binding. (Akutsu *et al.*, 2016; Fujita *et al.*, 2013; Papadopoulos *et al.*, 2017).

AUTOPHAGY AND DISEASES

The autophagic functioning can decrease for different reasons. The first is the aging, which leads to a decrease in transcription, translation and post-translational modifications of elements involved in autophagy (Mizushima, 2010). Another cause is the malfunctioning of the components of the autophagic pathway due to direct interaction with protein aggregates (Wang *et al.,* 2009). Finally, the decrease in activity may be due to mutations in genes that encode protein involved in the pathway.

Several gene mutations associated to ALS alter the normal functioning of autophagy. Studies have shown that mutated SOD1 interacts with Beclin-1 (co-factor in the *initiation* phase) destabilizing its interactions with other co-factors (Hara *et al.*, 2006). Another autophagic protein associated to ALS is dynactin which is found mutated in few cases of fALS. Dynactin mutants

prevent the retrograde transport of autophagosomes and subsequently enhance accumulation of aggregates (Münch *et al.*, 2004; Puls *et al.*, 2003). Finally, mutations in VCP and CHMP2B (Charged Multivesicular Body Protein 2B a subunit of ESCRT-III) are shown to prevent the fusion between autophagosome and lysosome (Parkinson *et al.*, 2006; Skibinski *et al.*, 2005).

Another protein involved in autophagy dysfunction is TDP-43 mutants. Recent studies have shown that loss of function of TDP-43 mutants, leads to destabilization of Atg7 mRNA resulting in a reduced expression of Atg7 (Bose *et al.*, 2011). This may prevent the autophagosome formation since Atg7 has an important role in the *initiation* phase.

Autophagy seems to have a fundamental role in the degradation of TDP-43 inclusions. Initially these aggregates are eliminated via UPS but when they become too large they are eliminated via autophagy (Andersen & Al-Chalabi, 2011; Ciechanover & Kwon, 2015).

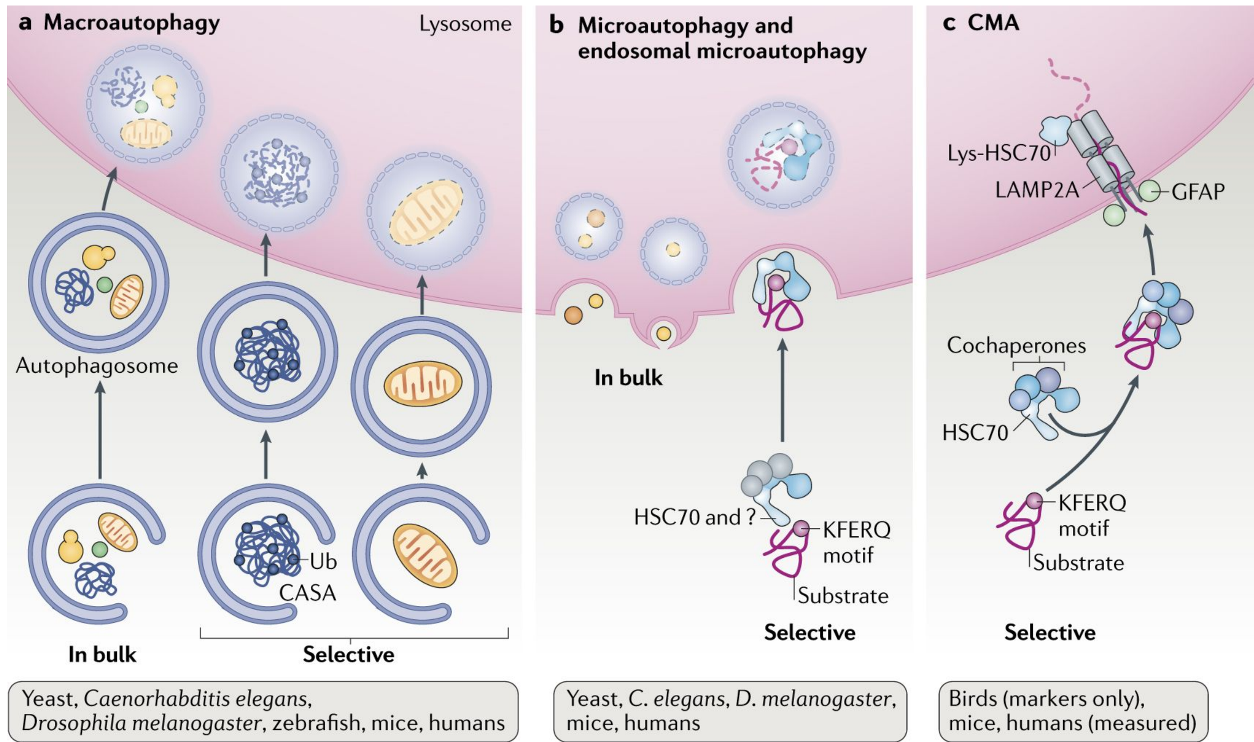


Figure 14 Different subtypes of autophagic pathway: macroautophagy, CMA and microautophagy. (Kaushik & Cuervo, 2018)

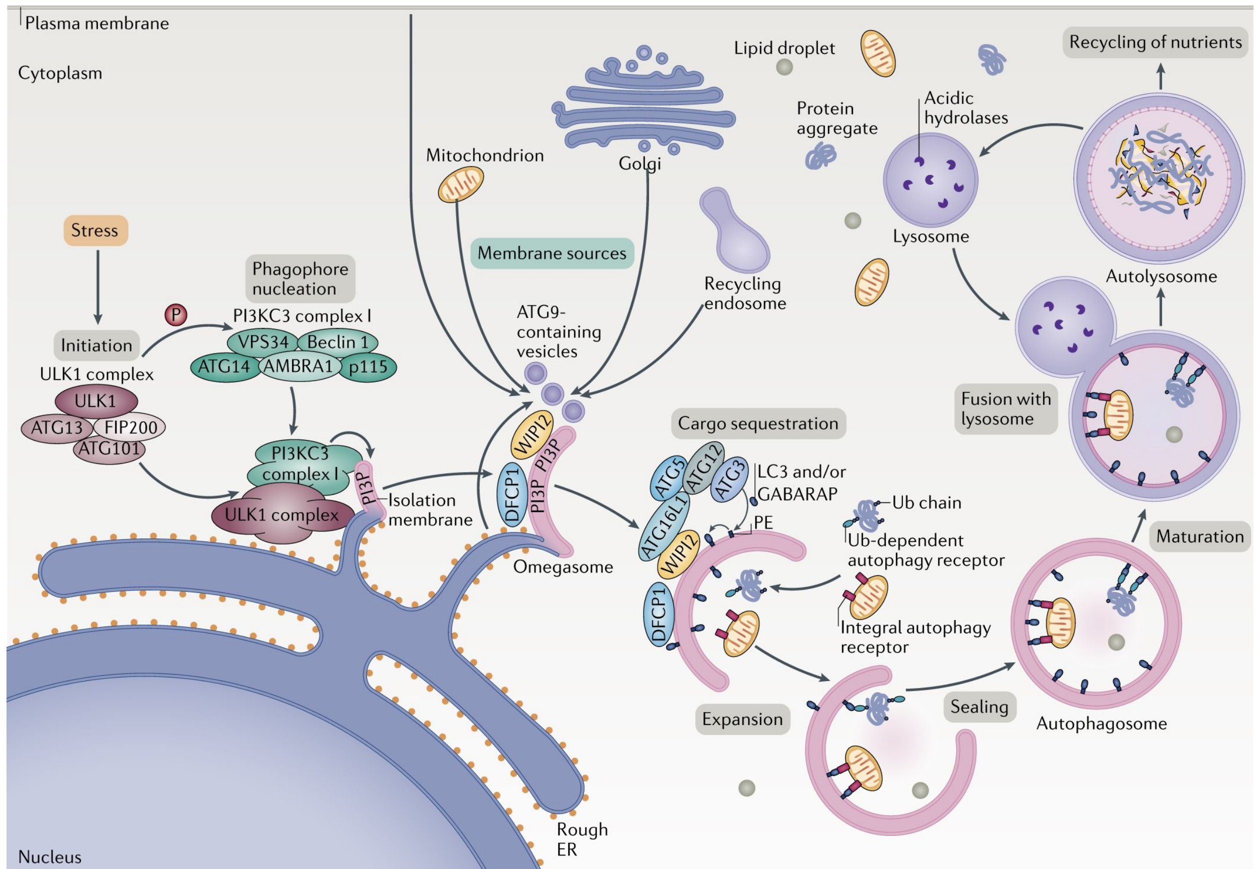


Figure 15 Regulation of autophagic pathway (Dikic & Elazar, 2018)

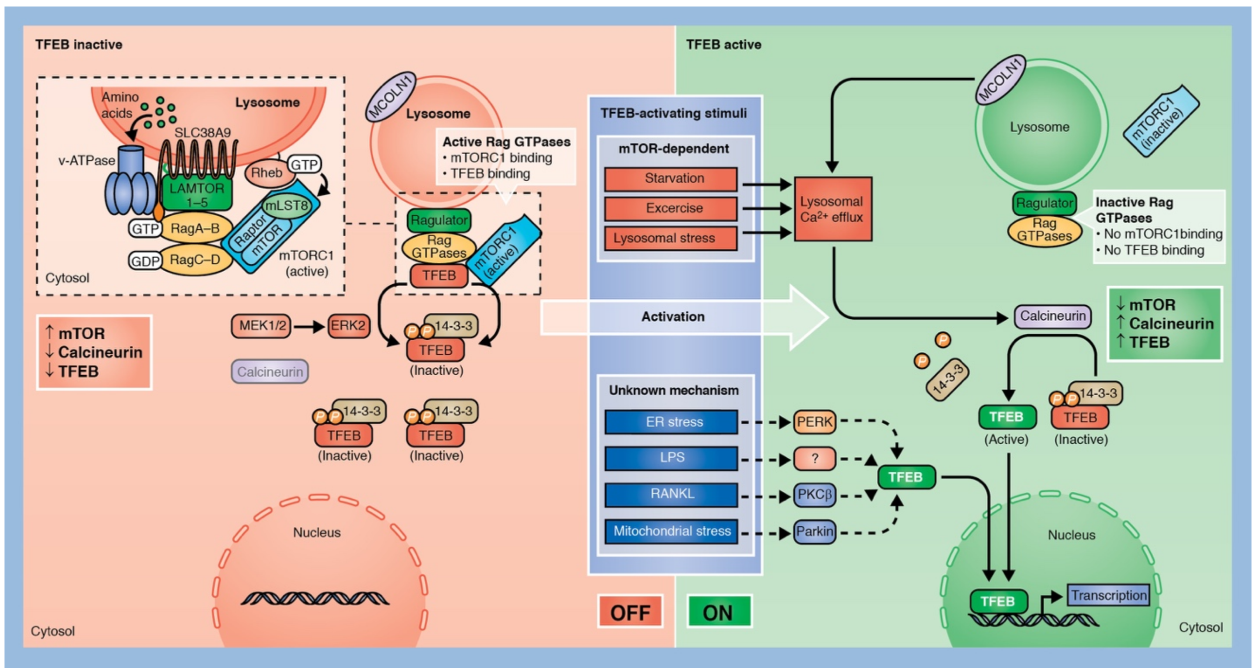


Figure 16 TFEB regulation (modified from Napolitano & Ballabio, 2016)

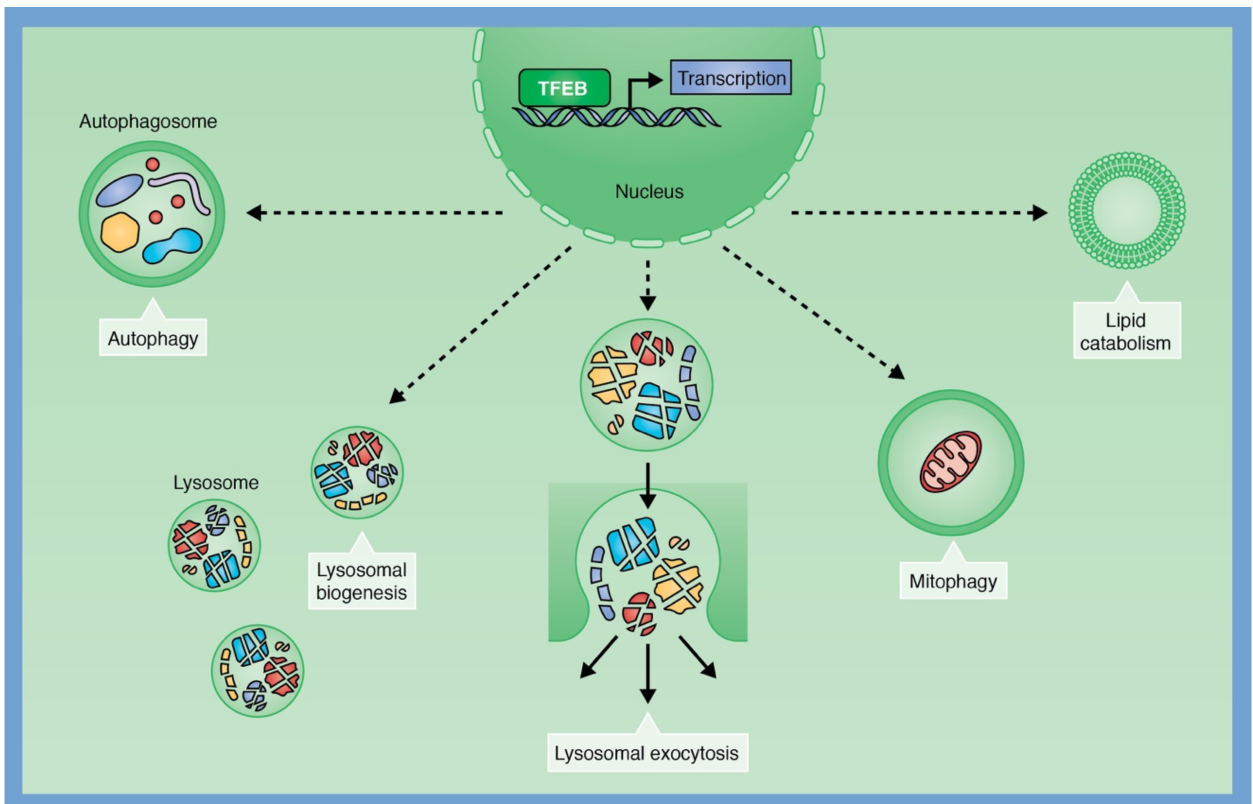


Figure 17 TFEB regulated pathways (modified from Napolitano & Ballabio, 2016)

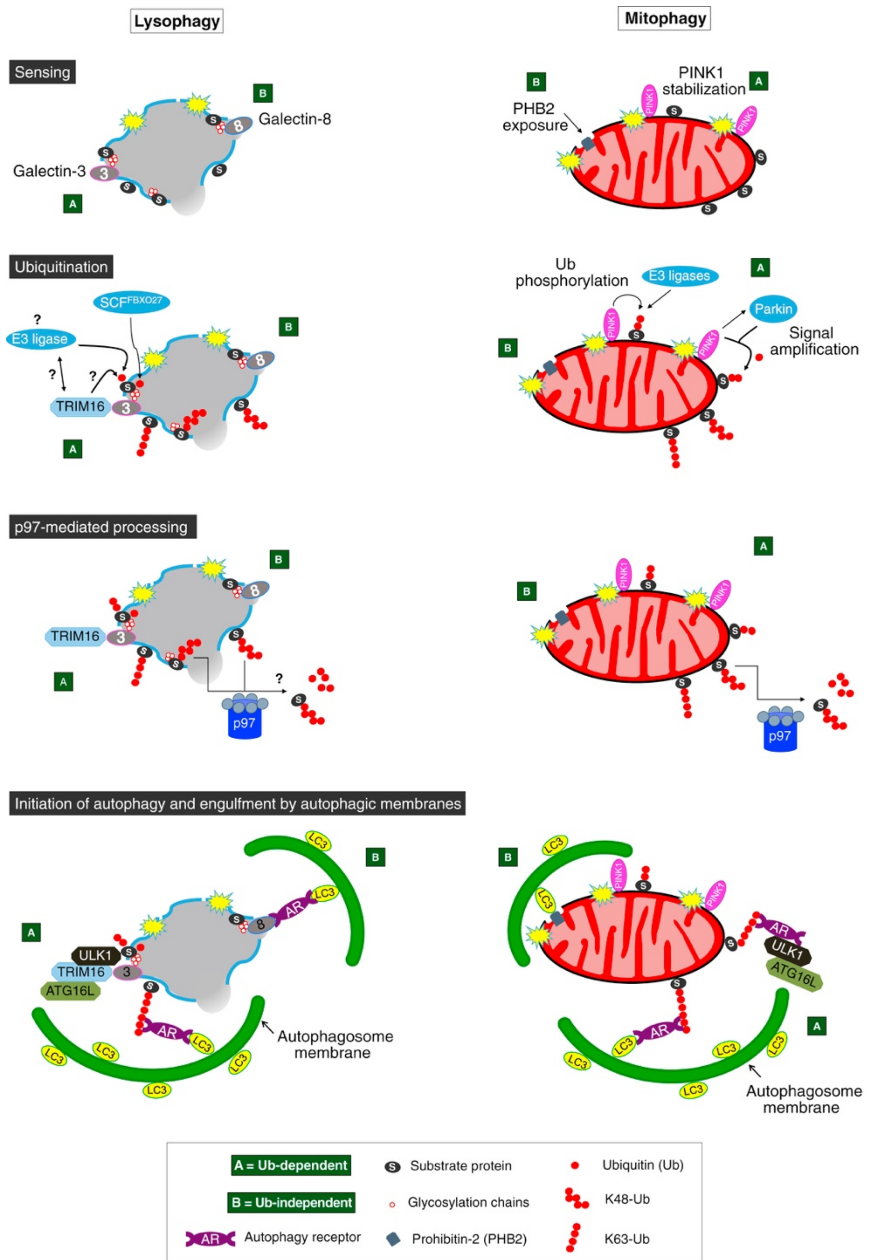


Figure 18 Lysophagy pathway (Papadopoulos & Meyer, 2017)

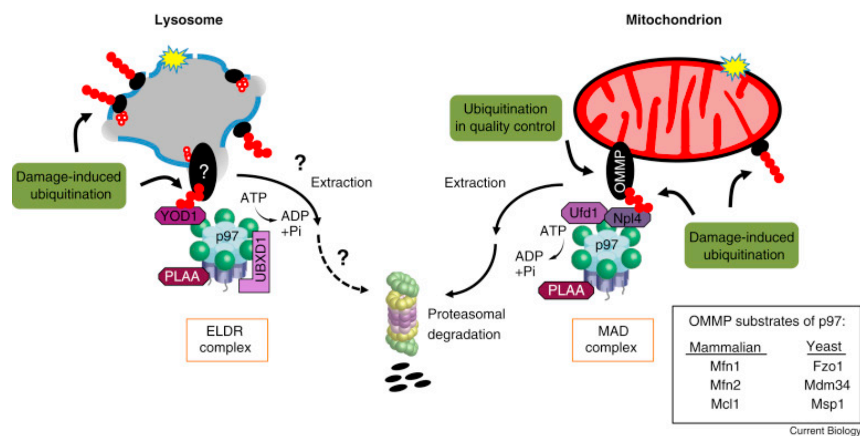


Figure 19 VCP role in lysophagy (Papadopoulos & Meyer, 2017)

MATERIALS AND METHODS

CELL CULTURES

To study ALS, a motor neuron disease, it was used *Neuroblastoma Spinal Cord* (NSC34), which is a mouse motor neuron immortalized cell line. NSC34 cells are routinely used in our lab. NSC34 are maintained in high glucose medium (Euroclone, Pero, MI, Italy) added with glutamine 1mM (Euroclone), with the antibiotics: penicillin G 100 U/ml (SERVA, Electrophoresis GmbH, Heidelberg, Germany) and streptomycin 100 U/mL (SERVA), and 5% fetal bovine serum (Sigma-Aldrich). Cells grow at 37°C and 5% of CO_2.

To use a human cell line to compare results obtained on NSC34 it was used SH-SY5Y. SH-SY5Y is neuroblastoma human cell line with neuronal phenotype. SH-SY5Y are maintained in high glucose medium (Euroclone, Pero, MI, Italy) added with glutamine 1mM (Euroclone), with the antibiotics: penicillin G 100 U/ml (SERVA, Electrophoresis GmbH, Heidelberg, Germany) and streptomycin 100 U/mL (SERVA), and 10% fetal bovine serum (Sigma-Aldrich). Cells grow at 37°C and 5% of CO_2.

PLASMIDS AND siRNA

The plasmid used are the following:

- pFLAG-VCP WT: encodes for human VCP WT tagged in N-terminal with FLAG tag, kindly provided by Prof. Serena Carra (UNIMORE).
- pFLAG-VCP R155H: encodes for human VCP mutant, R155H, tagged with FLAG on N-terminal domain. This plasmid is kindly provided by Prof. Serena Carra (UNIMORE).
- pFLAG-VCP R191Q: encodes for human VCP mutant, R191Q, tagged with FLAG on N-terminal domain. This plasmid was obtained replacing Arginin-191 with a Glutamine on pFLAG-VCP WT (Eurofins Genomics).
- pSOD1 WT: encodes for human SOD1 WT.
- pSOD1 G93A: encodes for human SOD1 mutant, G93A.
- pGFP-SOD1 WT: encodes for human SOD1 WT tagged with GFP fluorescence protein.
- pGFP-SOD1 G93A: encodes for human SOD1 G93A tagged with GFP fluorescence protein.
- pGFP-SOD1 A4V: encodes for human SOD1 A4V tagged with GFP fluorescence protein.
- pGFP-GAL3: encodes for Galectin 3 (GAL3), a marker of lysosomal damage: in physiological condition it is found diffused in the cytoplasm, after a lysosomal damage it colocalized with lysosomal raptured membrane . GAL3 is tagged with a GFP .
- pGFP-TFE3: encodes for TFE3 tagged with GFP.

- pCDNA3: Addgene plasmid, used as a transfection control.
- pSVIP: encodes for the functional domain of an isoform of SVIP a cofactor of VCP that is involved in ERAD and autophagy activation. Kindly provided by Prof. Serena Carra (UNIMORE). We used this plasmid in the first set of experiments, to analyze if it could contribute in enhancing VCP WT functioning, but the size and conformation of the partial domain of SVIP encoded, made it impossible to detect the expressed protein using our techniques. Moreover, the data where more complex than what it was firstly supposed so it was decided to leave aside SVIP contribute and to concentrate on explaining other data.
- pEGFPN1: Addgene plasmid that encodes for GFP protein, it is used as transfection control

The following siRNA duplex were used for silencing mPPP3CB endogenous expression: siRNA sense: 5′ UGAC AGAAAUGUUGGUAAAUU 3′ and antisense: 5′ UUUACCAA CAUUUCUGUCAUU 3′. As a control was used a non-targeting siRNA sense: 5′ UAGCGACUAAACACAUCAAUU 3′ and antisense: 5′ UUGA UGUGUUUAGUCGCUAUU 3′ (Dharmacon).

CHEMICALS

Cells were treated with:

- Z-Leu-Leu-Leu-al or MG132 (Sigma-Aldrich, Merck, Darmstadt, Germany) at 10μM for 16 hrs to inhibit the proteasome.
- 3-methyl-adenine or 3MA (Selleckem, Houston, TX, U SA) at 10mM for 48 hrs, directly diluted in medium. 3MA inhibits autophagy preventing autophagosome formation.
- N2,N4-dibenzylquinazoline-2,4-diamine or DBeQ (Sigma-Aldrich, Merck, Darmstadt, Germany) at 2.5 μM for 16 hrs a potent and specific inhibitor of VCP. It is reported that it inhibits the degradation of ubiquitinated proteins, the ERAD, and autophagosome maturation. DMSO was used as control (Sigma-Aldrich, Merck, Darmstadt, Germany).
- D-(+)-trehalose dihydrate (trehalose) (Sigma-Aldrich, T9531) used at 100 mM for 24hrs to study cytoplasmatic/nuclear translocation of TFEB and TFE3, and for different periods to study lysosomal damage: 18hrs, 6hrs, 2hrs. Recently it was demonstrated that trehalose treatment induced lysosomal damage and enhanced autophagy through the activation of TFEB in an mTORC1 independent pathway. (Rusmini *et al.*, 2019).
- NH_4Cl was used at 4μM for 90min. NH_4Cl is a late autophagy inhibitor, in particular it alters lysosomal pH preventing its functioning.

TRANSFECTION PROCEDURE

NSC34 cells were transfected with Lipofectamine® Transfection Reagent (Invitrogen, Thermo Scientific Life Sciences Research, Waltham, MA, USA), using manufacturer protocol: plasmid DNA is previously incubated with transferrin (Sigma-Aldrich, Merck, Darmstadt, Germany) and then mixed with Lipofectamine. SH-SY5Y cells were transfected with Lipofectamine 3000® Transfection Reagent (Invitrogen, Thermo Scientific Life Sciences Research, Waltham, MA, USA) using manufacturer protocol: plasmid DNA is previously incubated with p3000 in OPTIMEM and then mixed with Lipofectamine.

Experiments of Figure 20 were carried out in 12-well plates and the following quantities of plasmids were used: 0.5µg of pSOD1 WT or of pSOD1 G93A and 0.6 µg of pFLAG-VCP WT/pFLAG-VCP R55H/pFLAG-VCP R191Q or pCDNA3 as a control.

Experiments of Figure 21 on protein evaluation were carried out in 12-well plates and the following quantities of plasmids were used: 0.5µg of pSOD1 G93A and 0.6 µg of pCDNA3. MTT experiments where were carried out in 24-well plates and the following quantities of plasmids were used: 0,5mg of pCDNA3.

FLoIT experiments of Figure 22 were carried out in 24-well plates and the following quantities of plasmids were used 0.2µg of pGFPN1/pGFP-SOD1 WT/pGFP-SOD1 G93A/pGFP-SOD1 A4V and 0.3µg of pFLAG-VCP WT/pFLAG-VCP R55H/pFLAG-VCP R191Q or pCDNA3.

For experiments concerning VCP role in lysosomal damage, WB and FTA analysis the following quantities of plasmids were used: 1µg of pFLAG-VCP WT/pFLAG-VCP R55H/pFLAG-VCP R191Q or pCDNA3 as a control.

For Nuclear-Cytoplasmic analysis quantities were doubled.

For Immunofluorescence analysis cells were transfected with 0.2µg of pGFP-TFE3, and 0.3µg of pFLAG-VCP WT, pFLAG-VCP R155H, pFLAG-VCP R191Q or 0.5µg of pFLAG-VCP WT, pFLAG-VCP R155H, pFLAG-VCP R191Q.

For Galectin Puncta Assay cells were transfected with 0.2µg pGFP-GAL3, 0.2µg pSOD1 WT/pSOD1 G93A and 0.2µg of pFLAG-VCP WT, pFLAG-VCP R155H, pFLAG-VCP R191Q or 0.2µg pGFP-GAL3 and 0.3µg of pFLAG-VCP WT, pFLAG- VCP R155H, pFLAG-VCP R191Q.

For Electron Microscopy analysis cells were transfected with 0.8µg of pCDNA3, pFLAG-VCP R155H, pFLAG-VCP R191Q.

siRNA were transfected in NSC34 with Lipofectamine® 2000 Transfection Reagent (Invitrogen) following manufacturer instructions. 20 pmole of siRNA were incubated with Lipofectamine®

2000 reagent and the mix was added to cells directly in medium deprived of serum and antibiotics. After 5 hours medium was replaced with complete medium.

PREPARATION OF PBS PROTEIN EXTRACTS

NSC34 cells were plated in 12-well plate at 90,000 cell/ml. Whereas, SH-SY5Y cells were plated in 12-well plate at 120,000 cell/ml. After 48hrs from transfection and treatment cells were harvested, centrifuged (1,200 rpm for 5 min at 4°C) and resuspended in 75µL of PBS (Euroclone) with protease inhibitor cocktail 100X (IP) (Sigma-Aldrich). After sonication (3 hits at 10% of intensity) to lyse membranes, the total protein content of each sample was quantified with bicinchoninic acid (BCA) assay (Euroclone).

NUCLEAR-CYTOPLASM EXTRACTION

To analyze nuclear-cytoplasmic TFEB and TFE3 localization at the different condition, NSC34 were plated in 6-well plate at 90’000 cells/ml, transfected as explained, and treated for 24hrs with trehalose. Cells were harvested and were collected and centrifuged at 1’200 rpm for 5 min. at 4°C. Then pellets were lysed in lysis buffer (Tris-HCl 50 mM, pH 7.5, Triton X-100 0.5% (Sigma-Aldrich), NaCl 137.5 mM, glycerol 10% (Sigma-Aldrich), ethylenediaminetetraacetic acid 5 mM containing protease inhibitor cocktail (Sigma-Aldrich) to extract cytoplasmic fraction. Whereas, the supernatant, containing the nuclear fraction was transferred in a new tube, centrifuged 13’000 rpm for 15min at 4°C. The pellet was then resuspended in lysis buffer added with 0.5 % SDS (Sigma-Aldrich) and was sonicated (3 hits at 10% of intensity). Samples were then centrifugated and supernatant was transferred into a new tube. Protein quantification was then measured through the bicinchoninic acid (BCA) assay (Euroclone).
Samples were analyzed with WB assay.

FILTER TRAP ASSAY

Filter trap assay (FTA) is a technique that permits to quantify protein aggregates bigger than 0.22µm. utilizing the Bio-Dot SF Microfiltration Apparatus (Bio-Rad, Hercules, CA, USA) and specific antibodies. Samples containing 6µg of PBS soluble extracts from NSC34 and 9µg of PBS soluble extracts from SH-SY5Y, were loaded on a cellulose acetate membrane with pores of 0.22 µm. Then, thanks to a vacuum system, samples were filtered on the membrane. Proteins, trapped on the acetate membrane were then fixed by using methanol (10%). To analyze PBS-

insoluble proteins levels, the membrane was incubated first in blocking solution (5% dried non-fat milk (Euroclone) in T-BST 1X) for 1 hr, then with primary antibody in blocking solution for 1 hr. After two washes with TBS-T 1X of 10 min, the membrane was then incubated for 1 hr with HRP-conjugate secondary antibody diluted in TBS-T 1X. After a wash of 15 min. and four washes of 5 min. in TBS-T 1X signal was revealed with ClarityTM Western ECL Blotting Substrate (Bio-Rad) and optical densitometry was acquired using ChemiDoc XRS System (Bio-Rad). Results were finally analyzed using Prism 5.0, applying student T-test. Each result represents mean ± SEM of three biological replicates.

Primary antibodies used were: rabbit polyclonal SOD1 antibody (1:1000 Enzo Life Sciences) to analyze SOD1; mouse polyclonal anti-FLAG antibody (1:1000 Sigma-Aldrich) to analyze overexpressed VCP; mouse polyclonal anti-VCP antibody (1:1000 Abcam) to analyze total VCP expressed.
Secondary antibodies used were goat anti-rabbit HRP-conjugate secondary antibody (1:10’000 Santa Cruz Biotechnology) and goat anti-mouse IgG-HRP (1:10’000 Jackson ImmunoResearch).

WESTERN BLOT

Western blot is a technique that permits to evaluate total amount of SDS-soluble protein using specific antibodies. Experiments were carried out using 12% acrylamide gels. To analyze SOD1 protein, 15µg of NSC34 samples and 20µg of SH-SY5Y were loaded on gels. After electrophoresis, proteins were transferred with Trans-Blot Turbo (BIORAD) for 40 min at 25 V at RT on a nitrocellulose membrane with 0.45µm pores. Transfer with Trans-Blot Turbo is used to detect all proteins except for LC3. To detect LC3, proteins were transferred with Mini Trans-Blot® Cell (BIORAD) for 2hrs at 100V at 4° on a nitrocellulose membrane with 0.45µm pores. Membrane was then incubated for 1 hr at RT with blocking solution and then overnight at 4° with a primary antibody diluted in blocking solution. After two washes of 10 min with TBS-T 1X the membrane was incubated for 1 hr with secondary antibody diluted in TBS-T 1X.
Signal was revealed and acquired as for FTA.
The primary antibodies used were: rabbit polyclonal SOD1 antibody (1:1’000, Enzo Life Sciences), mouse polyclonal anti-VCP antibody (1:1’000, Abcam), mouse polyclonal anti-FLAG antibody (1:1’000, Sigma-Aldrich), rabbit polyclonal anti-p62 antibody (1:1’000, Sigma-Aldrich), rabbit polyclonal anti-GAPDH (1:1’000, Santa Cruz Biotechnology), mouse monoclonal anti-α-tubulin

(1:3’000, Sigma-Aldrich), rabbit polyclonal anti-LC3A/B (1:2’000, Sigma-Aldrich); rabbit polyclonal anti-TFEB (1:4’000, Bethyl Laboratories), rabbit polyclonal anti-TFE3 (1:3’000, Sigma-Aldrich), rabbit polyclonal anti-histone H3 (1:40’000, Abcam). The following secondary antibodies were used: goat anti-mouse HRP-conjugate secondary antibody (1:10’000, Santa Cruz Biotechnology), goat anti-rabbit HRP-conjugate secondary antibody (1:10’000, Santa Cruz Biotechnology), goat anti-rabbit IgG-HRP (1:10’000, Jackson ImmunoResearch), goat anti-mouse IgG-HRP (1:10’000 Jackson ImmunoResearch).

MTT ANALYSIS

NSC34 cells were plated in 24-well plate at 70’000 cell/ml, then were transfected as described before and finally were treated with DBeQ solution at different concentration. DBeQ solution was concentrated 1mM, 2.5 mM and 5mM and they were used as 1000X with a final concentration of 1μM, 2.5 μM and 5μM. DMSO was used as control.

MTT analysis was done after 16h hours of treatment. Cell medium was replaced with cell culture medium added with 1.5mg/1ml of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) powder and then cells were incubated for 30min at 37°C. During incubation period MTT was taken in cells and it was reduced to formazan, changing color from yellow to purple, by NAD(P)H-dependent cellular oxidoreductase enzymes present mitochondria of living cells. After incubation medium was taken away and it was added 2-propanol to stop the reaction and solubilize cells. Finally, samples absorption rate at OD 550 was read with spectrophotometer Enspire.

FLOW CYTOMETRIC ANALYSIS OF INCLUSIONS AND TRAFFICKING

Flow cytometric analysis of Inclusions and Trafficking (FLoIT) is a technique used to study protein inclusions and the trafficking of proteins between different cellular compartments. In this work it was used to study aggregates in particular for their identification and their quantification.

By using a cytofluorimeter, FLoIT technique allows to obtain: the number of inclusions ($\mathbf{n_i}$) present in a certain volume; the approximate number of cells by quantification of nuclei ($\mathbf{n_{nuc}}$) present in that volume; and transfection efficiency (γ) in the same volume. With these data it is possible to obtain the relative number of inclusions respect to the percentage of transfected cells. We defined the protocol for our model starting from the protocol described by Whiten and colleagues (Whiten *et al.*, 2016).

NSC34 cells were plated in 24-well plate at 60’000 cell/ml, were transfected as described above and were harvested after 72 hrs in PBS. Samples were centrifuged (1’200 rpm for 5 min at 4°C) and resuspended in 300µL of PBS (Euroclone) added with 5% of filtered fetal bovine serum (Sigma-Aldrich).

Then 30µl (A) of the resuspension was analysed with NovoCyte Flow Cytometer 3000 (ACEA Biosciences, San Diego, CA 92121, USA). This first measurement permitted to quantify transfection efficiency (**γ**). By using non-transfected samples, we could gate the GFP fluorescence eliminating cells’ autofluorescence. GFP fluorescence was measured using laser with excitation wavelengths of 488 nm and detector with band pass filter emission collection windows 515/10 nm.

In parallel, 150µl of LYSIS BUFFER 2x was added to 150µl of cell resuspension (B) and then was analysed with NovoCyte Flow Cytometer 3000. LYSIS BUFFER lysis cells membranes but cannot lyse aggregates and nuclei membrane. Aggregates and nuclei membrane resistance to LYSIS BUFFER permits to free them from cellular compartment and to quantify and analyse them individually. By using a control sample diluted in LYSIS BUFFER without DAPI (nuclei dye) it was possible to discriminate between nuclei population and non-nuclei population. DAPI fluorescence was measured using laser with excitation wavelengths of 407nm and detector with band pass filter emission collection windows 450/50 nm.

On nuclei population the number of nuclei present in the sample analysed where quantified ($\mathbf{n_{nuc}}$).

On non-nuclei population GFP signal was analysed. By using a sample transfected with pEGFPN1 (that it is known to encode for GFP soluble proteins) it was possible to discriminate between soluble GFP-events and aggregates GFP-events ($\mathbf{n_i}$) present in the samples.

Subsequently for each sample it was calculated the n° of inclusions (**i**) present in 100 transfected by using the formula:

$$\mathbf{i} = (\mathbf{n_i} / \mathbf{n_{nuc}} * \gamma) * 100$$

LYSIS BUFFER consists in PBS buffer added with protease inhibitor 100X, 0.5% of Triton X-100 (Sigma-Aldrich) and the nuclei dye, DAPI solution 20 mg/ml (Sigma-Aldrich) 10’000X.

IMMUNOFLUORESCENCE

NSC34 were plated on 13-mm coverslips in a 24-well plate at 70’000 cells/mL. Cells were transfected and/or treated as described before. Cells were then fixed using 4% paraformaldehyde solution and permeabilized using 10% TRITON X-100 in PBS solution. After the 1hr incubation at RT in blocking solution, cells were incubated with primary antibody overnight at 4°C and subsequently were incubated for 1 hr at RT with secondary antibody. Nuclei were stained with DAPI (1:10’000 in PBS) and coverslips were mounted on a support. Images were acquired using microscope Axiovert 200 (Zeiss, Oberkochen, Germany).

The following primary antibodies were used: mouse polyclonal anti-FLAG antibody (1:500, Sigma-Aldrich) and rabbit polyclonal anti-LC3A/B (1:200, Sigma-Aldrich). The following secondary antibodies were used: goat anti-mouse 549 Alexa Fluor® (1:1’000, Life Technologies, Thermo Fischer) and goat anti-rabbit 488 Alexa Fluor® (1:1’000, Life Technologies, Thermo Fischer). Fluorescence experiments in Figure 27 and Figure 28, were not processed with antibody after fixing. Nuclei were dyed with DAPI and coverslips were directly mounted on a support.

GALECTIN PUNCTA ASSAY

Galectin puncta assay was used to quantify lysosomal damage. To perform Galectin Puncta Assay, NSC34 were plated on 13-mm coverslips at 70’000 cells/ml in 24-well plates and transfected and treated as described. The cells were fixed as described for immunofluorescence assay, and cells with > 3 EGFP-GAL3 puncta were quantified by manual counting of 3 fields per sample and 3 samples per condition, using a PL 20X eyepiece with graticules (100 mm × 10 mm in 100-grid divisions). Fields were randomly selected (n = 3). Then cells that express green fluorescence was counted on the same field. To quantify positive GAL3 puncta cells on total transfected cells it was calculated the ratio between these parameters counted on each field. Then a statistical analysis was performed using Prism 5.0, applying student T-test. Each result represents the mean ± SEM of three biological replicates.

ELECTRON MICROSCOPY ANALYSIS

NSC34 cells were seeded at 90’000 cells/ml in a 2-well Nunc® Lab-Tek®Chamber SlideTM system (Nunc, C6682). The cells were transfected as described and after 48hrs were fixed using 2.5% glutaraldehyde (Sigma-Aldrich) in 0.1M sodium cacodylate (Sigma-Aldrich) pH 7.4 solution for 1 hr at RT. Then fixed cells were postfixed in osmium tetroxide (Electron Miscroscopy Science) for

2 hrs, and subsequently with 1% uranyl acetate (SERVA Electrophoresis) for 1 hr. Samples were then dehydrated using a graded ethanol series and then flat embedded in resin EMBED-812 (Electron Microscopy Science, 14120) for 24 hrs at 60°C. Ultrathin sections of 50 nm were then cut parallel to the substrate, stained with uranyl acetate 5% solution in 50% ethanol. Digital images were taken with a Megaview 3 camera using a CM10 electron microscope (Philips, Eindhoven, The Netherlands).

PART I: VCP ROLE IN AGGREGATES CLEARANCE IN A fALS MODEL

AIM

ALS is a deleterious disease with no cure to date. One of the main pathological hallmarks is the presence of insoluble aggregates/inclusions that may lead to cell toxicity and death. Cells activate various mechanisms to counteract aggregates toxicity by eliminating them. Previous studies from my lab have demonstrated the involvement of different chaperones complexes in the enhanced clearance of insoluble aggregates associated to fALS, sALS and to other motor neuron diseases.

In my work I focused on the functioning of VCP, a AAA$^+$ ATPase protein with an important role in disassemble large aggregates. Data demonstrate VCP co-localization with different type of aggregates. Moreover, recently it has been shown VCP involvement in the clearance of polyglutamate aggregates. In addition, VCP-mutants associated to degenerative diseases are correlated to the presence of inclusions positive to TDP-43 and to signs of alteration in the degradative systems. These findings show an important role of VCP in the clearance of insoluble aggregates.

In this context I analysed VCP involvement in the clearance of SOD1-mutants, a model of fALS. In particular, I studied VCP role on SOD1 G93A overexpressed in an immortalized motor neuron cell line.

In parallel, to confirm VCP role I compared its functioning with two different VCP-mutants (VCP R155H and VCP R191Q) associated to ALS, that should lose VCP disaggregating function. In fact, VCP R155H and VCP R191Q are both correlated to the presence of TDP-43 inclusions in the brain tissue of affected patients.

Moreover, as VCP-mutants are not dominant negative of VCP but only partially loose VCP functionality, I also studied SOD1 G93A aggregates in the presence of chemical inhibition of VCP, using DBeQ treatment.

RESULTS

VCP WT AND MUTANTS OVEREXPRESSION DECREASES THE LEVELS OF SOD1 G93A INSOLUBLE SPECIES

To study VCP WT chaperone like activity and its function on the disassembling of SOD1 G93A aggregates, SOD1 WT and SOD1 G93A were co-transfected in NSC34 with human VCP WT and VCP-mutants. (Figure 20 A, B). VCP-mutants were firstly used as a negative control. As I have explained VCP-mutants are associated to the presence of protein inclusions and of altered degradative systems.

To analyse SOD1 G93A insoluble species levels a FTA was performed; this technique permits to quantify the levels of PBS-insoluble species characterized by a size bigger than 0.22μm. To evaluate the overall levels of SOD1 protein which is SDS-soluble, I utilized a classical WB analysis. I used an anti-SOD1 antibody showed capable to recognize both the exogenous overexpressed human SOD1 (~23kDa, higher band) transfected in cells and the endogenous mouse SOD1 (~19kDa, lower band).

In WB (Figure 20 B) I found that the levels of the SDS-soluble fraction of SOD1 G93A were specifically decreased when co-transfected with VCP WT and mutants if compared to mock transfected cells. Also, SOD1 WT overexpressed levels decrease in presence of VCP WT showing a function of VCP in the clearance of SOD1 when its translation is increased. Moreover, WB showed that there is an increased trend of the overall p62 levels in presence of SOD1 G93A, which can be considered a signal of an alteration in the degradation pathways. GAPDH was used as loading control.

The FTA data (Figure 20 A) showed higher levels of the insoluble fraction of SOD1 G93A comparing to SOD1 WT levels (***=$p<0.001$). These data are in line to those reported in literature; indeed, SOD1-mutants tend to form insoluble aggregates whereas SOD1 WT is found mainly in soluble form. Moreover, in FTA I found a decrease in the levels of the insoluble fraction of SOD1 G93A in presence of overexpressed VCP WT (**=$p<0.01$). Surprisingly SOD1 G93A insoluble fraction decreased also in the presence of VCP-mutants overexpression (***=$p<0.001$, **=$p<0.01$).

In experiment described human VCP WT and mutants were overexpressed in mouse cell line. Therefore, I evaluated that the overexpressed human VCP could interact correctly with the

mouse endogenous VCP forming a proper homo-hexamer, and consequently that the decrease in the insoluble fraction of SOD1 G93A was due to the overexpressed VCP and not to the endogenous VCP WT (Figure 20 C). To this purpose, SOD1 G93A accumulation was tested in presence of VCP WT, VCP R155H, VCP R191Q in a human derived neuronal cell line: the SH-SY5Y cells. The results obtained were found to be very similar to those observed in the mouse NSC34 cell line. In fact, the FTA (middle inset) showed a decrease trend of SOD1 G93A levels in presence of VCP WT and a significant decrease in presence of VCP mutants (**=$p<0.01$, *=$p<0.05$). WB analysis (upper inset) performed using the SOD1 antibody, showed together endogenous and exogenous human SOD1 at comparable levels in all conditions tested. α-Tubulin (TUBA) was used as loading control.

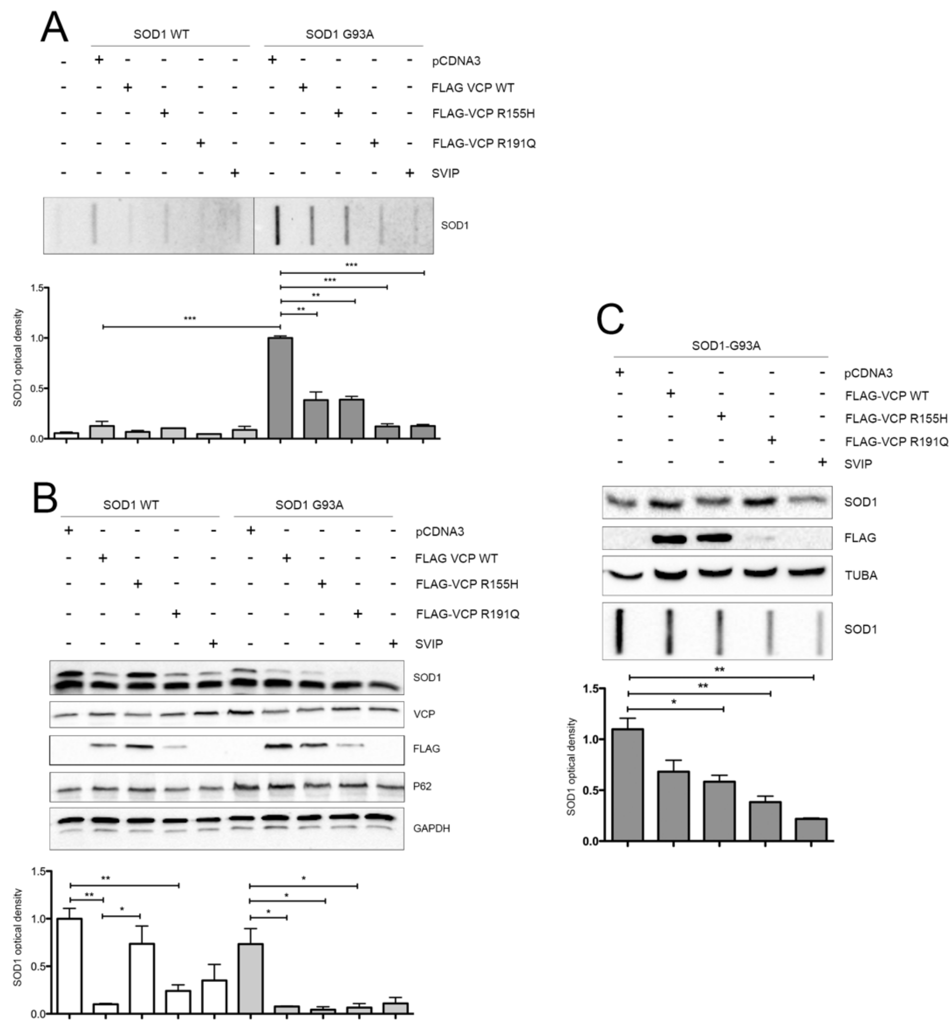


Figure 20 ***VCP WT and mutants overexpression decreases the levels of SOD1 G93A insoluble species****. (A) Filter TRAP assay (FTA) (upper inset) of PBS extracts and optical densitometry quantification of FTA (lower inset) ***=p<0.001, **=p<0.01; T-test. (B) Western blot (upper inset) and optical densitometry quantification of SOD1 in WB (lower inset) **=p<0.01, *=p<0.05; T-test. (C) Western blot (upper inset) and Filter Trap assay (FTA) (middle inset) of PBS extracts. Optical densitometry quantification of FTA (lower inset) **=p<0.01, *=p<0.05; T-test.*

VCP WT ENHANCES THE CLEARANCE OF SOD1 G93A AGGREGATES THROUGH THE UPS

Once I have found that overexpressed VCP WT decreases the levels of SOD1 G93A insoluble species I further analyzed VCP role in the clearance of SOD1 G93A by defining which degradative pathway was involved (Figure 21). In particular, I have studied if VCP enhances SOD1 G93A degradation through the UPS or the autophagic pathway. Thus, to evaluate which pathway VCP used to decrease SOD1 G93A insoluble fraction, I co-transfected SOD1 G93A and VCP WT and the corresponding mutants in NSC34 cells, then I chemically inhibited the two major degradation pathways: to inhibit the UPS I used MG123, an inhibitor of the proteasome and to inhibit the autophagic pathway I used 3MA, an early inhibitor of the autophagic pathway.

WB analysis (Figure 21 A upper inset, B, C) showed that MG132 treatment increased the levels of the SDS-soluble fraction of SOD1 G93A in all conditions. Moreover, WB showed that 3MA treatment increased the SDS-soluble fraction in mock cell condition, that is reverted only in presence of VCP WT (*=$p<0.05$). α-Tubulin (TUBA) was used as loading control.

In FTA (middle and lower inset), MG123 treatment brought to a significant increase in the levels of the SOD1 G93A insoluble species compared to untreated samples (**=$p<0.01$). The increased insoluble-fraction levels of SOD1 G93A were partially reverted by the presence of VCP WT (*=$p<0.05$) and VCP mutants (at non-significant trend).

The FTA also showed that 3MA treatment brought to an increase of the SOD1 G93A insoluble species compared to untreated control (**=$p<0.01$). The increased insoluble-fraction levels of SOD1 G93A were completely reverted by the overexpression of VCP WT (***=$p<0.001$). Whereas, the overexpression of VCP-mutants only partially reverted the increase (*=$p<0.05$). Moreover, SOD1 G93A insoluble-fraction levels in presence of VCP-mutants were significantly higher than SOD1 G93A insoluble-fraction levels in presence of VCP WT overexpressed (***=$p<0.001$, *=$p<0.05$). This brought to define a specific loss of function of VCP mutants in presence of autophagy inhibition.

Together, these data show that VCP WT works through the UPS. In fact, UPS inhibition prevented VCP WT functioning while autophagy inhibition did not influence VCP WT functionality. Moreover, inhibition of either degradative pathways prevented VCP-mutants activity in the decrease of SOD1 G93A insoluble fraction levels. In particular, these data show that the inhibition

of autophagy leads to a different behavior of VCP-mutants from VCP WT, underling a dependence of VCP-mutants on autophagy.

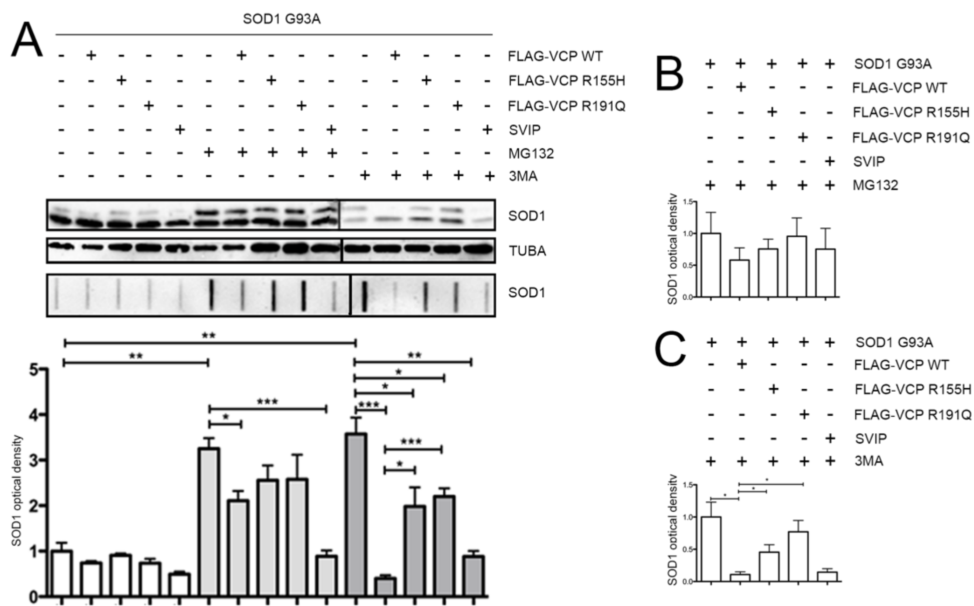


Figure 21 ***VCP WT enhances the clearance of SOD1 G93A aggregates through the UPS****. (A) Western blot (upper inset) and Filter Trap assay (FTA) (middle inset) of PBS extracts. Optical densitometry quantification of FTA (lower inset) ***=p<0.001, **=p<0.01, *=p<0.05; T-test. (B) Optical densitometry quantification of SOD1 in WB for MG132 treatment. (C) Optical densitometry quantification of SOD1 in WB for 3MA treatment; *=p<0.05; T-test.*

VCP INHIBITION HAS NO EFFECTS ON SOD1 G93A AGGREGATION

Previous data showed that SOD1 G93A insoluble fraction clearance is dependent from VCP WT and that VCP WT overexpression triggers its degradation. To define if VCP WT has an exclusive role in the degradation of SOD1 G93A aggregates, I studied SOD1 G93A aggregation levels in condition in which VCP was chemically inhibited. In particular, I used DBeQ, which is a specific inhibitor of VCP acting by preventing the functioning of both ATPase domains. The DBeQ treatment was performed on NSC34 cells overexpressing SOD1 G93A.

I firstly tested different concentrations of DBeQ treatment in our model to find the highest concentration that did not influence cells viability (Figure 22 A). NSC34 cells were transfected pCDNA3 and treated for 16hrs with different concentration of DBeQ: 1μM, 2.5μM and 5μM. MTT analysis showed a decrease in cell viability only with 5μM DBeQ treatment (**=$p<0.01$), so 2.5μM is the highest concentration used that did not alter cell viability.

Once defined DBeQ concentration, NSC34 cells were transfected with SOD1 G93A and treated with 2.5μM DBeQ, using DMSO as control (Figure 22 B, C). WB analysis and quantification (Figure 22 B), showed an increase of p62 levels that confirmed the efficiency of DBeQ treatment. Moreover, WB showed comparable levels of the total SOD1 G93A. GAPDH was used as loading control. FTA (Figure 22 C) confirmed that VCP inhibition did not influence SOD1 G93A levels as it was shown by WB analysis. In fact, FTA showed that insoluble-fraction levels of SOD1 G93A in presence of DBeQ treatment were comparable to the insoluble-fraction levels of SOD1 G93A treated with DMSO.

Data obtained showed that VCP role is not exclusive in the removal of SOD1 G93A aggregates. In fact, VCP inhibition did not result in an increase of SOD1 G93A levels that would have reported a dependency from VCP activity. Indeed, DBeQ treatment did not change neither total levels of SOD1 G93A, as WB analysis showed, nor the insoluble-fraction levels of SOD1G93A, as FTA showed.

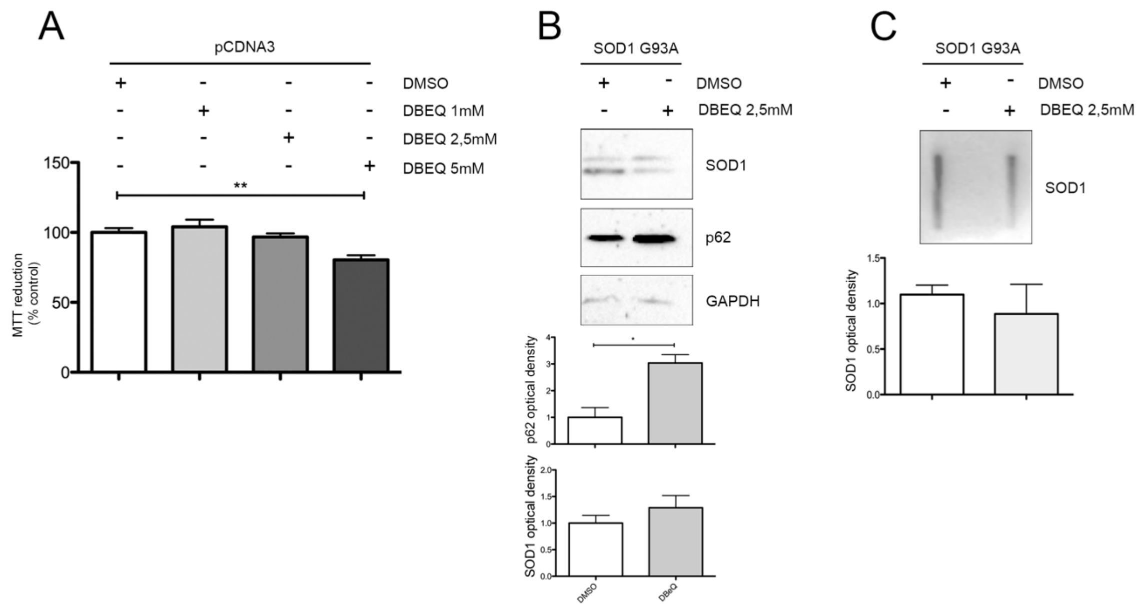


Figure 22 ***VCP inhibition has no effects on SOD1 G93A aggregation*** *(A) MTT analysis, **=p<0.01; t-test. (B) Western Blot (upper inset). Optical densitometry quantification of P62 (middle inset); *=p<0.05; t-test. Optical densitometry quantification of SOD1 (lower inset). (C) Filter Trap assay (FTA) (middle inset) of PBS extracts. Optical densitometry quantification of FTA (lower inset).*

FLoIT QUANTIFICATION OF SOD1 G93A INSOLUBLE AGGREGATES IN PRESENCE OF VCP WT AND MUTANTS

To better quantify SOD1 insoluble levels and confirm VCP contribute in SOD1 G93A aggregates clearance I defined and applied FLoIT technique in our model. FLoIT is a novel technique used to study protein inclusions and their trafficking between different cellular compartments. In particular, I used FLoIT to quantify and evaluate modifications in the levels of SOD1 G93A aggregates in presence of overexpressed VCP WT and VCP-mutants.

Using FLoIT I was able to quantify the total number of inclusion relative to transfected cells. Firstly I defined samples transfection efficiency, quantifying in a certain volume of not lysed sample the total number of cells present and the number of cells that expressed GFP protein transfected (Figure 23 A). Then, after lysing samples as described in "Materials and methods chapter", I quantified for each sample the number of DAPI-positive nuclei (considered the number of cells) and the number of GFP-positive inclusions (Figure 23 B). Finally to quantify for each sample the number of inclusions relative to transfected cells I divided the number of inclusions quantified by the total number of transfected cells quantified in the same sample.

In Figure 23 A, B and C samples analysed were NSC34 cells transfected with pEGFPN1, pGFP-SOD1 WT, pGFP-SOD1 G93A and pGFP-SOD1 A4V. Figure 23 A shows the analyses done on samples not lysed to evaluate their transfection efficiency. In the first column through SSC and FSC parameters cell population was identified. On cell population using FITC detector (that collects GFP fluorescence) I first defined a gate using a non-transfected sample then I counted GFP-positive cells for each sample (second column of Figure 23 A). Figure 23 B shows data collected of each sample after it had been lysed to permit the individuation and quantification of each insoluble aggregate that cannot be detected in an un-lysed cell. By using PASIFIC BLUE detector (that collects DAPI fluorescence) and a sample lysed with a LYSIS BUFFER w/o DAPI, I firstly defined a gate that discriminated between DAPI-positive and DAPI-non positive populations (not shown). DAPI-positive population was quantified to define the number of nuclei present in the sample. Then on DAPI-non positive population (in second column of Figure 23 B) using FITC detector and pEGFPN1 sample I defined a gate that discriminated in GFP-positive and GFP-non positive population. GFP-positive population was quantified to define the number of inclusions present in the sample. The graphic in Figure 23 C, shows the total number of inclusions relative to transfected cells quantified for each condition, (all conditions were reported to the condition

expressing pEGFPN1). The figure shows an increase in SOD1 mutant aggregates compared to SOD1 WT (***=$p<0.001$, **=$p<0.01$) as it was reported in literature. Figure 23 D shows inclusion quantification and condition comparison of samples co-transfected with pSOD1 G93A and pFLAG-VCP WT and mutants. Surprisingly, SOD1 G93A inclusions significantly decreased only in the presence of VCP R191Q (**=$p<0.01$).

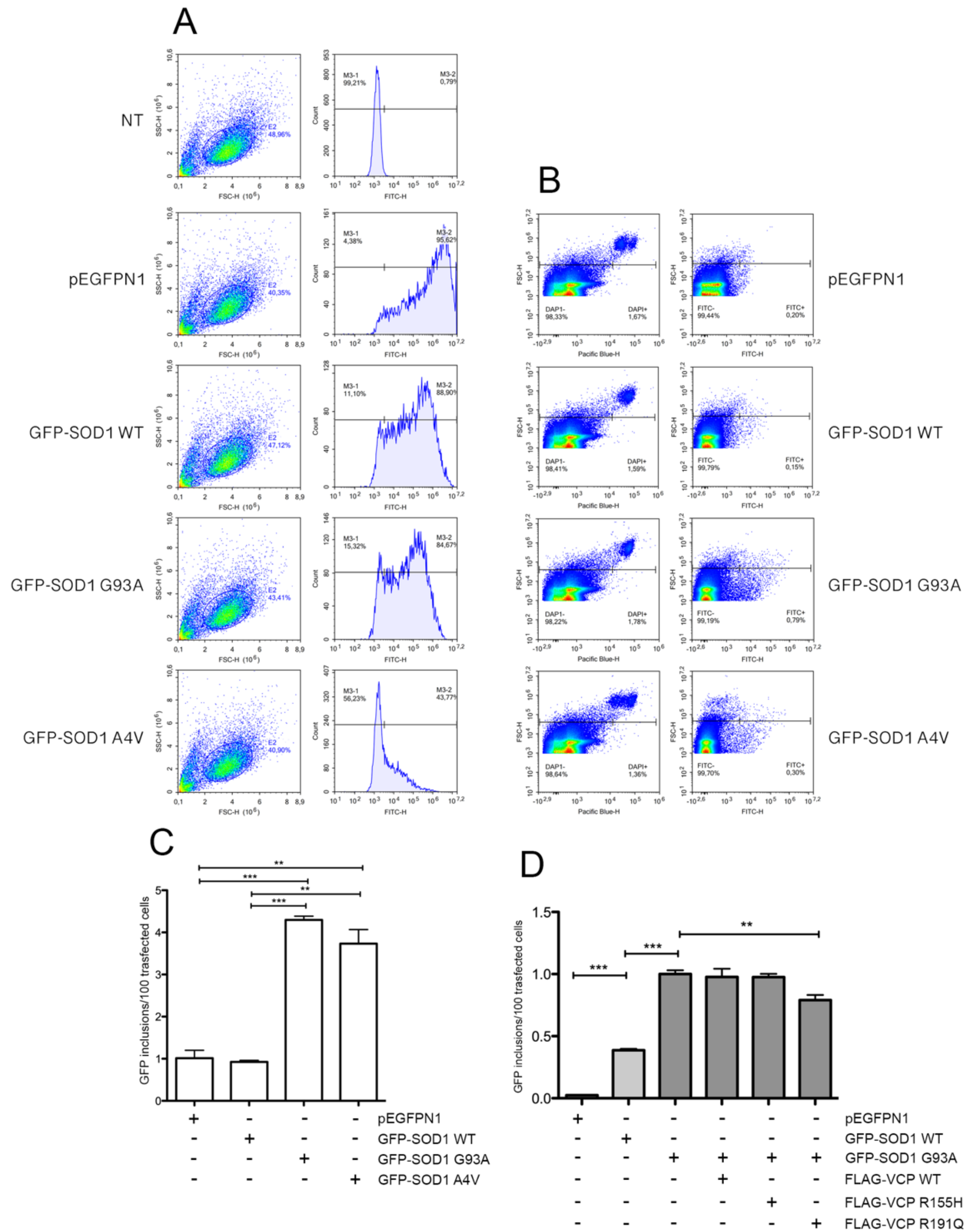


Figure 23 ***FLoIT quantification of SOD1 G93A insoluble aggregates in presence of VCP WT and mutants.*** *(A) Definition of transfection efficiency. (B)Definition of nuclei and non-nuclei populations and GFP aggregates. (C) Quantification of relative number of GFP positive inclusions respect to the percentage of transfected cells. ***=p<0.001, **=p<0.01. (D) Quantification of relative number of GFP positive inclusions respect to the percentage of transfected cells. ***=p<0.001, **=p<0.01.*

DISCUSSION

Amyotrophic Lateral Sclerosis (ALS) is a motor neuron disease with an incidence of 4-5 cases over 100'000 per year, for which no cure is available. Different pathogenic mechanisms have been identified to concur to ALS onset. One of the main mechanism is the alteration proteostasis. In fact, ALS is characterized by the presence of insoluble inclusions found in the brain of affected individuals. Moreover, various mutated genes as *SOD1, TDP-43, FUS* and *C9ORF72,* express proteins that misfold and form aggregates. If not removed, aggregates may become toxic to cells concurring to their death (Hirakura & Kagan, 2001; Kourie & Shorthouse, 2000; H. Lin *et al.*, 2001; Ross, 2002). To prevent aggregates toxicity, cells activate different processes that are part of the PQC system. PQC system activates different chaperone complexes to eliminate misfolded proteins aggregating prone and aggregates. VCP is an AAA^{+} ATPase protein with a key-role in many pathways of the PQC system. One of its role is to disassemble protein aggregates (Gallagher *et al.*, 2014; Ghosh *et al.*, 2018). In particular, it has been shown to co-localize with misfolded protein aggregates and to concur in the clearance of polyglutamine protein aggregates.

In my lab different pathways have been studied as targets to enhance aggregates clearance (Crippa *et al.*, 2010; Crippa *et al.*, 2016; Cristofani *et al.*, 2017, 2018). In this work I studied VCP contribute in the removal of aggregates in a fALS model. In particular, I studied VCP role in presence of overexpressed SOD1 G93A in NSC34 cell line.

First, FTA analysis showed a decrease in SOD1 G93A insoluble-fraction in presence of overexpressed VCP WT. Moreover, by inhibiting UPS and the autophagic pathway, that are the degradation pathway where VCP is involved, I could define that VCP WT enhanced the clearance of SOD1 G93A insoluble-fraction through UPS. In fact, when the UPS was blocked VCP WT overexpression only partially decreased SOD1 G93A insoluble-fraction levels whereas, the inhibition of autophagy did not alter VCP WT functioning that completely reverted SOD1 G93A aggregation. These data confer a novel role to VCP in the removal of ALS-associated aggregates, confirming VCP chaperone activity previously shown in relation to other models as Huntingtin-mutant model (Ghosh *et al.*, 2018). Moreover, I further analysed if VCP contribute was essential for SOD1 G93A insoluble-fraction degradation by chemically inhibiting VCP, using DBeQ treatment. Results showed that chemical inhibition of VCP did not change SOD1 G93A insoluble-fraction levels. These data suggest that VCP WT contribute in the removal of SOD1 G93A inclusions is not essential, but there are other pathways involved. In fact, in my lab it has been previously demonstrated that the modulation of another chaperon (HSPB8), could promote

SOD1-mutant clearance through the autophagic pathway (Crippa *et al.*, 2010). Thus, VCP WT modulation could be an alternative pathway UPS-dependent.

In parallel, I studied VCP mutants (VCP R155H and VCP R191Q) contribute in the removal of SOD1 G93A in NSC34 cell line. In brain tissue of patients, VCP R155H and VCP R191Q were both correlated to the presence of insoluble inclusions and signs of alteration in degradation pathways (Johnson *et al.*, 2010). These data suggested VCP mutants general misfunctioning in aggregates clearance. For these reasons I decided to study these mutants in the condition previously described, comparing them to VCP WT functioning. Surprisingly, FTA analysis showed that VCP mutants overexpression led to a decrease in the levels of insoluble species of SOD1 G93A. As VCP mutants behaviour was not in line with my hypothesis, I tested the same condition in a human cell line to be sure that the decrease in SOD1 G93A levels was due to the overexpressed human VCP that could interact correctly with human endogenous VCP. Data in human cell line were in accord with data observed in NSC34, confirming VCP-mutants contribute in decreasing SOD1 G93A aggregates. Moreover, also VCP mutant functionality was studied in condition of inhibition of the degradation systems. With proteasome inhibition, VCP mutant behaviour was similar to VCP WT. Conversely, VCP mutant behaviour differed from VCP WT when the autophagic pathways was inhibited. In fact, in this condition VCP WT completely reverted SOD1 G93A increased inclusion levels, whereas VCP mutant only partially reverted SOD1 G93A increased inclusion levels. Thus, SOD1 G93A insoluble fraction levels in presence of VCP mutants were significantly higher compared to the presence of overexpressed VCP WT. This unexpected activity of VCP-mutants, which both presented in patients tissue signs of altered activity, was further investigated in the second part of my work as I will describe in "Part II: VCP mutants enhance lysosomal damage and activate autophagy" chapter. I speculated that the presence of VCP-mutants could induce cellular-stress that could activate degradation pathways as it shown for SOD1-mutants (Morimoto *et al.*, 2007).

Finally, in this part, I defined a protocol to better evaluate SOD1 mutant inclusion levels and to better appreciate changes in aggregation levels. FLoIT technique resulted very reliable and reproducible to visualize SOD1 G93A inclusions, so I retested the same condition previously described. Using FLoIT, VCP WT and VCP R155H overexpression showed a not significant influence on SOD1 G93A inclusion levels. Conversely VCP R191Q overexpression showed a partial decrease in SOD1 G93A insoluble inclusions levels.

PART II: VCP MUTANTS ENHANCE LYSOSOMAL DAMAGE AND ACTIVATE AUTOPHAGY

AIM

As shown, ALS is associated to alteration in proteostasis. For this reason ALS is considered a proteinopathy. Among the different features, proteinopathies are characterized by the presence of misfolded protein aggregates and alteration PQC system. ALS-associated VCP-mutations are correlated at cellular level, to the presence of cytoplasmic TDP-43 insoluble aggregates and to the presence of altered degradation pathways. Alteration of proteostasis associated to VCP-mutants can be correlated to VCP mis-functioning. In fact, VCP is involved in several pathways of the PQC system. Recently studies demonstrated that VCP plays an essential role in regulating damaged lysosome degradation through the autophagic pathway. Lysosomes damage is deleterious for cells in different ways. Firstly, for their loss of function, that prevents the correct functioning of autophagic mechanisms. Moreover, lysosome damage results in leakage of lumen proteinases, cathepsins and Ca^{2+} that induce cellular toxic effects.

In this part of my thesis, lysosomal-damage response was analysed in presence of overexpressed VCP WT, and its ALS-associated mutants (VCP R155H and VCP R191Q) in NSC34 cells. To study VCP role in this mechanism, I biologically and chemically induced lysosome damage.

Moreover, as I observed that VCP-mutants overexpression led to lysosome alteration, I analysed if and which pathway resulted activated in these conditions.

RESULTS

VCP-MUTANTS FORM INSOLUBLE SPICIES IN NSC34

The first part of my work showed an unexpected behavior of VCP-mutants in relation to their chaperone-like activity. In fact, data from literature show that VCP-mutants are associated to the formation of intracellular inclusions and altered degradation pathways, signals of a loss of functionality. Whereas, I observed a functionality of VCP-mutants in the removal of SOD1 G93A aggregates. For these reasons I decided to further investigate VCP ALS-associated mutants behavior in ALS *in vitro* model. To perform this study, I overexpressed VCP WT and VCP-mutants (VCP R155H and VCP R191Q) in NSC34 cells.

Firstly, I analyzed VCP-mutants tendency to aggregate in this model using FTA technique (Figure 24 A and B). To study the insoluble fraction of overexpressed VCP, FTA was processed with FLAG-antibody (figure 24 A) which recognizes the FLAG tag present in the N-terminus of the recombinant VCP protein overexpressed. FTA showed a significant increase of VCP-mutants insoluble-fraction compared to VCP WT (*=$p<0.05$). Moreover, VCP R191Q showed higher increased levels compared to VCP R155H (*=$p<0.05$). Then, I analyzed whether exogenous VCP-mutants altered total VCP tendency to aggregate by processing FTA with VCP-antibody, which can recognize both endogenous and exogenous overexpressed VCP (Figure 24 B). VCP R191Q insoluble-fraction levels are higher compared to VCP WT and to VCP R155H (*=$p<0.05$). In particular VCP insoluble-fraction in the samples overexpressing VCP R191Q was increased of an average of three times respect to the samples overexpressed VCP WT, whereas in the case of the samples overexpressing the VCP R155H mutant I did not find any significant increase of the overall amount of the VCP insoluble-fraction.

WB analyses (Figure 24 C) processed with FLAG antibody showed that overexpressed VCP WT and mutants SDS-soluble fraction have comparable levels. Moreover, using VCP antibody, the WB analysis showed that total levels of VCP expressed are comparable in all conditions. Thus, VCP mutants affected total VCP insoluble-fraction, as FTA showed, but did not alter the SDS-soluble fraction. GAPDH was used as loading control.

These data showed that VCP mutants have an aggregate propensity in a neuronal model in line to what is shown in literature. Moreover, their increased tendency in aggregating did not influence the levels of the total VCP expressed.

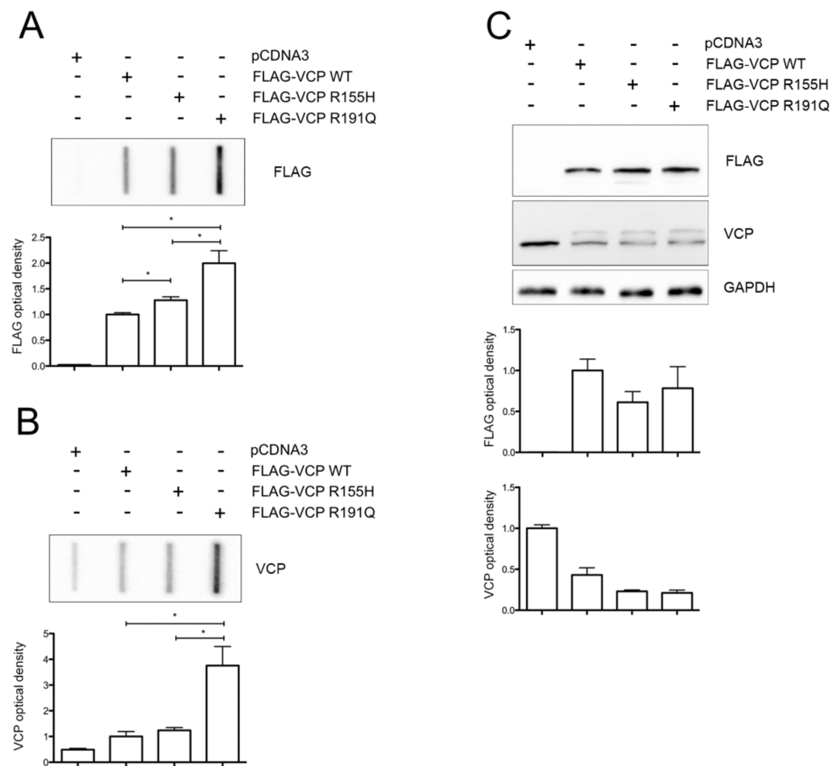


Figure 24 ***VCP-mutants form insoluble species in NSC34.*** *(A,B) Filter Trap assay (FTA) (upper inset) of PBS extracts. Bar graphs represent the FTA mean relative optical density computed over three independent biological samples for each condition (n=3) ± SD *=p<0.05; T-test. (C) Western blot of PBS extracts (upper inset). Optical densitometry quantification of FLAG in WB (middle inset). Optical densitometry quantification of VCP in WB (lower inset).*

VCP-MUTANTS LEAD TO LYSOSOME DAMAGE IN NSC34

The persistence of protein aggregates alters cellular homeostasis. In particular data show sequestration of protein and RNA preventing their functionality and alteration in organelles membranes that leads to loss of ions and lumen content.

In these contexts, I decided to investigate if VCP-mutants aggregation could lead to cell alteration in our model. In particular, to study VCP-mutants impact on organelles, NSC34 cells overexpressing VCP mutants were evaluated using Electron Microscopy analysis (Figure 25 A). Images acquired showed that in presence of VCP-mutants lysosomes lose their physiological structure. In fact, in these conditions, lysosomes were characterized by different dimension, morphology and luminal content. The overexpression of VCP mutants resulted associated with larger and empty lysosomes, that presented a darker membrane indicating a different lipid-composition.

As electron microscopy analysis showed altered lysosomes in presence of VCP-mutants, I performed a Galectin Puncta Assay to evaluate, and eventually quantify, lysosomal damage in presence of VCP-mutants (Figure 23 B). Galectin Puncta Assay is a technique that, using microscopy, quantifies cells that present number of damaged lysosome, relative to total number of transfected cells present in the same area considered. In this technique GAL3 is used as a marker of lysosome damage; indeed, GAL3 in physiological condition can be found diffused in cytoplasm, conversely, when a damage in lysosome membrane occurs, GAL3 binds to damaged lysosomes showing a dotted like cytoplasmatic localization. Galectin Puncta Assay was performed on NSC34 cells transiently overexpressing GFP-GAL3 and pCDNA3, FLAG-VCP WT, FLAG-VCP R155H, FLAG-VCP R191Q. Galectin Puncta Assay showed a significant increase of lysosomal damage in presence of both VCP-mutants compared to VCP WT (**=$p<0.01$ *=$p<0.05$). Moreover, lysosomal damage level in presence of VCP R155H was significantly higher compared to lysosomal damage level in presence of VCP R191Q (*=$p<0.05$).

Finally, fluorescence microscopy analysis (40 magnification) was performed on NSC34 cells transiently overexpressing GFP-GAL3 and pCDNA3, FLAG-VCP WT, FLAG-VCP R155H, FLAG-VCP R191Q. GFP-GAL3 (green), anti-FLAG antibody (red) and nuclei were stained with DAPI (blue). In presence of control or overexpressed VCP WT, GAL3 was homogeneously diffused in cells, showing no signs of lysosomal damage. On the contrary, in presence of VCP-mutants GAL3 showed its classical punctate staining which is associated to the presence of lysosome damage, since GAL3 redistributes into lysosomes in these conditions. In these conditions GAL3 signal is in

not anymore diffused in cells, but it was dotted and this punctated distribution was particularly evident in the case of NSC34 cells overexpressing VCP R155H. Since red immunoreactivity is associated to the FLAG signal and shows overexpressed VCP, the cells considered showed both red-FLAG and green-GFP signals to ensure that variation in GAL3 localization was due to the presence of overexpressed VCP.

Altogether, these data show that the presence of VCP-mutants lead to lysosomal structure alteration and membrane damage.

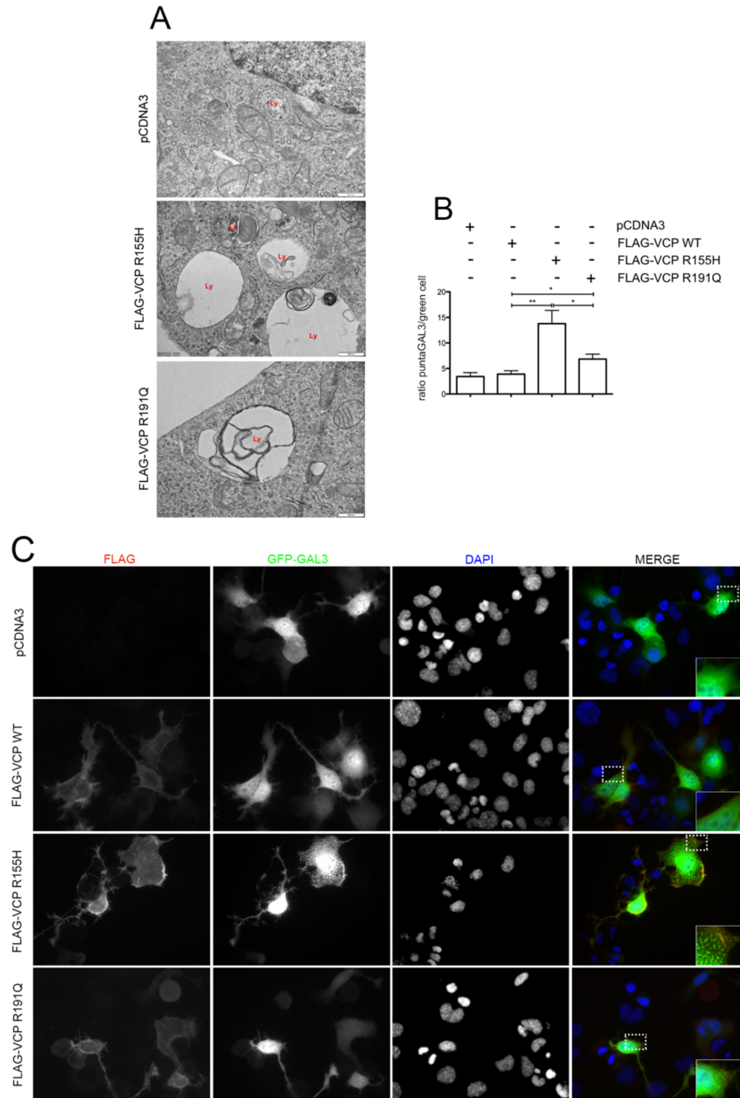


Figure 25 ***VCP-mutants lead to lysosome damage in NSC34.*** *(A) Electron microscopy analysis of NSC34 Ly, lysosome. Scale bar: 500 nm. (B) Quantification of percentage of the ratio of cells with GFP-GAL3 puncta/green cells computed over 5 independent biological samples for each condition (n=5) ± SD **=p<0.01, *=p<0.05. (C) Microscopy analysis (40 magnification). GFP-GAL3 (green), anti-FLAG antibody (red) and nuclei were stained with DAPI (blue). A 2x magnification of selected areas is shown.*

OVEREXPRESSION OF VCP-MUTANTS LEADS TO THE CONVERSION OF LC3-I IN LC3-II AND ACTIVATES AUTOPHAGY

Different studies demonstrated that lysosomal damage activates the autophagic pathway in order to stimulate lysophagy and the clearance of these damaged organelles, restoring the proper cell homeostasis. As the previous data showed that VCP-mutants induced lysosome damage, I decided to study whether the presence of the mutants could modulate the autophagic flux. To study autophagy activation, I analyzed LC3 activation, a marker of autophagy, in presence of VCP-mutants. LC3 activation can be quantified measuring LC3-II/LC3-I ratio. Moreover, to evaluate if the increase of LC3-II/LC3-I ratio was due to activation or inhibition of the autophagic flux, NSC34 cells were treated with NH_4Cl, a late inhibitor of autophagy which prevents the fusion of autophagosomes with lysosomes, leading to an accumulation of the LC3-II which normally is cleared from cells when the flux is properly working. Thus, by inhibiting autophagy in its final steps, LC3 conversion is not prevented, but I should see if the pathways was already blocked in its initial step or not. In fact, if autophagic flux was previously blocked NH_4Cl treatment would not show any further significant increase in LC3-II/LC3-I ratio. Conversely, if autophagic pathway was activated, NH_4Cl treatment would prevent LC3-II degradation further increasing LC3-II/LC3-I ratio.

To analyze LC3-II/LC3-I ratio, WB analysis was performed on NSC34 cells transiently expressing pCDNA3, FLAG-VCP WT, FLAG-VCP R155H or FLAG-VCP R191Q. Cells were treated with NH_4Cl 4μM for 2hrs. In WB analysis LC3 antibody detects LC3-I, higher signal, and converted LC3-II, the lower signal. WB blot showed an increase in LC3-II signal in samples treated with NH_4Cl. In particular, LC3-II was increased in presence of VCP R155H. In Figure 26 B, C, D, E and F I also performed the quantification of WB analysis of 5 independent biological samples for each condition (n=5) ± SD analyzed with student t-test.

In figure 26 B I quantified the mean of LC3-II/LC3-I ratio of each condition. In untreated samples, VCP R155H mutant led to a significant increase of LC3-II/LC3-I ratio compared to the control (**<0.01). NH_4Cl treatment led to an increase of LC3-II/LC3-I ratio in presence of VCP-mutants respect to LC3-II/LC3-I ratio in presence of VCP-mutants without treatment (*=p<0.05). Moreover, VCP-mutants led to an increase of LC3-II/LC3-I ratio compared to treated control (**=p<0.01). These data indicate that the activation of the autophagic flux is enhanced by VCP-mutants overexpression. Figure 26 C shows LC3-I mean level for each condition. T-test analysis did not reveal any relevant variation between each condition. Conversely, Figure 26 D indicates

an increase of LC3-II levels in presence of VCP-mutants, in particular VCP R155H mutant led to a significant increase of activated LC3 (*=$p<0.05$). LC3 total level, quantified in figure 26 E, showed an increased trend in samples treated with NH_4Cl in particular in presence of VCP-mutants. Finally, p62, another marker of the autophagic flux, is quantified in Figure 26 F. P62 quantification showed an increased trend in presence of VCP-mutants treated with NH_4Cl. P62 is also a marker of the autophagic flux as it is implicated and cleared by autophagy. When the autophagic flux is inhibited in its last steps, an accumulation of p62 indicates the presence of an increased number of active autophagosomes, sign of activated autophagic flux. Thus, an increased trend in p62 levels in presence of VCP-mutants and NH_4Cl treatment, is in line with the increased LC3-II/LC3-I ratio in these conditions, confirming that VCP-mutants presence is associated to an activation of autophagic flux.

Finally, immunofluorescence analysis (63 magnification) was performed on NSC34 cells transiently overexpressing pCDNA3, FLAG-VCP WT, FLAG-VCP R155H and FLAG-VCP R191Q and treated with NH_4Cl (Figure 26 G). LC3 localization and distribution analyzed in presence of VCP-mutants and autophagy inhibition. As Figure 26 G shows, VCP-mutants in presence of late autophagy inhibition induced a robust increase in LC3 puncta indicating an enhance in the formation of autophagic vesicles. These data confirmed LC3 activation that was quantified in WB in the same condition. In fact, VCP mutant presence when autophagic pathway was inhibited in its last steps showed, by WB analysis, a significant increase of LC3-II/LC3-I ratio, that is in line with the increase of LC3 puncta showed by microscope analysis.

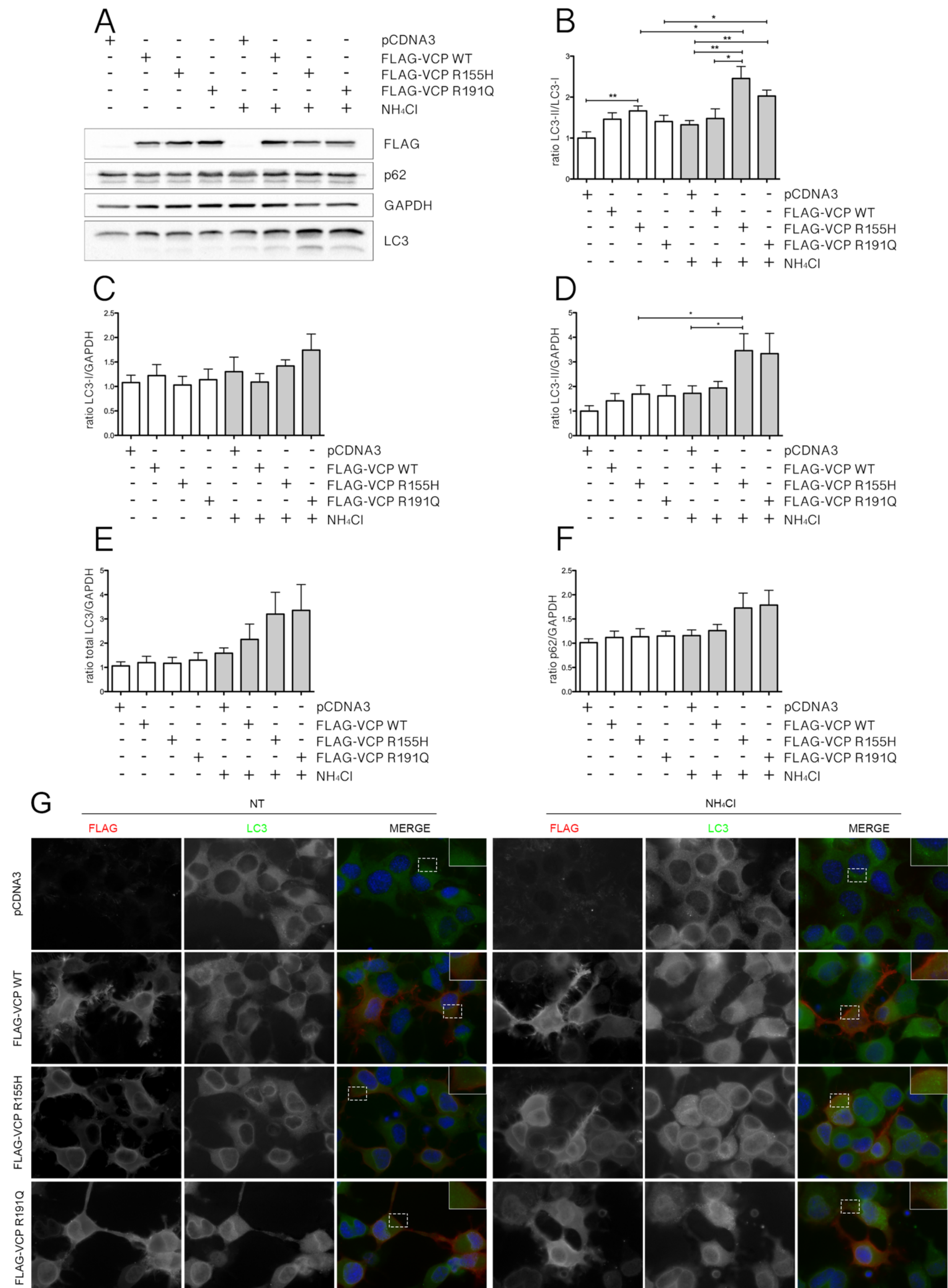


Figure 26 ***Overexpression of VCP-mutants leads to the conversion of LC3 I in LC3 II activating autophagy.*** *(A) WB analysis of PBS extracts. Cells were treated with NH4Cl 4μM for 2hrs. (B) Bar graph represents mean ±SD for n=5 independent samples LC3-II/LC3-I ratio (**<0.01, *p<0.05 T-test) (C)The bar graph represents mean ±SD for n=5 independent samples LC3-I/GAPDH ratio. (D) The bar graph represents mean ±SD for n=5 independent samples LC3-II/GAPDH (ratio *p<0.05 T-test). (E) The bar graph represents mean ±SD for n=5 independent samples totalLC3/GAPDH ratio. (F) The bar graph represents mean ±SD for n=5 independent samples p62/GAPDH ratio. (G) Microscopy analysis (63 magnification). Anti-LC3 antibody (green), anti-FLAG antibody (red) and nuclei were stained with DAPI (blue). A 2x magnification of selected areas is shown.*

OVEREXPRESSION OF VCP-MUTANTS SPECIFICALLY ACTIVATES TFE3 NUCLEAR TRANSLOCATION

Once defined that VCP-mutants overexpression enhanced the activation of the autophagic pathway, I analysed the mechanisms through which VCP-mutants activated autophagy. Lysosomal damage, induced by VCP-mutants, could activate autophagy in different ways. In particular I focused on the activation of transcription factors involved in lysosome and/or autophagy regulation. Firstly, I focused on TFEB, a key regulator of autophagic gene and secondly I extended my analysis to TFE3, another transcription factor that is involved in activation of autophagy and lysosomes biogenesis. To define VCP-mutants pathway in activating autophagy pCDNA3, FLAG-VCP WT, FLAG-VCP R155H, FLAG-VCP R191Q were overexpressed in NSC34 cell line. Trehalose treatment for 24hrs was used as control of TFEB activation. Literature data showed that trehalose treatment induces lysosomal damage that leads to TFEB activation and its nuclear translocation (Rusmini *et al.*, 2019). Trehalose-induced lysosomal damage is visible after 6hrs of treatment; whereas TFEB nuclear levels significantly increase after 18hrs of trehalose treatment. WB analysis was carried out on cytoplasmic (C) and nuclear extracts (N) (Figure 27 A). α-Tubulin (TUBA) was used as loading control of the cytoplasmic fractions, while Histone 3 (H3) was used as loading control of nuclear fractions. WB analysis showed TFEB translocation from cytoplasmic to nuclear fraction in presence of trehalose treatment, as it is shown in literature. However, TFEB nuclear levels did not increase neither in presence of overexpressed VCP WT, as expected, nor in presence of VCP-mutants. Conversely, WB analysis showed TFE3 translocation in different conditions. First, WB showed that trehalose treatment induced TFE3 nuclear translocation. Moreover, I could also observe that the presence of both VCP-mutants led to an increase in the nuclear TFE3 fraction if compared to mock transfected cells. Figure 27 B, C, D, E and F are the quantification of WB analysis of the mean of 5 independent biological samples for each condition (n=5) ± SD analyzed with student t-test. TFEB fractions analysis are shown in figure B and C. Cytoplasmic fraction did not have any relevant changes between each condition. Whereas TFEB nuclear fraction significantly increased only when cells were treated with trehalose (*=$p<0.05$). In the presence of VCP-mutants or VCP WT overexpressed no significant increase in TFEB nuclear levels were detectable. In Figure 27 D it was quantified the cytoplasmatic fraction of TFE3, by normalizing TFE3 with TUBA. It showed no significant changes of TFE3 levels in any condition compared to the control. While, in Figure 27 E it was evaluated TFE3 nuclear fraction, normalizing nuclear TFE3 with H3. Figure showed an increased trend of nuclear TFE3 in

presence of trehalose (*=p<0.05), which therefore, has a similar effect towards both transcription factors analyzed. Moreover, Figure 27 E showed that nuclear TFE3 levels increased in presence of both VCP-mutants, in particular it resulted significant in presence of VCP R191Q compared to the control (*=p<0.05). Finally, in Figure 27 F it was quantified nuclear/cytoplasmic TFE3 ratio that indicates the TFE3 translocation from cytoplasmatic to nuclear compartment. The graphic showed an increased trend in nuclear/cytoplasmic TFE3 ratio in presence of both VCP mutants and trehalose treatment, whereas the overexpressed VCP WT nuclear/cytoplasmic TFE3 levels are comparable to the mock transfected cells.

To confirm WB analysis on nuclear/cytoplasmic extracts I did a microscopy analysis (63 magnification) of NSC34 cells transiently overexpressing GFP-TFE3 and pCDNA3, FLAG-VCP WT, FLAG-VCP R155H, FLAG-VCP R191Q. GFP-TFE3 (green) and nuclei were stained with DAPI (blue) (Figure 27 G). Microscopy analysis permitted to evaluate TFE3 localization and distribution in presence of VCP-mutants. Paralleling the data of WB analysis, IF analysis showed that trehalose treatment, used as control of TFE3 activation, resulted as expected in TFE3 translocation. In the control and in presence of overexpressed VCP WT, TFE3 is found diffused in cytoplasm. Whereas when VCP mutants were overexpressed TFE3 partially translocated and localized in the nuclei and partially was found retained in cytoplasmic compartment. TFE3 activation and translocation enhanced by VCP-mutants presence showed by microscope analysis, is in line to TFE3 increased nuclear level in presence of VCP-mutants showed by WB analysis and quantification.

Overall these data show that autophagy flux activated by VCP-mutants presence is specifically mediated by transcription factor TFE3.

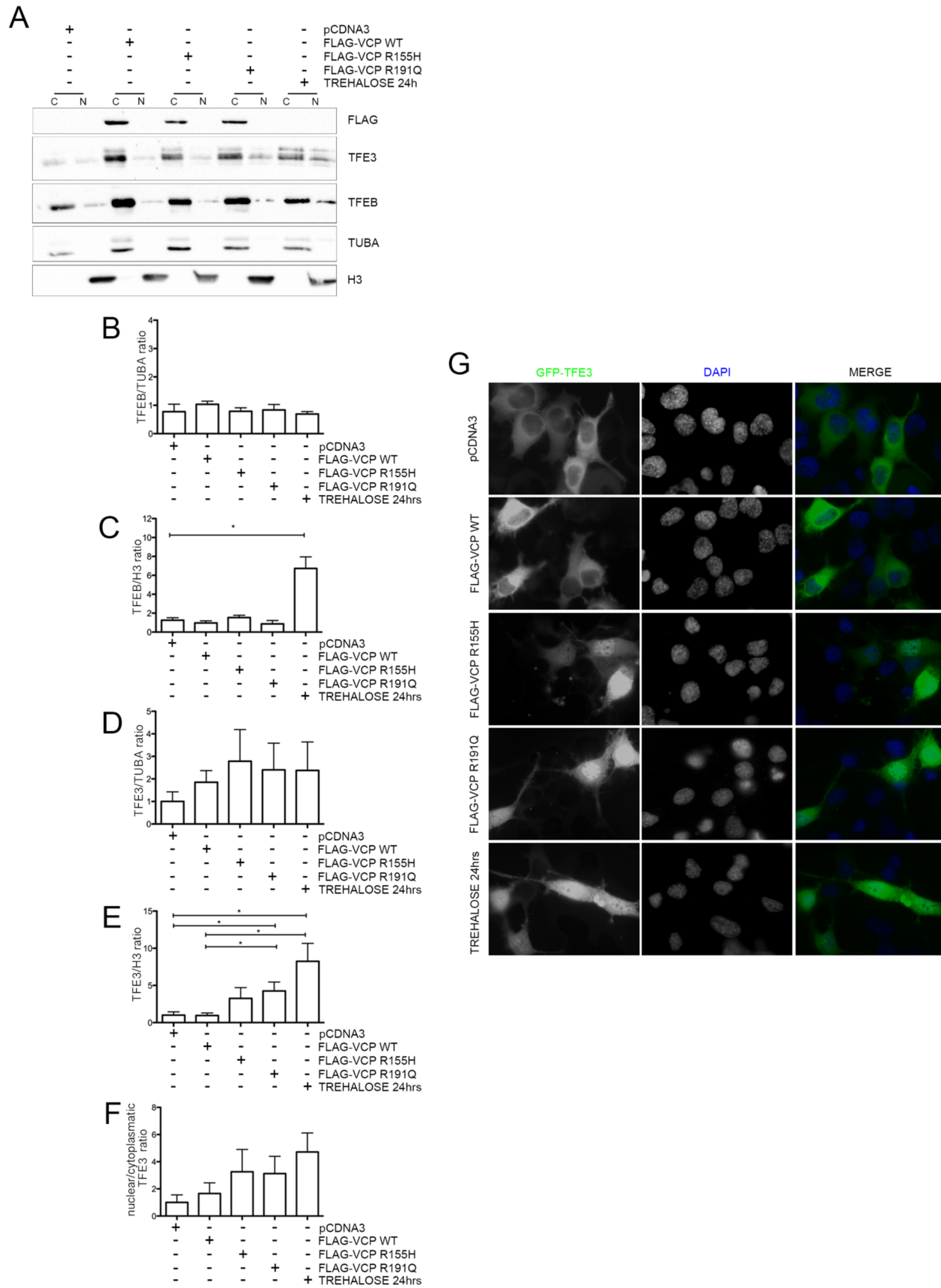


Figure 27 ***Overexpression of VCP-mutants specifically activates TFE3 nuclear translocation.*** *(A) WB analysis of cytoplasmic (C) and nuclear extracts (N). (B) The bar graph represents mean ± SD for n=5 independent samples of cytoplasmic TFEB/TUBA ratio. (C) The bar graph represents mean ± SD for n = 5 independent samples of nuclear TFEB/H3 ratio (*p<0.05 T-test). (D) The bar graph represents mean ± SD for n=5 independent samples of cytoplasmic TFE3/TUBA ratio (*p<0.05 T-test). (E) The bar graph represents mean ± SD for n=5 independent samples of nuclear TFE3/H3 ratio (*p<0.05 T-test). (F) The bar graph represents mean ± SD for n=5 independent samples of nuclearTFE3/cytoplasmaticTFE3 ratio. (G) Microscopy analysis (63 magnification). GFP-TFE3 (green) and nuclei were stained with DAPI (blue).*

VCP-MUTANT-MEDIATED TFE3 ACTIVATION IS CALCINEURIN DEPENDENT

Once it was determined that TFE3 nuclear translocation mediates VCP-mutants autophagy activation, I analyzed TFE3 activation pathway. Thus, to better define VCP pathway I analyzed if TFE3 activation was regulated by calcineurin (PPP3). PPP3 is calcium and calmodulin dependent serine/threonine protein phosphatase that is involved in activation of different transcription factors as TFEB and TFE3. Some studies demonstrated that PPP3 is activated by lysosomal Ca^{2+} release. Thus, in condition of lysosomal membrane rupture there is a Ca^{2+} release, which can activate PPP3 and promotes autophagic activity. To analyze if PPP3 mediates TFE3 activation in presence of VCP mutants, I silenced PPP3 in NSC34 cells overexpressing GFP-TFE3 and pCDNA3, FLAG-VCP WT, FLAG-VCP R155H or FLAG-VCP R191Q. In these conditions I carried out a microscopy analysis (63 magnification) (Figure 28). GFP-TFE3 (green) and nuclei were stained with DAPI (blue). NSC34 cells transfected with non-targeting siRNA were comparable with microscope analysis shown in Figure 26 G: in NSC34 cells analyzed in control condition or in presence of overexpressed VCP WT, GFP-TFE3 is retained in cytoplasmic compartment; in contrast, in presence of VCP-mutants, GFP-TFE3 is mostly found in the nucleus and only in a smaller fraction it is retained in the cytoplasm. On the other hand, NSC34 cells transfected with siRNA PPP3CB presented TFE3 localized exclusively in cytoplasmic compartment.

These data showed that TFE3 activation induced by VCP-mutants presence is mediated by calcineurin.

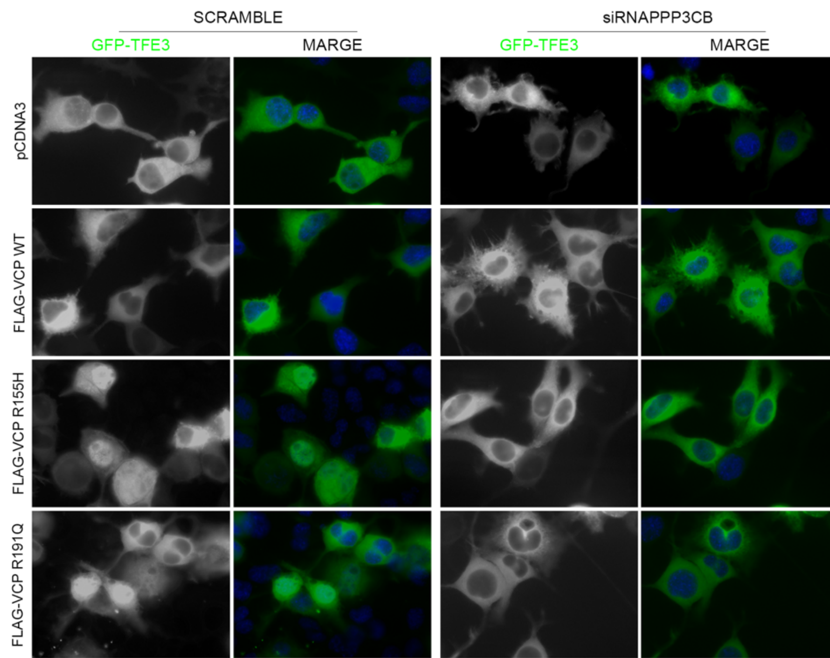


Figure 28 ***VCP-mutants TFE3 activation is calcineurin dependent.*** *Microscopy analysis (63 magnification). GFP-TFE3 (green) and nuclei were stained with DAPI (blue).*

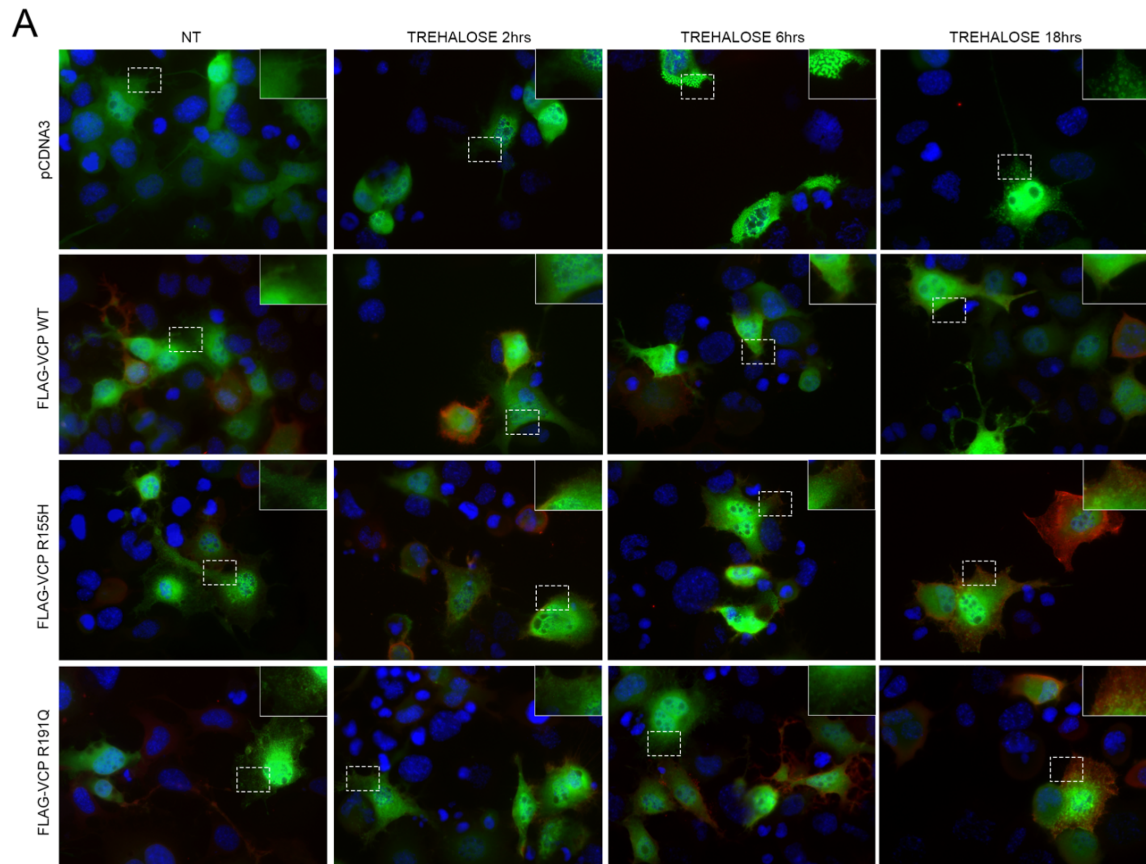
VCP WT OVEREXPRESSION IN NSC34 CELLS DECREASES LYSOSOME DAMAGE INDUCED BY TREHALOSE

In parallel to the study of VCP-mutants induced lysosomal damage, I analyzed VCP WT and VCP-mutants contribute in the clearance of damaged lysosomal. Lysosomal damage was chemically induced using trehalose treatment at different times (figure 29 A and B).

To study VCP WT and VCP-mutants role in chemical induced lysosomal damage I used microscopy analysis (40 magnification) of NSC34 cells transiently overexpressing GFP-GAL3 and pCDNA3, FLAG-VCP WT, FLAG-VCP R155H, FLAG-VCP R191Q and treated with trehalose at 2hrs, 6hrs and 18hrs. GFP-GAL3 (green), anti-FLAG antibody (red) and nuclei were stained with DAPI (blue). In the untreated condition, IF showed GFP-GAL3 localization similar to what it was shown in Figure 23 F: GFP-GAL3 is diffused, showing no lysosomal damage with control and VCP WT overexpression; VCP-mutants overexpression showed lysosomal damage with a GFP-GAL3 punctate distribution. Moreover, IF showed that trehalose treatment induced lysosomal damage in presence of VCP mutants and control. Whereas VCP WT overexpression prevented lysosomal damage induced by trehalose treatment. In fact, GFP-GAL3 can be observed in dotted-like distribution in all condition treated with trehalose excepts when VCP WT is overexpressed.

In Figure 26 B, Galectin Puncta Assay was performed on the same condition described in Figure 26 A, to quantify the effect of VCP WT overexpression on lysosomal damage induced by chemical treatment. The bar graph shows the mean of the quantification of percentage of cells with GFP-GAL3 puncta (n=9). In the case of the untreated condition I found that this is comparable to what was shown in Figure 25 E. Conversely, trehalose treatment for 2 hrs led to a significant increase of lysosomal damage in all conditions compared to the untreated except from VCP R155H overexpression condition ($^{++}p<0.01$, $^{+++}$ $p<0.001$). In fact, VCP R155H induced lysosomal damage levels present in condition of not treated with trehalose were comparable to lysosomal damage levels after 2hrs of treatment. The overexpression of VCP WT significantly decreased lysosomal damage compared to control ($^{*}p<0.05$). Trehalose treatment for 6hrs resulted in a great increase of lysosomal damage in the control and in presence of VCP R155H mutant compared to the untreated samples and to the trehalose treatment for 2hrs ($^{+++}$ $p<0.001$, °° $p<0.01$, °°° $p<0.001$). Lysosomal damage present in control condition was prevented with VCP WT overexpression (*** $p < 0.001$) and partially prevented with VCP R191Q mutant expression ($^{*}p<0.05$). Trehalose treatment for 18hrs resulted in a significant decrease of lysosomal damage levels in control condition compared to trehalose treatment for 6hrs, in line with our previously reported data

(Rusmini *et al.*, 2019) (^ $p<0.05$), but lysosomal damage levels were still significantly higher than the ones in untreated condition or after 2hrs of treatment ($^{+++}$ $p<0.001$, °° $p<0.01$). As I have seen for data obtained with trehalose treatment for 6hrs, VCP WT overexpression significantly prevented lysosomal damage compared to the to mock transfected cell (control condition) (*** $p < 0.001$) while VCP R191Q mutant overexpression partially decreased lysosomal damage compared to control condition (**$p<0.01$). With all different treatments tested, VCP R155H mutant overexpression led to lysosomal damage levels comparable to mock transfected cells. While VCP R191Q mutant overexpression in parallel with trehalose treatment for 6 and 18 hrs led to lysosomal damage levels significantly higher compared to levels present in presence of VCP WT overexpression (*$p<0.05$, **$p<0.01$). Summarizing, the data shown in Figure 29 B demonstrate that VCP WT overexpression prevented chemically-induced lysosomal damage. Moreover, VCP R155H mutant completely lost VCP functioning in this context: in fact, lysosomal damage levels in presence of overexpressed VCP R155H mutant, when lysosomal damage has been chemically-induced with trehalose, was always comparable to the control condition. Finally, VCP R191Q mutant partially reverted lysosomal damage chemically induced with trehalose, but it partially lost VCP functionality if lysosomal damage levels were compared to the levels in presence of overexpressed VCP WT used as control.



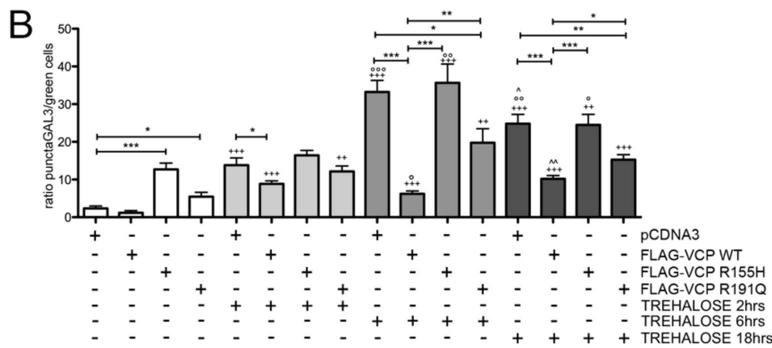


Figure 29 ***VCP WT overexpression in NSC34 decreases lysosome damage induced by trehalose.*** *(A) Microscopy analysis (40 magnification) GFP-GAL3 (green), anti-FLAG antibody (red) and nuclei were stained with DAPI (blue). (B) Quantification of percentage of the ratio of cells with GFP-GAL3 puncta/green cells computed over 9 independent biological samples for each condition (n=9) ± SD (*p<0.05, **p<0.01, *** p < 0.001; ++p<0.01, +++ p<0.001, against NT; ° p<0.05, °° p<0.01, °°° p<0.001,against 2hrs trehalose treatment; ^ p<0.05, ^^ p<0.01 against 6hrs trehalose treatment; T-test).*

VCP WT OVEREXPRESSION IN NSC34 DECREASES LYSOSOME DAMAGE INDUCED BY MISFOLDED PROTEINS

After studying VCP WT overexpression contribute in presence of lysosomal damage chemically induced with trehalose, I decided to evaluate also VCP WT overexpression contribute in presence of lysosomal damage biologically induced with misfolded proteins. Recent data showed that the presence of protein aggregates led to lysosome membrane breakage. Thus, I decided to evaluate if SOD1 G93A overexpression enhanced lysosomal damage and if VCP WT overexpression could influence damage levels (Figure 30).

To study these conditions, I performed a Galectin Puncta Assay and I quantified VCP contribute in lysosomal damage biologically induced with misfolded proteins. Galectin Puncta Assay was performed on NSC34 cell line transfected with pCDNA3, FLAG-VCP WT, FLAG-VCP R155H, FLAG-VCP R191Q and SOD1 WT or SOD1 G93A. The bar graph shows the mean of the quantification of percentage of cells with GFP-GAL3 puncta (n =7).

Firstly, I demonstrated that SOD1 G93A induced lysosomal damage. In fact, the figure shows a significant increase in lysosomal damage in presence of SOD1 G93A compared to the overexpressed SOD1 WT (**$p<0.01$).

Moreover, lysosomal damage induced by SOD1 G93A was reverted only by the overexpression of VCP WT. In fact, lysosomal damage levels in presence of VCP WT overexpressed were significantly lower respect to lysosomal damage levels in presence of mock transfection (*$p<0.05$). Conversely, the presence of VCP mutants did not decrease lysosomal damage level induced by SOD1 G93A presence. Indeed, lysosomal damage levels in presence of VCP-mutants were comparable to the levels in presence of SOD1 G93A.

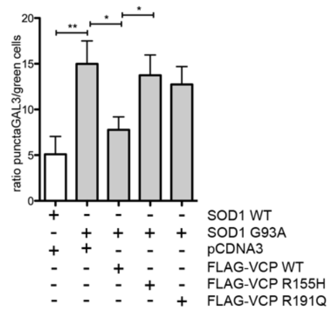


Figure 30 ***VCP WT overexpression in NSC34 decreases lysosome damage induced by SOD1 G93A overexpression.*** *Quantification of percentage of the ratio of cells with GFP-GAL3 puncta/green cells computed over 7 independent biological samples for each condition (n=7) ± SD (*p<0.05, **p<0.01 T-test).*

DISCUSSION

All ALS forms are characterized by the presence of insoluble protein aggregates present in the brain tissue of affected patients. In fact, various mutations in genes lead to the expression of proteins that misfold and aggregate. Moreover, there are many genes involved in protein regulation that are found mutated. One of these genes associated to ALS encodes for VCP. VCP ALS-mutants are correlated with altered proteostasis. In fact, VCP-mutants are associated to ubiquitin positive inclusions, TDP-43 positive inclusions and accumulation of vacuoles-like structure, a sign of altered degradation pathways (Johnson *et al.*, 2010).

VCP has many roles in the regulation of proteostasis. Recent studies demonstrate that VCP is also involved in the removal of altered organelles like lysosomes (Papadopoulos *et al.*, 2017). The alteration of lysosomes is deleterious for cell; firstly, for its key role in proteostasis and secondly lysosome-damage leads to massive lysosomal leakage that causes cell toxicity and death (Aits & Jäättelä, 2013; Wang *et al.*, 2018). There are different mechanisms that can be activated to maintain the lysosomal activity. The most studied is lysophagy where VCP has been found involved.

In the second part of the work VCP contribute to lysosomal damage was analysed by overexpressing VCP WT and VCP ALS-associated mutants (VCP R191H and VCP R191Q) in NSC34. Firstly, it was determined by FTA that VCP-mutants significantly aggregate, in particular VCP R191Q mutant. FTA analysis showed an increase in the insoluble fraction of the overexpressed VCP mutants compared to the wild-type. WB analysis showed that the increased soluble fraction was not correlated and explained with an increase of the total protein expression, therefore it can only be explained with an increased aggregating capacity. These data confirms to what is observed in VCP-mutants associated patient tissue which presents VCP-positive inclusions (Hübbers *et al.*, 2007).

Aggregates if not removed lead to cell toxicity, thus I decided to verify if VCP-mutants aggregation could alter cellular organelles by analysing NSC34 cells organelles morphology in presence of VCP-mutants. Electron-microscopy analysis showed that VCP-mutants presence altered lysosomal morphology, which presented increased size and empty lumen. To prove that VCP-mutants associated to ALS altered lysosomes and also caused membrane rupture and leakage I performed a Galectin Puncta Assay. As expected lysosomal damage quantification showed increased levels in presence of VCP-mutants compared to the levels in presence of the overexpressed VCP WT. In particular, VCP R155H mutant expression showed a sensible increase

in lysosomal damage levels compared to the overexpressed VCP WT but also to VCP R191Q expression. These data were then confirmed by immunofluorescence analysis. In fact, it was observed that in control and overexpressed VCP WT condition Galectin 3 signal was diffused, whereas in presence of VCP-mutants Galectin 3 signal had a dotted-like localization that resembles Galectin 3 recruitment to damaged lysosomes. The alteration of lysosomal morphology, size and membrane confers a new insight to VCP-mutants pathological mechanisms.

Different studies demonstrate that lysosomal damage activates the autophagic pathway to enhance damaged lysosomes clearance (Aits *et al.*, 2015; Rusmini *et al.*, 2019). VCP WT and mutants contribute in regulating the autophagic flux is very debated in literature, so I decided to determine if lysosomal damage induced by VCP-mutants influenced the autophagic pathway activation in the condition that I was studying. To study this, LC3 conversion was analysed. Firstly, it was observed an increase in LC3 conversion in presence of VCP R155H mutant that further increased when the autophagic flux was blocked in its end stage (fusion of autophagosomes to lysosomes), demonstrating an activation of the autophagic flux. In fact, an inhibition of autophagy in its late steps permits to discriminate if the increase of LC3 conversion is due to autophagic inhibition or to an increase of autophagic flux. By blocking the autophagic flux in its last steps, I could also define that VCP R191Q mutant determined an increase of the autophagic flux. The activation of the autophagic flux was also confirmed by IF analysis where the presence of VCP-mutants and autophagic late inhibition led to puncta like localization of LC3 indicating autophagic vesicles formation.

Once it was defined that VCP-mutants positively regulated the autophagic flux, I analysed which pathway could have been implicated and activated by VCP-mutants. Lysosomal damage activates autophagy flux through different pathways. In particular, it enhances the activation and translocation of different transcription factors that regulate expression of autophagic genes and lysosomal related genes. I started analysing TFEB activation as it is the main transcription factor generally involved. By analysing cytoplasmic and nuclear TFEB fraction in presence of VCP WT and mutants overexpressed, I could determine that TFEB was not involved in the pathway. Thus, I extended my research to TFE3 an analogous of TFEB. By IF and nuclear-cytoplasm extraction analysis I determined that VCP mutants, in particular VCP R191Q mutant, increased TFE3 nuclear level and subsequent gene regulation. Moreover, TFE3 translocation in presence of VCP mutants was prevented by PPP3 silencing. PPP3 is a calcium-dependent phosphatase that activates

various transcription factors among which TFEB and TFE3. Thus VCP-mutants, in particular VCP R191Q mutant, specifically increased nuclear levels of TFE3 and not TFEB. More studies are needed to determine the reason of this specific modulation.

In the last part of my work, I analysed if VCP WT overexpression could positively regulate lysosomal damage degradation. In fact, it was recently demonstrated that VCP has a key role in damaged lysosome degradation and that VCP inhibition or VCP mutants are correlated to the persistence of damaged lysosome impeding their clearance (Papadopoulos *et al.*, 2017).

To study VCP WT overexpression contribute in this pathway I chemically and biologically induced lysosome damage in NSC34 cells overexpressing VCP WT and mutants. I chemically induced lysosome damage treating cells with trehalose. Using Galectin Puncta Assay and IF I demonstrated that VCP WT overexpression prevented chemical lysosomal damage in all different time treatment. Also, VCP R191Q mutant overexpression moderately prevented lysosomal damage chemically induced with trehalose, but it partially lost VCP functionality. In fact, chemical induced lysosomal damage level, in presence of VCP R191Q mutant is significantly higher compared to chemical induced lysosomal damage level in presence of overexpressed VCP WT. Finally, I could determine that VCP R155H mutant completely lost VCP WT functioning; in fact, both IF and Galectin Puncta Assay showed that chemically induced lysosomal damage is not influenced by overexpressed VCP R155H mutant. In this condition, lysosomal damage levels were always comparable to lysosomal damage levels in mock transfection condition.

To biologically induce lysosomal damage, I determined that overexpression of SOD1-mutant could lead to lysosome damage. Thus, inducing lysosomal damage overexpressing SOD1-mutant I could confirm VCP WT overexpression role in presence of induced lysosomal damage. Indeed, data show that the overexpression of VCP WT prevented lysosomal damage induced by SOD1-mutant presence. Conversely VCP-mutants lost VCP functionality. In fact, VCP-mutants presence could not revert lysosomal damage biologically induced by SOD1-mutants. These findings open VCP as a target to positive regulate the clearance on damaged lysosome restoring cellular homeostasis.

CONCLUSIONS

During my PhD period, my worked focused on the role of Valosin Containing Protein (VCP) in different pathways of the protein quality control (PQC) system. I analysed VCP wild type overexpression to study if enhancing its activity could ameliorate pathological conditions correlated to ALS. Moreover, I focused my attention on two VCP ALS-associated mutants to determine and define their pathological contribute on PQC system. My work can be divided in two parts.

In the first part, I studied VCP contribute in the disassemble and clearance of misfolded protein aggregates associated to ALS, in particular focusing to SOD1-mutants aggregates. I could determine that VCP WT overexpression led to SOD1-mutant clearance through UPS. Moreover, I determined that VCP WT contribute in SOD1-mutant clearance was not essential, but there are other pathways that are involved. These data show that promoting VCP activity could be a target for the clearance of ALS-associated inclusions. It would be interesting to determine if VCP modulation could trigger the clearance of other type of ALS-associated inclusion.

In this part, I could also demonstrate that VCP-mutants did not lose functionality in SOD1-mutants clearance in basal conditions, but they partially lost their functionality when autophagy was inhibited, showing a dependency to this degradation pathway. Finally, as SOD1-mutants aggregates were very instable and there were many conditions that could alter their aggregation levels, data where difficult to obtain. Thus, starting from a protocol used by Whiten and colleagues, I developed a more reliable and reproducible technique that better defined and quantified SOD1 mutant inclusion levels.

In the second part of my work, I focused on VCP WT and mutant role in lysosomal damage. Firstly, I found that VCP-mutants aggregated and that their expression led to lysosome morphology alteration and damage, giving a new insight on VCP-mutants pathological mechanisms. The modulation of autophagy in presence of VCP-mutants is very debated. Here I demonstrate that VCP-mutants induced lysosome damage led to specifically increase of TFE3 nuclear levels, resulting in activation of the autophagic flux. In fact, in presence of VCP-mutants, LC3-II/LC3-I ratio significantly increased. Moreover, I found that TFE3 activation induced by VCP-mutants presence was mediated by PPP3.

In parallel, I studied if overexpressing VCP WT could promote damaged lysosome clearance via lysophagy, when the damage was triggered chemically or biologically with trehalose or misfolded

protein, respectively. To biologically induce lysosome damage, I firstly demonstrated that overexpress SOD1 G93A led to lysosome membrane breakage. It resulted that VCP WT overexpression prevented lysosome damage when it was both chemically and biologically induced. This data show another possible pathway where VCP is found implicated, that can be modulated and could positive ameliorate cellular pathological condition. Moreover, studying lysosome induced damage in presence of VCP-mutants I found that: VCP R155H mutant completely lost VCP activity in preventing lysosome damage; while VCP R191Q mutant only partially lost VCP functionality. In fact, VCP R191Q mutant reduced in part lysosomal damage levels chemically induced with trehalose.

Connecting data of part-I and part-II on VCP-mutants, I could speculate an explanation for VCP-mutants capacity in removing SOD1-mutants aggregates. In fact, data from part-I showed that VCP-mutants could decrease insoluble SOD1-mutants fraction, but that by inhibiting either the UPS or autophagy they lost their functionality. Interestingly, VCP-mutants had a different behaviour from VCP WT with autophagy inhibition: VCP WT activity was not influenced by autophagy inhibition; VCP-mutants activity was partially lost with autophagy inhibition, showing a correlation with this pathway. In the second part it was shown that VCP-mutants aggregation and induced lysosomal damage led to autophagy activation. This could explain VCP-mutants unexpected functionality in removing SOD1-mutants aggregates (Figure 31).

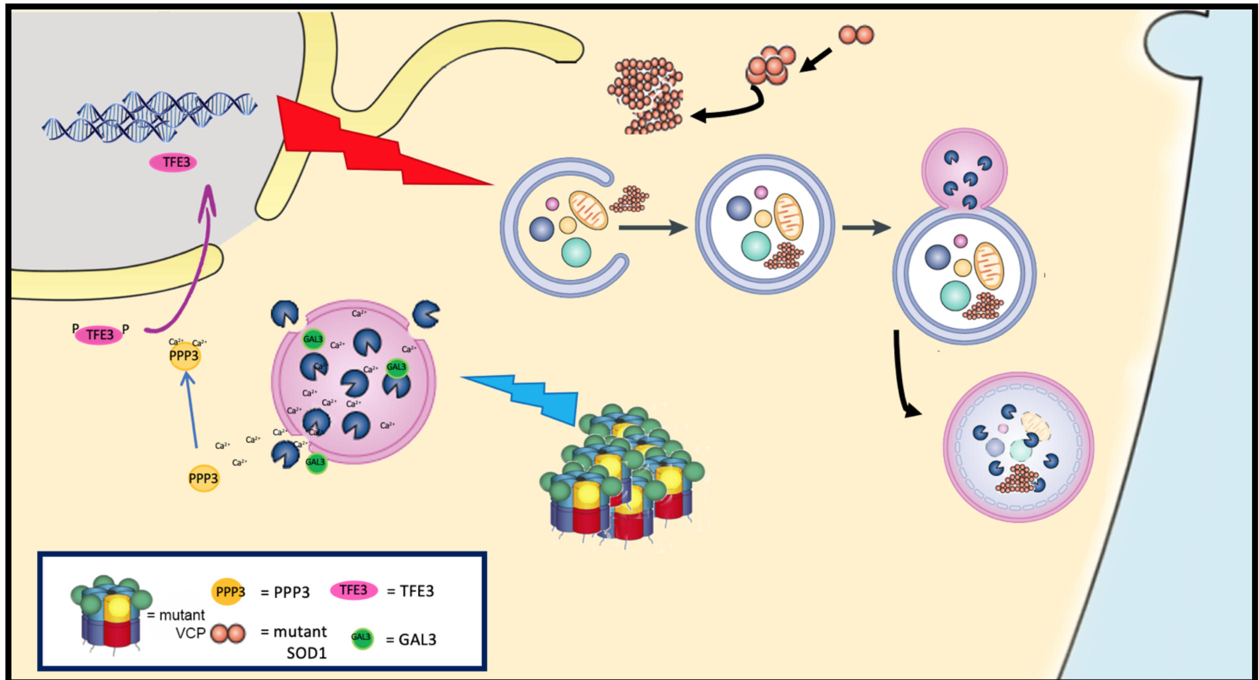


Figure 31 VCP mutants aggregate inducing lysosomal damage and autophagy activation. Autophagy activation resulted in SOD1 G93A enhanced clearance.

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