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Epigenetics and neurodegeneration: physiological relevance of TDP-43/HDAC1 interaction

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ABSTRACT

TDP-43 pathology is a disease hallmark that characterizes both sporadic and familial amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD-TDP). TDP-43 has been implicated in transcription, RNA metabolism and transport, and different TDP-43 post-translational modifications, spanning from phosphorylation to acetylation, can regulate its activity. In the present PhD thesis we provide evidences that TDP-43 interacts with histone deacetylase 1 (HDAC1), both in vivo and in vitro. By biochemical assays, performed in SH-SY5Y cells, we demonstrated that HDAC1, as well as HDAC6, can modify TDP-43 acetylation, that occurs mainly on amino acid residues K142 and K192, located in the RRM1 and RRM2 domains, necessary for the interaction . Interestingly, HDAC1 overexpression modulates TDP-43 transcriptional activity on CHOP promoter, but not TDP43 splicing activity on polymerase delta interacting protein 3 [POLDIP3] gene. Finally, both in cell culture and in Drosophila, HDCA1 reduced level (genomic inactivation or siRNA) or treatment with pan-HDAC inhibitors, reduce WT or pathological mutant TDP-43 toxicity, suggesting TDP-43 acetylation as a new potential therapeutic target.

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INTRODUCTION

1. Amyotrophic Lateral Sclerosis (ALS)

1.1 Epidemiology

Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disorder characterised by progressive muscular paralysis reflecting degeneration of motor neurones in the primary motor cortex, brainstem and spinal cord.

The word "Amyotrophy" refers to the atrophy of muscle fibres, which are denervated as their corresponding anterior horn cells degenerate, leading to weakness of affected muscles and visible fasciculations; on the other hand "Lateral sclerosis" refers to hardening of the anterior and lateral corticospinal tracts, as motor neurons in these areas degenerate and are replaced by gliosis[1]. The death of motor neurons is associated with the activation of astrocytes, microglia and intracellular accumulation of ubiquitinated skein-like inclusions in the axons and cell bodies of the remaining atrophic motor neurons[2]. This leads to generalised fasciculations, muscle weakness, speech and swallowing disabilities, muscle atrophy, progressive paralysis and, ultimately, death caused by respiratory failure.

The crude prevalence of ALS is estimated at 4–6/100,000 population. The prevalence of ALS increases with age, reaching a peak in the 60–75 years old age-group at 33/100,000 for men and 14/100,000 for women[3,4]. The incidence rate of ALS is 1–3/100,000 person year and increases with age. A peak incidence rate of 10.5 and 7.4/100,000, in males and females, respectively, is observed in the 55–75 years old age-group; these values are three times higher in Sardinian population[5]. In fact Sardinia, the second largest Mediterranean island, represents a genetic isolation characterized by a higher frequency of ALS cases, in particular associated with TARDBP A382T mutation[6,7]

ALS is a multifactorial pathology caused by both genetic and environmental factors. Degeneration of motor neurons is driven by the alteration of molecular processes responsible for the maturation and transport of RNA and proteins, mitochondrial and

axonal dysfunction, lack of fundamental growth factors underlying normal neuronal trophic development and alteration of calcium and glutamate loading processes.

1.2.Genetic factors

The idea of the involvement of genetic factors in the development of the disease has ancient origins, in fact pathological cases with family history and inheritance have been observed as early as 1850[8]. Although most cases are classified as sporadic ALS (sALS), about 10% some cases of ALS are inherited, and therefore classified as familial cases (fALS), with multiple autosomal dominant and recessive forms. The sporadic and familial ALS shares clinical and neuropathological manifestations, and both types of patients, show a certain degree of heterogeneity regarding the symptoms, age of onset and duration of the disease. All the genes found mutated in fALS cases were found mutated in sALS[2,9]. Since fALS and sALS are clinically indistinguishable, with the exception of the time of the onset of the disease, that is found to be earlier in cases of family ALS[1], several studies have been carried out in order to understand the role of genes associated with fALS in sporadic cases.

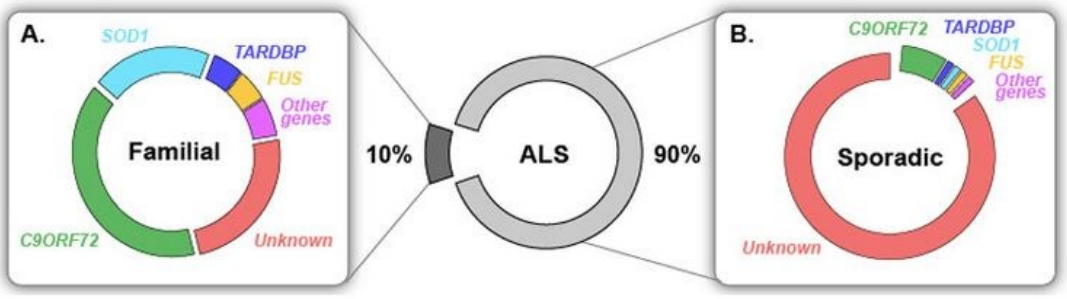

Figure 1. Known genetic causes in familial and sporadic ALS. *Most ALS cases are sporadic (sALS) and only 10% are inherited, called familial (fALS). (A) 20% of fALS are caused by mutations in SOD1, which is the first known ALS-linked gene, identified in 1993*[1].

Over the years, a number of investigations have been carried out, leading to the identification of a large number of genetic factors associated with ALS (Table 1), which are categorized according to the risk of developing the disease in two large groups:

- causative genes associated with high risk of ALS outbreaks, such as SOD1, FUS,

TARDBP, C9ORF72, UBQLN2, which are by no chance the ones that are most frequently mutated in pathologic subjects;

- low-risk sALS, so-called susceptibility factors such as NEFH[11] (Table 1);

Up to now, about 20 genes explain most of the cases of familial sALS, but only a minority, about 10%, of sporadic cases of illness[12,13].

LOCUS	GENE, PROTEIN	Disease Mechamism	ONSET	AD/AR
ALS 1	SOD1, Cu/Zn SOD1	Oxidative stress	A	AD/AR
ALS 2	ALS2, Alsin	Endosomal trafficking	J	AR
ALS 3	?	--	A	AD
ALS 4	SETX, Senataxin	RNA metabolism	J	AD
ALS 5	SPG11, Spatacsin	DNA damage repair and axon growth	J	AR
ALS 6	FUS, Fused in sarcoma	RNA metabolism, DNA repair	J/A	AD/AR
ALS 7	?	--	A	AD/AR
ALS 8	VAPB, Vescicle-associated membrane	Endoplasmatic reticulum stress	A	AD
ALS 9	ANG, Angiogenin	RNA metabolism	A	AD
ALS 10	TARDBP, TAR DNA-binding protein	RNA metabolism	A	AD
ALS 11	Phosphoinositide-5-phosphatase	Endosomal trafficking	A	AD
ALS 12	OPTN, Optineurin	Autophagy	A	AD/AR
ALS 13	ATXN2, Ataxin 2	RNA metabolism	A	AD
ALS 14	VCP, valosin-containing protein	Autophagy	A	AD/AR
ALS 15	UBQLN2, Ubiquilin 2	UPS (ubiquitin-proteasome system) and Autophagy	J/A	X-linked
ALS 16	SIGMAR1, sigma non-opioid intracellular receptor 1	UPS (ubiquitin-proteasome system) and Autophagy	J	AR
ALS 17	CHMP2B, Charged multivesicular body protein 2B	Endosomal trafficking	A	AD
ALS 18	PFN1, Profilin 1	Cytoskeleton	A	AD
ALS 19	ERBB4, Neuregulin-ErbB4	Neuronal development	A	AD
ALS 20	HNRNPA1, heterogeneous nuclear	RNA metabolism	A	AD

	ribonucleoprotein A1			
ALS 21	MATR3, Matrin 3	RNA metabolism	A	AD
ALS 22	TUBA4A, Tubulin a4A	Cytoskeleton	A	AD
ALS-FTD1	C9orf72, guanine nucleotide exchange C9orf72	RNA metabolism and autophagy	A	AD
ALS-FTD2	CHCHD10, coiled-coil-helix-coiled-coil-helix domain-containing 10	Mitochondrial maintenance	A	AD
ALS-FTD3	SQSTM1 , sequestosome 1	Autophagy	A	AD
ALS-FTD4	TBK1, serine/threonine-protein kinase TBK1	Autophagy	A	?
Susceptibility factors	PRPH, peripherin gene	Cytoskeleton	-	-
Susceptibility factors	DCTN1, Dynactin Subunit 1	Neuronal development , Endosomal trafficking	A	AD
Susceptibility factors	NEFH, Neurofilament Heavy Polypeptide	Cytoskeleton	-	-

Table 1.Principal gene locus involved in ALS and relative mutations: *Genetic factors at the base of the SLA. Abbreviations: AD: dominant autosomal; AR: autosomal recessive; A: adult; J: youthful. (Data extracted from OMIM database: http://omim.org/phenotypiSeries/105400).*

Superoxide dismutase 1 (SOD1) is the first ALS-linked gene that was identified in 199314 and, for almost fifteen years, ALS research has been focused on mutant forms of this protein15,16. Since 2008, starting with the discovery of ALS-linked mutations in DNA/RNA-binding proteins TDP-43 and FUS, an era of unprecedented genetic discoveries in ALS begun.

Although the genetic cause of most sALS is not known, recent studies have shown that mutations causing fALS are also able to cause illness in sporadic cases. In fact, only a small part of sALS patients showed that they had de novo mutations in known ALS-causing genes[17-20]. C9ORF72 repeat expansions, which were also found in a substantial fraction (~7%) of apparently sporadic ALS patients, most likely are not occurring de novo[21,22], but rather represent cases with insufficient family history or incomplete penetrance. Taken together, ~10% of apparently sporadic ALS cases are caused by known genetic mutations, while the aetiology of the rest ~90% sALS remains unknown (figure 1).

The pathological hallmark of ALS is the presence of ubiquitin-positive inclusions, consisting of misfolded protein aggregates in affected motor neurons and glial cells of the spinal cord and motor cortex. The most frequent protein component of these inclusions, in most ALS cases, is TDP-43[23,24]. Notable exceptions are patients with mutations in SOD1 and FUS, which lack TDP-43 inclusions, but show misfolded SOD1[25,26] or FUS[27,28] protein accumulations. Moreover, patients with C9ORF72 expansions are affected by the most complex pathology form, characterized by typical TDP-43 inclusions, atypical TDP-43 negative inclusions[29-31], consisting in abnormal dipeptide proteins[32,33], as well as nuclear repeat RNA foci[29,32,34] (figure 2 and 3).

ALS-associated genes code for proteins implicated in different cellular processes, and various mechanisms have been suggested as major contributors to neurodegeneration in fALS and sALS[35].

There are two typical expressions of the onset of the disease: about 70% of patients present limb atrophy and muscular weakness at the trunk level at the initial stage of the disease , while the rest of the patients exhibit a symptomatology that ,during the first stage of the pathology, affects musculature of the tongue and swallowing[36]. Nearly 85% of patients with spinal-onset ALS, however, exhibit bulbar changes with disease progression. Approximately 50% of all patients diagnosed with ALS show cognitive and language impairment, while 10% of patients have clear signs of Frontotemporal dementia (FTD).

Some chromosomal loci containing ALS-causing genes are inherited as dominant while other as recessive autosomal traits, and it can be seen that there are at least 4 chromosome loci containing genes predisposing the pathology (Table 1). For the majority of ALS subtypes, the onset period is adulthood, with the exception of subtypes ALS2, ALS4, ALS5, whose period of onset is strictly juvenile.

1.3 Environmental and lifestyle factors

Environmental factors may play a decisive role in the onset of ALS disease. Among these risk factors the exposure to pesticides, fertilizers and heavy metals (such as selenium and mercury) has surely a significant role, especially for some specific occupational classes

(farmers, foundry workers)[37]. Exposure to secondary metabolites of cyanobacteria[38], such as non-neurotoxic amino acid β-N-methylamino-L-alanine (BMAA), may also have a primary role as a possible causative agent of ALS[39]. Cigarette smoking would appear to be an etiologic agent, with a directly proportional association between exposure time and exposure intensity[40]. Exposure to this set of chemical substances would result in cellular oxidative stress phenomena, increasing levels of reactive oxygen (ROS) and blood species in cerebral tissue, yielding to progressive neurodegeneration[41]. It also seems that, among all environmental factors, alcohol abuse[42], excessive physical activity and mechanical trauma associated with it[43], self-immunity phenomena, as well as latent infections mediated by retrovirus and enterovirus[44], play a key role in the onset of ALS. In fact, this disease, has been reported at a higher frequency among groups of athletes with respect to general population, although it is unclear whether physical activity is a risk factor or is simply a marker of underlying athletic prowess[45].

2. Pathogenic mechanisms in ALS

ALS is a heterogeneous disease, to which great diversity in genetic and environmental causes correspond a complex network of molecular pathogenic mechanisms that haven't been completely understood yet. Nevertheless neuropathological hallmark of disease is the aggregation and accumulation of ubiquitinated proteinaceous inclusions in motor neurons.

ALS pathogenic mechanisms, studied in many different experimental models and confirmed in ALS patients, outline a complex model in which non-competing mechanisms, including not only gene mutations and environmental factors but also impairment of protein homeostasis, aberrant RNA metabolism, impaired DNA repair, oxidative stress, mitochondrial dysfunction, dysregulation of nucleocytoplasmic and endosomal transport, neuroinflammation, excitotoxicity, axonopathy, are likely to converge in various unfortunate patterns to mediate selective motor neuron degeneration46 (Figure 2).

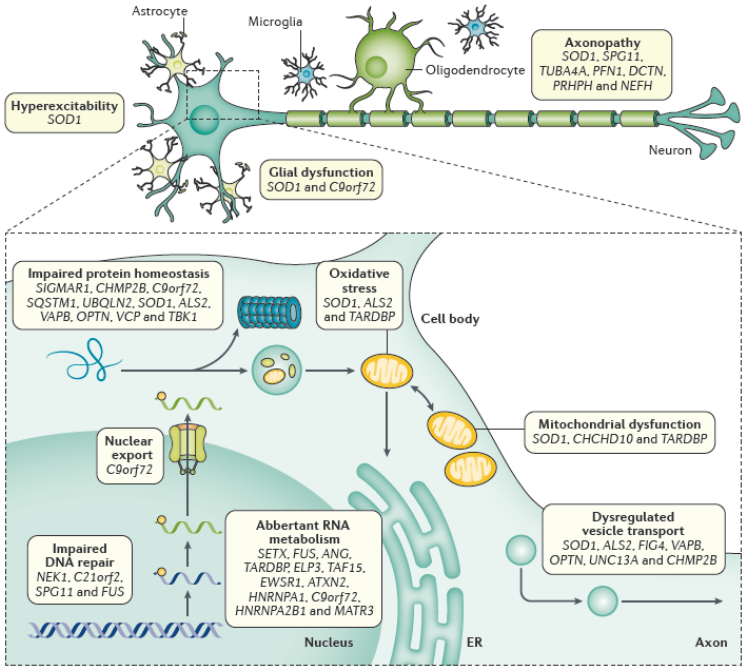

Figure 2. Molecular mechanisms of motor neuron injury in ALS. *ALS is a complex disease involving activation of several cellular pathways in motor neurons, and dysregulated interaction with neighbouring glial cells*[2].

2.1 Impaired protein homeostasis

Pathological protein aggregates, identified as compact or skein-like ubiquitinated inclusions, are a cardinal feature of ALS[48-50]. The identification of TDP-43 as the most abundant protein constituent of these inclusions, initiated a major shift in our understanding of the pathobiology of ALS[23]. Under normal conditions, TDP-43 is predominantly localized in the nucleus, and loss of nuclear TDP-43 staining is seen in nearly all cells containing TDP-43-positive cytoplasmic inclusions[23]. TDP-43 inclusions are not restricted to motor neurons, and it seems that cytoplasmic redistribution of TDP-43 is an early pathogenic event in ALS. In 2008, when mutations in TARDBP, the gene encoding for TDP-43, were discovered in several fALS pedigrees, the evidences for TDP-43 dysfunction in ALS were consolidated, thus establishing this protein as a crucial player in both sporadic and familial disease[51].

Although TDP-43 is the most abundant component of protein inclusions in 97% of ALS patients, different aggregates have been identified in various types of ALS, highlighting

the heterogeneity of the disease (Figure 3).

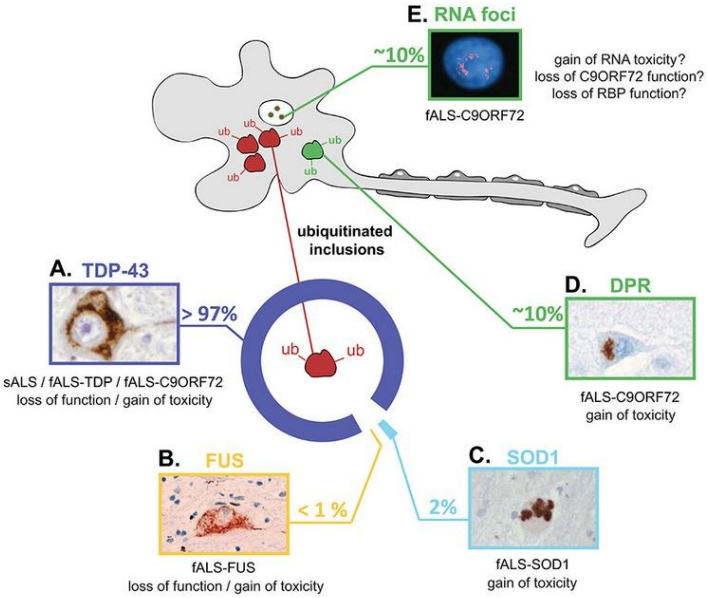

Figure 3: Pathogenic hallmarks of ALS and the underlying factors and mechanisms.

(A) In the majority of ALS cases, including all sporadic and most familial cases, the ubiquitinated inclusions contain TDP-43. When TDP-43 accumulates in the cytoplasm, it is excluded from the nucleus, where it normally resides in healthy cells. (B) In 0.5% of fALS that carry mutations in FUS, the cytoplasmic inclusions do not contain TDP-43, but they contain FUS protein. Similar observations to that of TDP-43, with partial nuclear clearance of FUS suggest that its neurotoxity may be the result of either loss of function or gain of toxicity. (C) In fALS patients with SOD1 mutations, misfolded SOD1 accumulates and becomes toxic to neurons. (D) In contrast, patients with hexanucleotide repeat expansions in C9ORF72 show typical TDP-43, similar to those found in sALS. (E) In addition to TDP-43 and DPR pathology, C9ORF72 patients accumulate nuclear RNA foci that contain sense and antisense hexanucleotide repeats in neuronal and glial cells. Insert images reproduced with permission from[1].

SOD1 inclusions are found in motor neurons of patients with fALS, as well as in mouse and cellular models expressing SOD1 mutations[52]. Monoclonal antibodies that are specific for epitopes of misfolded SOD1, very common in inclusions motor neurons of patients with SOD1 fALS[53], and seem to form similar structures in some patients with sALS[54]. Similarly, cytoplasmic inclusions containing mutant fused in sarcoma (FUS) protein have been observed in some patients with FUS-related fALS[55,56]. Proteins found in aggregates in ALS provide several important clues about the disease pathogenesis. Loss of nuclear

TDP-43 and/or aggregation of the protein in cytoplasmic inclusions, may be key of pathogenic processes in both sALS and fALS.

Protein misfolding and aggregation, in ALS, indicate both a defect in the activity of protein quality control (PQC) system, which includes molecular chaperons that lead protein folding and aggregation, and degradation systems (proteasome and autophagy). In fact, many ALS causing genes code for proteins that can promote dysfunction of the UPS (ubiquitin-proteasome system) such as SOD1, TDP-43 and VAPB, or proteins that are key regulators of both autophagy initiation, such as C9orf72 and optineurin, and execution, such as SQSTM1 and TBK1.

2.2 Oxidative stress

Several studies focused their attention on the role of mitochondria in the pathogenesis of neurodegenerative diseases such as ALS[57]. Mitochondrial dysfunctions, such as abnormal mitochondria and morphological defects, have been identified in skeletal muscles and intramuscular nerves of sALS patients[58,59]. In these cases, biochemical analysis revealed defects in muscular respiratory chain (complexes I and IV)[60], which leads to/evolves/ends in (occhio a quale scegli per lasciare o togliere in) damage to motorneuron metabolism and alteration to mitochondrial membrane pores permeability, and high levels of mitochondrial calcium in muscle and spinal cord[61]. In transgenic mice, the main evidence of mitochondrial alteration is the presence of mitochondria with vacuoles. For example, in SOD1 G93A mice, there have been observed vacuous originated from the detachment between the internal and external mitochondrial membranes, leading to a remarkable increase in number and volume as pathology progresses[62-64].

The expression of mutated SOD1 in neuronal cell lines or in primary motor neuron cultures, causes depolarization of mitochondria, alteration of calcium homeostasis and reduces ATP production, similarly as it happens in SOD1 G93A transgenic mice[65-67].

Mitochondrial alterations may contribute to the generation of oxidative stress conditions, as they appear to mediate cell death by releasing calcium into the cytoplasm and by weakening the respiratory chain, leading to the release of cytochrome-c and pro-apoptotic factors that activate the cascade of caspases and trigger apoptosis[68,69].

Oxygen is essential for life, but paradoxically, as a sub-product of its metabolism produces reactive oxygen species (ROS), which causes oxidative stress. Post-mortem analysis on brain tissues of patients with neurodegenerative diseases, such as ALS, clearly indicates an increase in the ROS index, in brain regions affected by the disease. The role of oxidative stress, and consequently of ROS in the onset of ALS, is still being discussed, since it is not clear yet whether they are the cause or a consequence of neuronal cell loss. However, ROS contribution to the events associated with neurodegeneration is evident[70].

Oxidative stress, in addition to mitochondrial damage, may be associated with an aberrant chemistry of SOD1. This appears to be a consequence of alterations in the shape of the active site that allow copper to interact with atypical substrates. In fact, modified SOD1, in contrast to normal activity, can act as peroxidase, resulting both in production of hydroxyl radicals and in inactivation of the enzyme itself, or can react with peroxynitrite causing tyrosine nitration[71]. Both peroxynitrite and hydroxyl radicals are strongly reactive species and can, therefore, cause oxidative damage to proteins, lipids, and DNA.

Such damage can cause alteration of protein conformation, destruction of the active site of enzymes, and modification of cell membrane properties by oxidation of unsaturated fatty acids and introduction of DNA mutations[72]. While earlier this mechanism was referable only to aberrant mutations of SOD1, recent studies investigate the possible involvement of FUS and TDP-43: it appears that TDP-43 mutations may be responsible for the production of reactive oxygen species and may alter the functioning of the mitochondrial respiratory chain[73]. The sum of these events seems to be directly correlated to motorneurons degenerative process in patients with ALS.

2.3 Aberrant RNA metabolism

Many different RNA-binding proteins (RBPs) have been implicated in human diseases, ranging from cancer to neurodegeneration, and alteration of mRNA processing is a key event in ALS pathogenesis. There are more than one hundred genes associated with ALS[74], a handful of which encode proteins that control RNA processing; table 1 reports RBPs known to be mutated in ALS patients.

There are many commonalities among these proteins. TDP-43, hnRNP A1, and hnRNP

A2/B1 are structurally similar, as they contain two RNA recognition motifs (RRMs) and a Gly-rich C-terminal domain[75]. FUS, EWSR1 and TAF15, which form the FET family, share a similar structure: a zinc finger and RRM domain that facilitates DNA and RNA binding, respectively, an N-terminal low complexity, prion-like domain that mediates protein interactions and self-assembly[76-78], multiple C-terminal Arg-Gly-Gly (RGG) domains that facilitate non-specific RNA binding and protein interactions, and an atypical Pro-Tyr nuclear localization signal (PY-NLS) that is recognized by transporters that control nuclear–cytoplasmic shuttling[79]. Those proteins are predominantly multifunctional nuclear proteins widely expressed in most cell and tissue types, since they are implicated in a broad range of cellular processes.

Whether motorneuron damage is caused by loss of normal nuclear function of TDP-43 and FUS in RNA processing, or by toxic gains of function, or both is unknown[79]. TDP-43 and FUS contain two RNA recognition domains—structures that are common to many RNA-interacting proteins, including those that are involved in mRNA transport. TDP-43 and FUS may form part of such RNA transport complexes and, when mutated, could thereby contribute to motor neuron injury through loss of axonal mRNA transport.

Fibroblast cell lines derived from patients with TARDBP-related ALS, showed the expected loss of nuclear expression of TDP-43, together with widespread changes in RNA splicing, including changes in transcripts of other RNA-processing genes and genes that have been previously implicated in ALS; hence, splicing disruption associated with the loss of nuclear TDP-43, is likely to contribute to ALS pathophysiology. Several in vivo models of TDP-43 dysfunction, including both knockout models and models overexpressing the wild-type and mutant forms of the protein, have recently been generated in Mouse, Rat, Drosophila melanogaster, *Zebrafish* and *Caenorhabditis elegans*[80,81].

A note of caution relating to genetic studies is that, although gene structures are often similar between species, intronic sequences show great variability. Given the key role of TDP-43 in binding to long intronic sequences, the intraspecies variability in introns may hinder accurate modelling of human TDP-43 proteinopathies in other species, including rodents[82,83].

2.4 DNA repair

Impaired DNA repair was suggested to have a role in ALS pathophysiology since the identification of FUS mutations, although the exact role of DNA repair failure in ALS remains to be clarified[84]. Interestingly both FUS and TDP-43 have been shown to be recruited by DNA damage; in fact, Wang et al. (2013) demonstrated that FUS is important in the mediation of DNA repair and DNA damage response (DDR) in post-mortem neurons, thus explaining how FUS mutations can alter the response to DNA damage leading to pathogenesis of neurodegenerative diseases[84].

TDP-43, like FUS, plays a role in the prevention and repair of damages associated with DNA as well as damages associated with DNA loops, thus helping both DNA stabilization and damages related to its transcription. Specifically, TDP-43 is localized in foci before the damage of endogenous DNA and its presence increases even more following DNA damage, suggesting the existence of some shared functions for FUS and TDP-43[85].

Mutations in NEK1 and C21orf2, which both encode for proteins involved in DNA repair, can cause ALS[86-88], although the biological pathways associated with their causal role needs further confirmation.

2.5 Nucleocytoplasmic and endosomal transport

Perturbation of nucleocytoplasmic transport has been identified as a central mechanism underlying ALS-FTD, particularly those cases caused by mutations in C9ORF72, but is also involved in other neurodegenerative diseases as well as normal aging. The observation that most cases of ALS are associated with mislocalization of TDP-43 was, in hindsight, the first clue that the pathomechanism of ALS might involve an abnormality in nuclear transport. The temporal relationship among mislocalization of TDP-43, nuclear transport defects, and neurotoxicity in ALS remains unclear. It is possible that a primary defect in nuclear transport causes mislocalization and subsequent aggregation of TDP-43. Another hypothesis is that accumulation of cytoplasmic aggregates of TDP-43 drive a secondary defect in nuclear transport. Despite the accumulation of experimental data indicating impairment of nucleocytoplasmic transport through the nuclear pore complex (NPC) in disease models, evidence of such defects in patients with ALS and other related diseases

is thus far limited to redistribution of NPC components in end-stage disease. In addition, the relative pathogenic contribution of nucleocytoplasmic transport deficiency, compared to impairments in other cellular processes, has not been fully evaluated. Moreover, the relative roles of toxic species causing damage to nucleocytoplasmic transport machinery, whether toxic RNA or toxic DPRs, remain unresolved. While challenging, we anticipate that these issues will be addressed soon in this rapidly moving field[89].

2.6 Neuroinflammation and Excitotoxicity

Activated microglia and infiltrating lymphocytes indicate an inflammatory component in CNS pathology of ALS[90]. Proinflammatory mediators, including monocyte chemoattractant protein 1 and IL-8[91], are present in the (cerebrospinal fluid) CSF of patients with ALS, and biochemical indices of immune-response activation are present in the blood[92]. Reduced counts of CD4+CD25+ regulatory T (TREG) cells and monocytes (CD14+ cells) are detected early in ALS, suggesting recruitment of these cells to the CNS early in the neurodegenerative process. TREG cells interact with CNS microglia, attenuating neuroinflammation by stimulating secretion of anti-inflammatory cytokines[93]. Consistent with this scenario, double transgenic mice, carrying mSOD1 and lacking CD4, develop a more aggressive ALS phenotype, which is reversible by bone marrow transplantation[94]. Neuroinflammation is closely linked to activation of the immune response. A recent study identified CD40L — a ligand expressed by T cells that activates the immune response when bound by CD40 on antigen-presenting cells — as a promising therapeutic target[95]. Proinflammatory mediators, including monocyte chemoattractant protein 1 and IL-8[91], are present in CSF of patients with ALS, and biochemical indices of immune-response activation are present in the blood[92]. Reduced counts of CD4+CD25+ regulatory T (TREG) cells and monocytes (CD14+ cells) are detected early in ALS, suggesting recruitment of these cells to CNS early in neurodegenerative process. Excitotoxicity is a particular neuroinflammation process that is due, at least in part, to the excessive activation of glutamate N-methyl-D-Aspartate receptors, which causes an increased calcium input through the ionic channel associated with the receptor, thus resulting in production of free radicals, such as NO and ROS, which trigger numerous

pathways related with the onset of ALS. A body of evidence implicates excitotoxicity as a mechanism contributing to motor neuron injury in ALS, although clear evidence that it is a primary disease mechanism is lacking. In some patients with ALS, levels of CSF glutamate are elevated[96], and the expression and activity of EAAT2 are reduced in pathologically affected areas of the CNS[97-99], although whether if this is a cause or a consequence of neuronal loss is unclear[100]. Electrophysiological studies in humans have shown hyperexcitability of the motor system in presymptomatic[101] or early stages[102] of ALS. Evidence suggests that the calcium permeability of AMPA receptors in the spinal ventral horn may be dysregulated by abnormal editing of the GluR2 AMPA receptor subunit[103]. In addition, a recently identified ALS-linked gene encodes d‑amino acid oxidase (DAO). This enzyme is responsible for the oxidative deamination of d‑amino acids, one of which — d-Serine — is an activator and co-agonist of N‑methyl‑d-Aspartate (NMDA) receptors[104]. Mutations in DAO could potentially contribute to excitotoxic motor neuron injury. Excitotoxicity in ALS pathology leads to many events, including altered electrophysiological properties and increased sensitivity of motor neurons to excitotoxicity[105], altered AMPA receptor subunit expression, reduced expression and activity of EAAT2[106], increased glutamate efflux from spinal cord nerve terminals[107], a reduction in motor neuron inhibitory–excitatory synaptic ratio[108], and loss of regulation by astrocytes of the expression of GluR2 by neighboring motor neurons[109]. Ameliorating excitotoxicity is the only strategy that has, so far, slowed disease progression in ALS. Riluzole, which has several effects, including inhibition of presynaptic glutamate release[110], causes a modest increase in survival[111].

3. Structure and function of TDP-43

3.1 TDP-43 genetics

TDP-43 or TARDBP (TAR-DNA Binding Protein-43) is a 43 kDa ribonucleoprotein, originally identified as transcriptional repressor of the HIV-1 TAR-DNA. The gene coding for TDP-43 protein, consisting of 414 amino acids, is located along the short arm of chromosome 1 and consists of 6 exons (exon 1 is represented by a non-coding sequence). TDP-43 shares

with the heterogeneous ribonucleoprotein family a series of key protein domains essential for its activity:

- A Nuclear Localization Signal (NLS) domain at the N-terminal;
- Two domains "RRM1 and RRM2" (RNA-recognition motifs): recognition of DNA and RNA;
- A Nuclear Exportation Signal (NES) within the RRM2 domain;
- The "Glycine-rich region" located at the C-terminal end: Glycine-rich domain for interaction with other proteins and to promote the exon skipping of some pre-mRNAs [112,113] (figure 4).

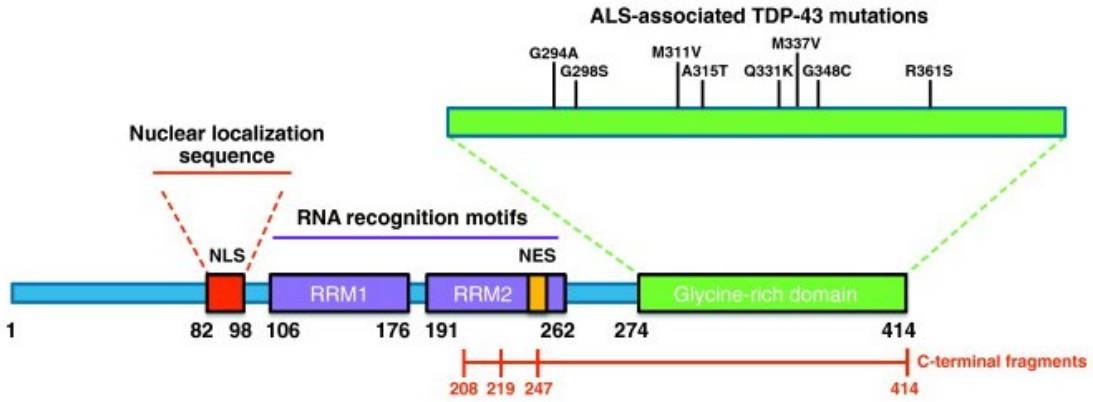

Figure 4: Graphic representation of TDP-43 functional domains of the protein. *TDP-43 have structural similarities with both harboring a Prion-like domain, RNA recognition motif(s) and nuclear localization signal[3].*

TDP-43 has a predominantly nuclear localization and, as FUS, is involved in the regulation of many biological processes. In fact, TDP-43 is a DNA/RNA binding protein that participates in transcription regulation by promoting binding DNA transcription factors, is involved in the alternative splicing process, in biogenesis and maturation of microRNAs, and in transport and localization of some pre-mRNAs115. Additionally, TDP-43 is particularly important in regulating axonal transport and cytoskeletal integrity. Mutants and aberrant protein forms are therefore associated with a wide range of cellular toxicity phenomena116. Since 2006, studies on the composition of protein aggregates in neuronal brain cells of patients with neurodegenerative diseases such as ALS have shown that TDP-

43 protein is the major constituent of such inclusions23. As for FUS, more than 40 mutations were identified in TARDBP gene, and TARDBP pathogenic variants prevalence is:

- 1.6% in all individuals with ALS (fALS and sALS)
- 3.4%, but ranges from 0% to 12% across studies[117,118] in fALS;
- 1.1% but ranges from 0% to 5% across studies in sALS[119,120].

Most mutations fall within exon 6, that codes for the rich glycine region at the C-terminal end of the protein, and are inherited as dominant autosomal traits (Table 2).

Allelic Variant	Genotype-Phenotype Correlations	Notes	Ref.
GLY298SER	Earlier age of onset (mean 50.7 years) and the most rapid rate of progression of disease (mean 27 months)	This mutation was identified in Chinese family	[110]
ALA315THR	Llater age at onset (mean 66 years) and much longer survival (mean 110 months)	The mutation occurs in a highly conserved residue	[106]
MET337VAL	Intermediate in their average ages at onset and survival times	Methionine at this position is invariant in human, orangutan, mouse, opossum, chicken, frog, and zebrafish	[47]
GLY348CYS	Intermediate in their average ages at onset and survival times	This mutant, which introduce a cysteine to the C-terminal hnRNP interaction region, was predicted to increase the propensity for aggregation through the formation of intermolecular disulfide bridges	[109]
ALA382THR	Intermediate in their average ages at onset and survival times	This mutant is very common in Sardinia due to a founder effect. It accounts for 80% of familial ALS and 9% of simplex cases	[111,112]
ASP169GLY	Increases the hydrophobic interactions in the RRM1 core, thus enhancing the thermal stability of the RRM1 domain	This mutant is more susceptible to production of the pathogenic C-terminal fragment TDP-35	[113]
LYS263GLU	The stability of the protein complex is less than that of the wild-type protein, indicating a reduced affinity for long nucleic acids	This mutant shown to increase thermal stability by up to 3 °C in the apo state, which was unexpected due to its location outside of the RRM2 structure	[114]

Table 2. Principal mutations of TDP-43 protein. *TDP-43 has several pathological mutations, represented in small part in this table, mostly located in the domain rich in wiccans, although there are many discreetly discovered findings that resurface in the RRM1 and RRM2 domains, mainly due to lysine.*

The pathological manifestation, in type 10 ALS patients, is characterized by a great heterogeneity in terms of age of onset, aggressiveness of the phenotype and geographical location. In fact, some pathological mutants are characteristic of delimited geographical areas: a clear example is represented by the mutant TDP-43 A382T (defined as the "Sardinian variant"), which accounts for approximately 30% of total ALS cases in Sardinia[121,122].

3.2 TDP-43 protein aggregation

As described in paragraph 3.1, TDP-43 is present in the protein aggregates of almost all cases of ALS. Pathologic TDP-43 is hyperphosphorylated, ubiquitinated and undergoes an abnormal proteolytic process, from which a 25KDa carboxy terminal fragment is generated, resulting in function loss. Neo-formed fragments are transported from nucleus to cytoplasm of neuronal and glial cells, where they tend to aggregate and precipitate[123]. Immunoprecipitation and immunoblotting experiments demonstrated how the cytoplasmic inclusions are subjected to ubiquitination and phosphorylation processes. The ubiquitination of misfolded proteins that aggregate in the cytoplasm and nucleus of neuronal cells, seems to be one of the key phenomena involved in the pathogenesis of neurodegenerative diseases and proteinopathies: in fact this phenomenon is appreciable in the most advanced stages of the disease. The formation of cytoplasmic inclusion bodies, therefore, would appear to be due to the presence of aberrant mutations to the functional protein in wild-type form[12].

3.3 TDP-43 as transcriptional modulator

TDP-43 was originally identified as a transcriptional repressor that binds to TAR DNA of human immunodeficiency virus type 1 (HIV-1) [124]. Consistent with its role in transcription, TDP-43 was found, in human brain and in cell culture systems, to associate with euchromatin and to bind the promoter of mouse acrv1 gene, coding for SP-10 protein, which is required for spermatogenesis, acting as transcriptional repressor[125]. Recent evidences indicate that TDP-43 acts as a repressor of Acrv1 gene in spermatocytes and post-translational modifications, most likely ubiquitination, induce conformational change, leading to release of repression in round spermatids[126]. A second example that

illustrates TDP-43 activity as a transcriptional repressor in vivo came from a recent report that identified rat and human VPS4B gene (vacuolar protein sorting 4 homolog B) as a TDP-43 target in brain, via direct binding to a TG-rich region in the promoter[127]. Moreover TDP-43 is associated with proteins involved in transcription, including methyl CpG-binding protein 2 (MeCP2)[128]. Beside this role as transcriptional repressor, TDP-43 has been indicated also as transcriptional activator, by luciferase assay or mRNA level determination for Bim promoter[129], NFB responsive elements[130], p53 binding elements[131] and CHOP promoter[132].

3.4 TDP-43 as modulator of RNA metabolism

Splicing process is strictly regulated and is essential for an appropriate gene expression. It is not surprising, therefore, that dysfunctional spliceosome is closely linked to neurodegeneration. For example, when TDP-43 is removed from mouse embryonic stem cells, multiple cryptic exon have been coupled. This phenomenon has also been observed in cells from human patients with ALS and FTD[133].

TDP-43 shows the ability to bind RNA in a very specific way through the presence of two RRM motifs within its sequence, preferably UG repeats and UG/GU-rich repeats interspersed by other nucleotides, and the acetylation of residues K145 and K192, located in these domains, leading to reduced binding activity[134,135]. These observations were subsequently confirmed by CLIP analyses[136,137], and a high resolution TDP-43 nuclear magnetic resonance structure that binds one of these sequences has been recently solved[85]. It should also be considered that the presence of a potential binding site does not always mean that it will play a fundamental role in a particular process[138].

In *C. elegans,* as well as in cell culture, TDP-43 loss induces the accumulation of double-stranded RNA (dsRNA) and the abnormal processing of ribosomal RNA. Since it remains to be clarified whether these changes in RNA metabolism play a role in ALS/FTD pathology, several research groups are currently examining the molecular mechanisms with which TDP-43 limits the dsRNA and are trying to determine whether the loss of FUS and MATR3 or the expression of hexanucleotide C9orf72 has similar effects on RNA metabolism.

Another important aspect is represented by cytoplasmic redistribution associated with TDP-43 disease, in particular in response to the expression of C9orf72 hexanucleotide expansion and the associated production of non-aggregated polypeptides[139].

3.5 Transposable Elements and TDP-43

The transposable elements (TEs) are highly abundant genetic elements that constitute a great part of the eukaryotic genomes. Retrotransposons, which are transposed via an RNA intermediate, account for about 40% of the human genome[140,141]. Although most TE copies do not work, a subset has retained the ability to mobilize and even the stationary copies can be expressed[142]. Due to their potential to copy and paste into new genomic positions, TEs represent a huge endogenous reservoir of genomic instability and cellular toxicity[141]. The impacts of these genetic parasites are usually stifled by strong cellular mechanisms involving small interfering RNAs acting through induced RNA (RISC) to inhibit expression of transposon[143]. Although most surveys naturally focus on germs, where new insertions are inheritable and favoured from the evolution of the transposon, somatic tissues also have an active effect transposon mute mechanism whose functional meaning is less understood. As found in literature, TEs are normally active in brain[144-147], and LINE and SINE (which are non-LTR retrotransponants) are even more expressed. LTR elements, on the other hand, have been associated with several neurodegenerative agents disturbances [148-154]. With regard to this, it was evaluated by Wang group[155] if TDP-43 RNA targets include transposon derivatives transcripts. Several recent studies used deep sequences for RNA analysis targets that co-purify with immunoprecipitated mouse, rat or human TDP-43, and also for profiling gene expression in mouse after TDP-43 abatement or excess expression[156-159].

From data in literature, it can be stated that TDP-43 is generally destined to derive from TE transcripts, including many short interspersed nuclear elements (SINE), long interspersed nuclear elements (LINE) and long terminal repeat elements (LTR) classes as some DNA elements. Secondly, the association between TDP-43 and TE-derived RNA targets is reduced in patients with FTLD with respect to healthy subjects, consistent with the idea that loss of TE control could be a part of the onset of the disease[160,161,162].

3.6 The role of TDP-43 in mitochondrial translation

Several research paper reported that, in ALS patients, TDP-43 co-localizes with various neuronal organelles in both human spinal cord and frontal cortex tissue samples, oppositely to what physiologically happens in age-matched normal individuals.

In fact, it was demonstrated that in control cases, both in spinal cord motor neurons and in cortical neurons, TDP-43 mainly localizes in nucleus, while both ALS motor neurons and FTD cortical neurons showed characteristically higher levels of cytoplasmic TDP-43 accumulation.

Notably, cytoplasmic TDP-43 co-localizes with mitochondrial markers in many ALS spinal cord motor neurons or FTD cortical neurons, but minimally overlaps with markers of Golgi, endoplasmic reticulum, lysosome, autophagosome, endosome or peroxisome. Despite its low abundance in cytoplasm, TDP-43 co-localizes with mitochondria in motor neurons and cortical neurons in human control samples. Consequent sub-mitochondrial fractionation analysis, detected that mitochondrial TDP-43 was exclusively present in the inner mitochondrial membrane but not in outer mitochondrial membrane, intermembrane-space or matrix. Also Immuno-electron microscopy analysis of isolated mitochondria and biopsied human cortex confirmed the principal localization of TDP-43 in inner mitochondrial membrane cristae.

Further studies on TDP-43 localization, showed that disease-associated mutations, such as A382T, influenced its mitochondrial localization too.

It remains to be clarified whether TDP-43 deficiency alone is sufficient to cause neuronal loss, or if, with an inactivated nuclear localization signal, it causes a significant co-localization of this protein with mitochondria, indicating mitochondrial localization as an intrinsic property of cytoplasmic TDP-43[160].

Since mitochondrial dysfunction precedes TDP-43-induced neuronal death, and since mitochondria are involved in almost all types of cell death, including apoptosis and necrosis, it could be of great interest understanding whether mitochondrial dysfunction is induced by TDP-43 or not[161,162].

4 Epigenetics and neurodegeneration

4.1 Epigenetic modifications

Epigenetics is defined as the set of potentially inheritable changes in gene expression that do not involve modifications in the nucleotide sequence of DNA[137,163]. The complexity of our organism clearly points out the existence in the genome of something more than DNA sequence that is capable of determining the great intra-inter-individual variability. The latter is also based on epigenetic modifications, which are responsible for the maintenance of chromatin stability and which are implicated in a wide range of neurodegenerative disorders[164]. To date, many molecular complexes are known to be responsible for chromatin remodelling, DNA-dependent enzymatic modification and DNA-related histone proteins, as well as substitution of the same histones. Epigenetics is, thus, the configuration by which regulation of transcription and, hence, gene expression within our cells is realized and, in particular, it explains how genome written modifications are read, interpreted and removed, thus contributing to the stability and plasticity of nerve cells[164] (Figure 5).

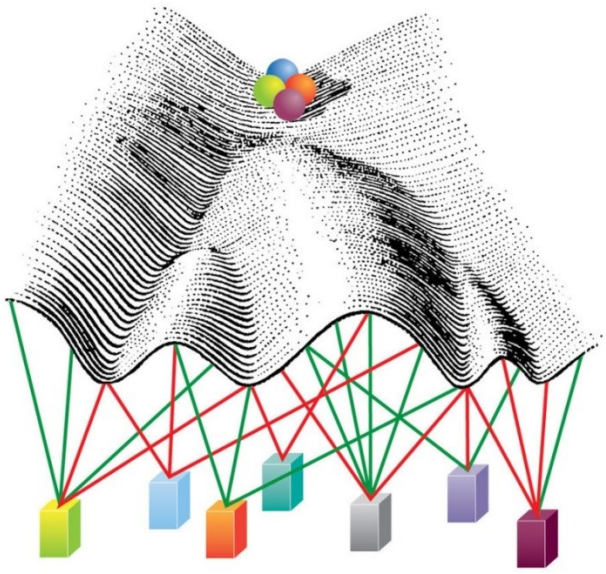

Figure 5: Waddington's vision of an epigenetic landscape. *This image shows that DNA provides the stable base on which our individual details are written in the form of chemical marks. Most likely, the timing, location, and persistency of the marks are some of the variables that will determine how important they will*

be in impacting such things as development and disease. Much remains to be elucidated, but the demonstration that external factors can alter the epigenome suggests that we can manipulate it, hopefully for good rather than evil.

Chromatin can be defined as a plastic substrate capable of responding to rapid,short-term and long-term changes within cells. Modulation and regulation of gene expression, at chromatin level, involve different epigenetic mechanisms, which can remodel or modify chromatin template[164-166]. Chromatin remodelling activities are ATP-dependent and increase DNA-binding proteins accessibility to genome by altering DNA–histone interaction non-covalently. Chromatin modifying activities are carried out by introducing covalent modifications on histone tails, histone core proteins or by DNA methylation. Histone modification complexes make post-translational changes of covalent nature at the N-terminal end of histone proteins, such as acetylation, methylation, phosphorylation, ubiquitation, sumoylation, glycosylation and ribosylation. These post-translational modifications, as well as the introduction of histone variants, co-operate in altering chromatin fiber[167,168], changing the degree of chromatinic compaction, which closely correlates to transcriptionally active or inactive state[169]. The histone DNA-code interactions control the activation/repression of gene transcription; therefore, in case of transcriptional activation, compact and inaccessible DNA is made available to DNA binding proteins by chromatin modification[170]. For example, high levels of histones H3 and H4 acetylation and H3 methylation on lysine 4 (H3K4me) are generally found in promoter regions of transcriptionally active genes, while high levels of H3 methylation on lysine 27 (H3K27me) correlates with polycomb-mediated protein transcriptional repression[171].

Of course, chromatin organization is designed to ensure that writing, reading and preservation of gene information is carried out within a well-defined spatial and temporal sequence, both during cell differentiation and during various stages of development[164]. It has long been suggested that histone modification patterns act as "recognition codes" for the recruitment of chromatin remodelling complexes and there are several experimental evidences that support the existence of the so-called histone code[168,172]. Histones N-terminal amino acids, when acetylated, are bound by proteins containing well-

characterized protein domains, such as bromodomain, found in many chromatin-associated proteins[173,174]. In addition, methylated lysine 9 of H3 is a specific recognition site for chromodomain, a domain typically involved in the formation of heterochromatin and in gene silencing; however, such residue may also be acetylated, and this modification is mutually exclusive with methylation[175]. In addition, H3 acetylation on lysine 14 from GCN5 HAT complex, is preceded by phosphorylation of serine 10 on the same tail; this suggests the existence of a regulatory cascade that controls the pathway, thanks to complexes capable of recognizing specific amino acid residues on the N-terminal codons[171].

4.2 Acetylation and deacetylation

Lysine acetylation and deacetylation in N-terminal histone tail, play a prominent role in regulating neuronal plasticity and transcriptional regulation[164,176]. The ability to influence neuronal vitality by modulating levels and activity of enzymes regulating nuclear homeostasis, such as HATs (Histone acetyl transferase) and HDACs (Histone deacetylase), has been demonstrated. It is now clear that the pattern of acetylation is strongly damaged in case of degeneration of nerve cells. Not unexpectedly one of the major causes of dysfunction and toxicity in cells is the imbalance in the presence of HATs and HDACs[177]. Acetyltransferases and deacetylases, combined with large multiproteinic complexes, catalyse opposite reactions on protein substrates, including core histones and transcription factors; in addition to regulation of transcriptional machinery, HAT-HDAC system is also involved in the modulation of replication, in DNA repair and in site-specific recombination[177]. Acetyltransferases and deacetylases, combined with large multiproteinic complexes, catalyse opposite reactions on protein substrates including core histones and transcription factors; in addition to regulation of transcriptional machinery, the HAT-HDAC system is also involved in the modulation of replication, and of DNA repair and site-specific recombination[177].

4.2.1 HATs

HATs are a large family of enzymes that catalyse acetylation reactions involving the

addition of acetyl groups derived from the Acetil-CoA. In particular, acetyl-transferase histones act on nucleosomal N-terminal tails and, in spite of the name, are also able to acetylate transcription factors such as p53, E2F and GATA1[177]. Acetylation of lysines neutralizes the positive charge of their ε-amine groups, thus reducing the affinity of these basic proteins to the acid DNA, altering histone core arrangement and relaxation of chromatin structure.

All core histones are subjected to acetylation, but the ones occurring on histones H3 and H4 are more characteristic; the main targets of HATs are lysine 9 and 14 on H3 and lysine 5, 8, 12 and 16 on H4-histone[178]. Acetylated lysine represent a surface on which many proteins with specific domains, such as bromodomain, can bind. Thus, chromatin is made more accessible to transcription factors. Acetylation of histones is consequently associated with transcriptional activation; on the contrary deacetylation closely correlates with gene silencing[179].

4.2.2 HDACs: functions, location and classification

Histone deacetylation is related with the removal of acetyl groups from amino acid residues of N-terminal histone tails, leading to an increase in chromatin packaging, compatible with gene silencing.

HDACs play a direct role in regulating transcription by deacetylation and regulation of TFs, or by interaction with transcriptional co-repressors. In *Homo sapiens*, 18 different HDACs have been identified, and have been divided into four categories based on their homology with yeast HDACs[180]: class I, class II, class III (sirtuine) and class IV HDACs (Figure 6).

Class	Isoform	Drosophila counterpart	Size	Cofactor	Location	Expression	Catalytic domain
(I)	HDAC1	dHDAC1	58	Zn^{++}	N	Ubiquitous	482 aa
	HDAC2	dHDAC3	59		N		488 aa
	HDAC3		50		N, C		428 aa
	HDAC8		44		N		377 aa
(IIa)	HDAC4	dHDAC4	120	Zn^{++}	N, C	Specific	1084 aa
	HDAC5		130		N, C		1122 aa
	HDAC7		110		N, C		952 aa
	HDAC9		160		N, C		1011 aa
(IIb)	HDAC6	dHDAC2	160	Zn^{++}	N, C	Specific	1215 aa
	HDAC10		70		N, C		(Inactive) 669 aa
(III)	SIRT1	dSir2	120	NAD^+	N	Variable	NAD 747 aa
	SIRT2		45		C		NAD 389 aa
	SIRT3		28		M		NAD 399 aa
	SIRT4		35		M		NAD 314 aa
	SIRT5		36		M		NAD 310 aa
	SIRT6		39		N		NAD 355 aa
	SIRT7		48		N		NAD 400 aa
(IV)	HDAC11	dHDACX	39	Zn^{++}	N	Ubiquitous	347 aa

***Figure 6. Table representing Class I, II and IV HDACs**. Class I (HDAC1, 2, 3 and 8), class IIa (HDAC4, 5, 7 and 9), class IIb (HDAC6 and 10), class III (sirtuin family) and class IV (HDAC11) HDACs with the Drosphila counterpart[181,182].*

Cellular localization, as well as site-specific expression, differs between various deacetylases. In order to carry out their biological activity, HDACs have to move to nucleus, where most of the target substrates are located.

Nuclear localization is guaranteed by the presence of a Nuclear Location Signal (NLS). Some HDACs are only nuclear, while others may be cytosolic, depending on the regulatory domains present. In particular, Class I HDACs, and a group of HDACs of class II (IIa), are the most expressed in cerebral areas associated with learning and memory[183].

Class I, II and IV HDACs have zinc-dependent active sites, while sirtuins require NAD^+ (nicotinamide adenin dinucleotide) cofactor to function. However, interaction between different classes of HDACs is necessary for their biological activity. All HDACs have a catalytic domain consisting in about 390 amino acids with a highly conserved amino acid sequence. The active site consists in a slightly curled tubular pocket with a wide bottom[184]. The removal of acetyl group occurs through a charge system, consisting in two adjacent histidine residues, two aspartate residues, located about 30 amino acids from

the histidines and separated by 6 amino acids, and a tyrosine residue, located 123 amino acids downstream of aspartate. A fundamental component of this system is zinc ion, retained by its binding site at the bottom of the pocket, although other cofactors are required for the activity of HDACs. The conserved tyrosine catalytic residue is substituted by a histidine in Class IIa HDACs. This substitution greatly reduces catalytic activity[185].

4.2.2.1 Class I HDACs

Deacetylases belonging to this class are expressed ubiquitously with a predominantly nuclear site. They show homology with the transduced yeast regulator RPD3 (Reduced Potassium Dependency 3) and are represented by HDACs 1, 2, 3 and 8[186]. These are largely expressed in brain, except for muscular-specific HDAC8, where they interact with key proteins that form part of large multi-unit complexes.

HDAC1 and 2, respectively constituted by 482 and 488 amino acids, exhibit high structural and functional similarity with a sequence homology equal to 82%, and participate in the formation of large transcriptional repression complexes consisting of SIN3A, NuRD and Co -REST[187], thus inactivating the expression of neuronal genes in non-nervous tissues[188].

In particular, such complexes act on multiple aspects, such as cell cycle regulation, maintenance of pluripotency of stem cells and cell differentiation[189]. HDAC1 and HDAC2 are located in nucleus by acting not only on histones, but also on different substrates, including MyoD, E2F, p53 transcription factors and retinoblastoma protein. The two deacetylases carry out their biological activity only when incorporated into a multiproteic complex, which is, in fact, composed of proteins necessary for modulation of their deacetylase activity, DNA binding, and recruitment of HDACs on promoter regions. In addition, their activity, as well as the formation of repression complexes, is modulated by phosphorylation. Precisely, both deacetylases are phosphorylated by casein kinase 2 (CK2) and, in addition, HDAC1 represents the target of other kinases such as PKA (cAMP-dependent protein kinase A) and cGMP-dependent G protein kinase[177]. In resting cells HDAC1 and 2 are phosphorylated at low levels; when hyperphosphorylation occurs, there is a significant increase in deacetylase activity. Analysis of HDAC1 reveals two crucial sites for modulation of deacetylation by phosphorylation, represented by Ser421 and Ser423.

When these residues are mutated, the formation of protein complexes is hindered and HDAC1 biological activity is reduced[190,191].

In ALS context, HDCA1 has been described as the interaction partner of FUS during DNA repair; in addition, ALS-causing FUS mutant displays a reduced binding activity[84].

4.2.2.2 Class II HDACs

Class II HDACs are homologous to yeast histone deacetylase 1 (HDA1), and represent a class that is further subdivided in two subclasses:

- Class IIa HDACs, represented by HDACs 4, 5, 7 and 9;
- Class IIb HDACs, represented by HDAC 6 and 10.

The former have, in addition to deacetylase domain, an extended N-terminal regulatory domain that regulates nucleotide-cytoplasmic shuttling and DNA-specific binding, while the latter have distinct C-terminal domains[192]. They both show cellular and tissue-specific distribution, with a narrower expression pattern than class I HDACs, suggesting their possible involvement in cell differentiation and development[193], but they are expressed abundantly in brain[194]; in particular, almost all move from nucleus to cytoplasm because of the presence of nuclear signal, while cytosolic retention depends on phosphorylation and interactions with proteins 14-3-3[195].

HDAC6, in particular, is expressed mainly in cytosol where it acts as an important constituent of cytoskeleton deacetylase, the α-tubulin[196,197], but other substrates have been identified, such as TDP-43[198]. HDAC6 is the only enzyme with two deacetylase active domains organized in tandem and one zinc fingers (HDAC6-USP3 and Brap2-related zinc finger motif) domain in the carboxyl terminus. This latter domain is a signal for ubiquitination, suggesting, therefore, that this enzyme is particularly prone to degradation[199]. Although it is localized predominately in cytoplasm where, by inducing α-tubulin deacetylation, regulates cell motility and microtubule-dependent cell adhesion[197], it was detected also at nuclear level in association with HDAC11. In addition, HDAC6 presents an activity independent from deacetylase functions[200]; in fact, by tying the ubiquitin, it participates, for example, at the autophagy process regulation and activity of HSF1 protein (heat shock factor 1).

4.2.2.3 Class III HDACs

Commonly known as sirtuins, because of its homology with SIR2 regulator identified in yeast[201], class III HDACs are represented by 7 different NAD^+-dependent enzymes expressed in the mammalian brain. In particular, SIRT1, 2, 6 and 7 are localized both in nucleus and in cytosol, while SIRT3, 4 and 5 exhibit mitochondrial localization[202,203]. Sirtuins catalyse the deacetylation of other targets than histones: for example, SIRT1 acts on targets such as TAF68 (TBP-associated factor 68), p53 and p300; SIRT2 has the ability to deacetylate α-tubulin exactly as HDAC6[204]; SIRT3, 4 and 5 determine the global level of acetylation in mitochondria by regulating energy metabolism, but also lipid metabolism and apoptosis[186].

In particular, many studies highlight the role of sirtuins in the maintenance of genomic integrity; in fact deficiency of their deacetylase activity is compatible with alterations in gene silencing, increased genomic instability and susceptibility to DNA damage. For example, it has been noted that SIRT6-deficient cells, accumulate a large number of chromosomal anomalies. Recent studies have also shown that this sirtuin is capable of affecting telomeric regions in human cells. Another important sirtuin, SIRT1, deacetylates factors associated with DNA repair mechanisms and is recruited on the same sites as a result of oxidative stress. Although this recruitment seems to have a protective role in genomic instability, it is accompanied by a depression of previously silenced genes, suggesting its implication in epigenetic silencing and chromatin modeling[205]. All sirtuins, except SIR2 and 5, have higher levels of expression in foetal brain with respect to adult, suggesting the crucial role of these molecules in initial brain development[202].

4.2.2.4 Class IV HDAC

The only representative of this class, HDAC11, has common traits with class I and II HDACs but its function and substrates are still unknown. It can be found mainly in nucleus and it is expressed during the development of central nervous system of mammals, including oligodendrocytes, and plays a key role in maturation of this cell type[206]. It is also possible that it plays a role in regulating inflammation, through its inhibitory effect on interleukin 10 expression[207,208].

4.3 HDACs regulation and deregulation in ALS

4.3.1 Zn-dependent HDACs in ALS

The implication of Class I HDACs in the onset of ALS was first shown by Janssen and colleagues[209], which showed that in affected patients there is an over-regulation of HDAC2 in the motor cortex and in the grey matter of the spinal cord[209], especially in the nuclei of motor neurons. Additionally, HDACs, in particular HDAC6, are increasingly implicated in ALS disease. A recent study reported that FUS and TDP-43 proteins, altered in many cases of ALS, interact with each other, forming a ribonucleoprotein complex that regulates the expression of HDAC6, by influencing the levels of its mRNA[210]. In addition, in nerve tissue of ALS patients, nucleus and cytoplasm of motor neurons present low levels of HDAC11 encoding mRNA[209]. In neurodegenerative context, HDAC6 plays a significant role in α-tubulin post-translational modification, which modifies the properties of cytoskeleton; HDACs strictly regulate the acetylation of a preserved lysine residue (K40) on α-tubulin protein, to regulate the movement of organelles within cells, mediated by motor proteins appearance[211]; this is especially important for those neurons who have to carry a large load for long distances.

The increase in acetylation levels in α-tubulin, following inhibition of the above-mentioned deacetylase activity, improves transport in primary neurons, thus preventing axonal degeneration[212,213]. Considering that reduction of acetylation levels in α-tubulin is a characteristic pathological marker, an approach based on the use of HDACs inhibitors for the treatment of neurodegenerative disorders with neurons with particularly long axons, has been attempted. In this respect, ALS is a significant example because axonal transport efficiency loss, in both upper and lower motorneurons, is significant in the onset of the disease[214]. A lot of experimental work on the role of α-tubulin acetylation in ALS was conducted in SOD1 G93A mice, where it has been observed that HDAC6 genetic ablation positively influences acetylated α-tubulin levels in the central and peripheral nervous system, and maintains integral axons. Moreover the potential for muscle activity and the number of neurons in ventral spinal cord horns are increased, suggesting that these events that are associated with increased cell survival[215].

Different studies also suggest that some of these HDACs regulate vitality and mortality of

nerve cells; first, HDAC1 assumes neurotoxic and neuroprotective character as it interacts with HDAC3 or HDAC9[216]. In fact, the role of HDAC1 in regulating neuronal vitality is quite controversial, since some studies show that such deacetylase is able to protect neurons from death, while other studies demonstrate that HDAC1 induces neurodegeneration and axonal death[217]. It, therefore, has a dual role, behaving differently depending on its molecular partner. Precisely, neurotoxic effect induced by HDAC1 requires interaction with HDAC3, which is toxic to motor neurons.

HDAC1 interaction with HDAC3 is quite strong both in vivo and in vitro, and HDAC3 repression suppresses HDAC1-induced neurotoxicity and vice versa. HDAC1 also interacts with HDRP, the truncated form of HDAC9, which is underrated in case of neuronal death. HDRP-HDAC1 interaction protects neurons from death, because high levels of such protein inhibit HDAC1-HDAC3 interaction, thus preventing neurotoxic action of both deacetylases[216]. In this regard, it was assessed the possibility that HDRP protects neurons from degeneration by preventing such interaction through the seizure of HDAC1; according to this hypothesis, HDAC1-HDAC3 association is strongly reduced when HDRP is expressed and, since HDAC3 does not interact directly with HDRP, it may be that the reduction in toxicity is due to the binding of the HDAC1. Overall, abovementioned considerations suggests that HDAC1 behaves as a kind of molecular switch between death and neuronal survival, although it remains to be clarified in which cellular compartment occurs the interaction between HDAC1 and HDAC3. HDCA1 has a nuclear localization signal and HDAC3 moves from nucleus to cytoplasm within neuron[216]. Although this may suggest a nuclear interaction, some recent studies indicate that in degenerative neurons HDAC1 moves to cytoplasm, where its presence is implicated in axonal alteration and degeneration of the cell[213]. In fact, the exposure of neurons to toxic stimuli, such as glutamate, promotes the passage of HDAC1 to cytosolic level mediated by CRM-1 nuclear receptor, thus inducing an alteration in mitochondrial transport. Specifically, the interaction of HDAC1 with cytosolic motor proteins, hinders their interaction with cargo proteins, resulting in the onset of swelling that appear as a succession of sagging and narrowing along the axon[210]. Neurofilaments, which represent the only axonal cytoskeletal component, are composed of different subunits and three of them are

phosphorylated in physiological conditions; phosphorylation levels correlate with the rapidity of axonal transport, probably affecting neurofilament-motor protein binding[218]. Conversely, hypoacetylated neurofilaments are more sensitive to calcium-activated calcium proteases, such as calpains and caspases, and exhibit greater tendency to aggregation[219]. Some studies show that high glutamate concentrations in neuronal cultures weaken transport along neurofilaments, and induce build-up of cytoskeletal and organoleptic proteins in localized granules and local destruction of axonal transport[220]. However, it remains to be clarified whether the input of calcium ions and the activity of HDAC1 are independent mechanisms associated with early or late axonal damage stages, although it has been observed that the passage of HDAC1 into the cytosol requires Ca^{2+}. Such translocation is permitted by the presence of a leucine-rich region, similar to nuclear signal (NES), which interacts with CRM-1 receptor. The effects of the transport of HDAC1 into cytosol on the instability of axon are not due to a loss of its nuclear function but it is due to the acquisition of a cytosolic function. In fact, the toxic effect of cytosolic HDAC1 is due to its ability to bind proteins such as KIF2A, KIF5 and α-tubulin, preventing them from forming complexes with cargo proteins such as dynamite[210].

4.3.2 NAD^+-dependent HDACs in ALS

Unlike other deacetylases, the activity of sirtuins in the SNC appears neuroprotective. Protective agents such as polyphenols, in particular resveratrol, selectively activate both SIRT1 and SIRT2[221]. Cortical neurons of SOD1 G93A transgenic mouse treated with resveratrol, appear to survive longer because of the activation of SIRT1 that deacetylates the FoxO3a transcription factor, which is responsible for determining cellular destiny in stress situations[222]. SIRT1 then leads to its activation or inhibition, depending on deacetylase residue or depending on cellular location of FoxO3a[223].

Moreover SIRT1 and SIRT2 have been described to be misregulated in G93A-SOD1 and G86R-SOD1 mice. SIRT1 decreases in the spinal cord, but increases in muscle during the progression of the disease, while SIRT2 mRNA expression increases in spinal cord, even if protein expression is substantially unchanged in all models examined[224].

4.4 Role of HDACs in Neuronal Development and Growth

It has long been known that HDACs are implicated in neurogenesis, a process that involves the formation of new neurons from non-mature cellular precursors. In particular, cell-specific expression of HDACs in central nervous system, suggests the specific roles in neuronal development; for example missing HDAC1-HDAC2 precursors doesn't allow cells to differentiate into mature neurons and are therefore confronted with death[226]. Although HDACs are enzymes generally associated with transcriptional repression, HDAC1 is also able to act as a positive transcript regulator during the neuronal development of the central nervous system of Zebrafish, since genes regulated by such deacetylase encode for transcription factors implicated in the promotion of neuronal specification[226].

HDAC3 is involved in the control of gene expression during neuronal development and maintenance of neuronal stem status, while HDAC5, as well as other molecules belonging to class II HDACs, is involved in differentiation of neurons[192]. In addition, histone deacetylase is involved in neurite formation as well as dendritic and axonal growth; these processes are dependent on cytoskeletal rearrangements[227] and largely dependent on microtubules and actin filaments deacetylation[228]. HDAC6 controls microtubular dynamics to optimize neurite formation through septin, which represents a scaffold that allows HDAC6 to perform its activity efficiently[229].

HDACs also play a key role in development of dendrites, as loss of HDAC6 in cerebellar and hippocampal neurons reduces both length and dendritic branching, thus highlighting its dendritogenic activity. However, this function is independent on its deacetylase activity and requires direct interaction with Cdc20-APC complex[230]. HDAC1, HDAC2 and HDAC9 are also implicated in dendritic growth.

Another role is the regeneration of axons damaged by reorganization of HDACs mediated microtubules[231,232]; the influence of calcium ion induced by active HDAC5 cell damage, which reduces tubulin acetylation levels, results in reduced microtubule stability. This is, in fact, necessary for the regeneration of axon[233]. Sirtuins are also implicated in neuronal development and in the protection of nerve cells; for example, during the neurite genesis, SIRT2 controls tubular acetylation, while SIRT1 plays a key role in stimulation of axonal regeneration, probably increasing actin-dependent motility deacetylating cortactin[234].

In general, gene expression is directly proportional to the level of acetic acid. This happens because acetylation pattern regulation is essential to maintain gene homeostasis. Thus, until acetylation and deacetylation events mediated by HATs and HDACs occur synchronously, modulation of gene expression is performed correctly. However, transcriptional dysfunction represents a mechanism involved in the death of motor neurons[235]. There are many evidences that deregulation of transcription may be or may be not the cause of many neurodegenerative disorders such as ALS, Parkinson's Disease, and Alzheimer's.

Although causal mechanisms and causal relationships are still under study, it has been hypothesized that protein inclusions of mutated forms, such as SOD1 and TDP-43, that are formed in pathological neurons, can cause toxicity, since sealing surfaces that can seize components are essentials for transcriptional machinery. It has been shown that inhibition of deacetylase prevents protein aggregation[236]. In particular, inhibition of HDACs in motor neurons cultures protects cells from excitotoxicity, which is a mechanism involved in the pathogenesis of ALS[237]. There are different ways in which changes mediated by HDACs are involved in neurodegeneration; in some cases, neurodegenerative disorder is caused by a HDAC-dependent transcriptional decreasing of a certain protein with consequent loss of function, whereas in other cases mutations are identified due to extensive transcriptional deficits along the genome[186]. In addition, HDACs do not only act on histones but also on a wide variety of targets, thus playing a wider role in cellular biology.

4.5 HDACs inhibitors

On the basis of considerations made in previous paragraphs, it is evident that pharmacological inhibition of HDACs can be an interesting therapeutic opportunity for ALS.

Various HDACs inhibitors (iHDAC), selective and non, synthetic and natural, used to stop proliferation in experimental neoplastic models[222], are available. Four large classes of inhibitors used in several clinical trials have been identified, some of which have already been approved by drug agencies in treating neoplastic diseases:

- Short chain fatty acids such as butyrate (NaB), phenylbutyrate (SPB) and valproic acid (VPA),
- Hydroxy acids such as trichostatin A (TSA) obtained from the fermentation of Streptomyces and oxamflatin,
- Ortho-aminoanilines, such as MS-275,
- Cyclic tetrapeptides, mostly derived from bacteria and fungi, such as trapoxin and FK228.

Most of them act as chelating agents against enzyme active site containing zinc, thus acting as competitive inhibitors and blocking the accessibility of acetyl lysates to the active site of HDAC. HDACs inhibitors have, in fact, a zinc atom with chelating activity into their active site, a linker region that accommodates the entry into the active site and a "hood" group, linked to the linker region via a small connection unit, to allow external surface interactions[238].

All inhibitors act by inhibiting reversible HDACs, with the exception of trapoxin and depudesine, which in fact bind the enzyme at the level of the epoxicone group covalently and irreversibly[222].

The non-specificity of such inhibitors is responsible for the contradictory effects found in different cell types, since inhibition of HDACs seems to be primarily protective in nerve cells and, in turn, deadly in cancer cells. From the beginning, most of the work on these small molecules was initially focused on apoptotic and anti-proliferative effects in tumor cell lines and it was, in fact, believed that the increase of histone acetylation following HDAC inhibition, activated the transcription of pro-apoptotic genes. In fact, it has been found that increased acetylation promotes also the expression of survival genes such as Bcl-2 and growth factors, thus providing protection to different cell types from neoplastic cells such as neurons[177].

Many studies, therefore, show neuroprotective effects induced by inhibitors of HDACs; as shown in Figure 7, treatment of neurons with such molecules results in histone hyperacetylation, which results in greater opening and accessibility of chromatinic conformation, resulting in recruitment of chromatinic remodeling complexes that bind acetyltransformed tails through protein domains of specific binding[164,166].

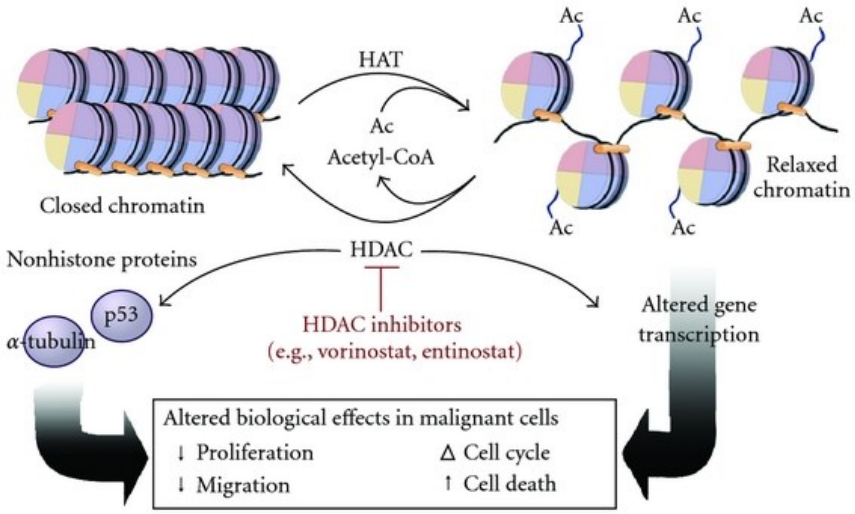

Figure 7. Effects of HDAC inhibitors*: When disabled, HDACs can not mediate chromatin condensation. This promotes the transcriptional activation of protective genes.*

In the presence of inhibitors of HDACs, an increase in acetylation levels is observed for H3 and H4 histones and other proteins such as p53[193].

HDACs inhibitors exhibit varying potency and selectivity, and it is possible to diversify selectivity by modifying the chelating region. Among non-selective inhibitors, TSA and Vorinostat are found to inhibit many zinc-dependent HDACs, including HDAC6, due to the ability to cross blood vessel barrier. Fatty acids such as NaB and SBP are able to inhibit most class I and II HDACs.

A fairly known inhibitor, VPA, binds to the active site by inhibiting both class I and IIa HDACs, but does not exhibit activity against Class IIb HDACs[217], although the susceptibility of class II HDACs to that inhibitor is five times lower than that of class I HDACs[193]. However, given the poor selectivity, recent attempts have been made to produce more selective inhibitors such as:

- MS-275, a synthetic benzamide that preferably inhibits HDAC1;
- romidepsin (FK228), a cyclic tetrapeptide that strongly inhibits HDAC1 and HDAC2;
- tubacin, which exhibits high selectivity for HDAC6;
- suramine and its structural analogues, which show selectivity for human SIRT1 and SIRT2;

- nicotinamide, precursor of NAD + and selective sirtuins inhibitor.

Many of these compounds were tested in transgenic mouse models SOD1. For example, treatment with TSA induces a modest improvement in motor capacity and protection against motor neuron and axonal degeneration, muscular atrophy and denervation of neuromuscular junction[239]. VPA treatment, however, does not result in an increase in survival or motor performance, but still induces an improvement in the acetylation pattern in spinal cord by restoring intracellular levels of CREB factor in motor neuron of mice, with pathogenic mice resulting in prevention of neuronal death in case of excitotoxicity and hypoxia[240]. NaB is also capable of extending survival and motor capacity in the aforementioned pathological animal models, attributed both to over-regulation of NF-B factor expression and other proteins involved in cell survival and stress response to reduced activation of caspase pathway[241]. The specific MS-275 inhibitor is particularly useful in presence of toxic stimuli leading to axonal damage; it, in fact, induces a calcium-dependent exporter of HDAC1, an enzyme that normally has nuclear localization. As already mentioned, its presence in cytosol interrupts the interaction of cargo and motor proteins resulting in loss of intracellular transport and cell death. MS-275 then acts by inhibiting the activity of the HDAC1 enzyme[210].

Some inhibitors such as VPA, NaB and SPB have been tested in different clinical trials that have demonstrated the ability of these molecules to induce an increase in histone acetylation.

However, there are several known undesirable effects associated with the intake of HDACs inhibitors; specifically, fatigue and weakness, gastrointestinal disorders, neurocortical manifestations, cardiac disorders, which result in non-specific electrocardiogram changes, and, in some studies, there is also a mild and transient thrombocytopenia. However, anaemic and neutropenic conditions are uncommon, and septic complications rarely occur[242]. In addition, the efficacy of HDACs inhibitors is closely dependent on their in vivo stability and, unfortunately, many of them do not have great stability and are therefore easily degraded by first-pass hepatic metabolism[243]. This is why many research fields are involved in identifying more potent and stable inhibitors that can

restore and normalize the pattern of acetylation in motor neuronss of patients with ALS.

5. Acetylated TDP-43 inclusions are linked to ALS

Lysine acetylation has emerged as a major covalent modification controlling diverse cellular processes and has been implicated in Alzheimer's disease (AD) and other neurodegenerative disorders[244-248]. For example, it has been demonstrated that acetylation of misfolded tau proteins marks mature neurofibrillary tangles (NFTs) in AD and related tauopathies, and represents a disease-specific marker of AD pathology[241,243,244]. In addition to tau, a global proteomics approach identified ~1,750 proteins that are subjected to lysine acetylation, including a distinct subset of RNA-binding proteins and associated factors.

Given the importance of TDP-43 binding with RNA, one of the most studied aspects is whether post-translational modifications, such as acetylation, may somehow alter its normal function especially in all those molecular pathways that result then later in ALS. It has been extensively demonstrated that if such modifications fall on residues contained in RNA-binding domains (RRMs), they alter the normal binding to RNA, and also promote the accumulation of insoluble TDP-43 aggregates. Further confirmation of how acetylation may be a distinctive alteration in ALS typical neurodegeneration processes, was obtained by analysing lesions of the spinal cord of ALS patients, where TDP-43 was re-used to be largely acetylated, thus providing the presupposition for further study of this modification for therapeutic purposes, not only for ALS, but for many other proteinopathies associated with TDP-43[198].

It seems that acetylation modifies both function and localization of TDP-43. In fact, TDP-43 in its WT form, normally located in the nucleus, seems to be subject to acetylation, although an higher cytoplasmic acetylation of TDP-43 have been detected, probably due to the different conformation of TDP-43 when it has cytoplasmic or nuclear localization, and because of different lysine accessibility. In addition, as TDP-43 primary cytoplasmic forms are observed in diseased brain and spinal cord, it is hypothesized that this increase in cytoplasmic cytotoxicity may be responsible, though only partially, for the formation of inclusion in TDP-43 proteinopathies.

In support of a pathological role, acetylation at K145 was sufficient to increase the pathogenicity of TDP-43 in cells, as demonstrated by both nuclear and cytoplasmic aggregation of TDP-43. Furthermore its C-terminus is widely phosphorylated and there is a consequent loss of its normal nuclear function. Based on all these scientific considerations, it can be hypothesized that aberrant acetylation of TDP-43 can act in a pathogenic way and that this can trigger the aggregation and loss of TDP-43 function. However, it remains to be investigated whether TDP-43 acetylation is limited to ALS or if this modification causes a wider range of TDP-43 proteopathies, thus expanding the clinical and therapeutic relevance of this phenomenon[198].

Acetylation also does not significantly alter steady-state TDP-43 protein levels in cell-based system[135].

When the effect of acetylation on binding with target mRNAs is evaluated, particularly acetylation on K145 and K192 within the RRP domains of TDP-43, by means of mass spectrometry assay, obtained data suggest that even a single acetylation event is sufficient to compromise the binding and splicing functionality of RNA, thus demonstrating how modification on K145 causes a critical change in the TDP-43 function. Other data reported by Bhardwaj, show that non-specific K145 acetylation, induced in vitro using acetic anhydride, is more effective in the absence of RNA, suggesting that the RNA binding protects K145 from acetylation[249]. According to these experimental data, TDP-43 acetylation within the RRMs is sufficient to compromise normal RNA binding, thus forming a fast and efficient regulator switch to modulate TDP-43 association with downstream target RNAs[198].

In pathologic conditions, TDP-43 is abnormally phosphorylated on serine C terminal terminals (Ser-403/404 and Ser-409/410) by multiple kinases[250-253], which is a highly specific marker of disease[26,198,201]. Although phospho-TDP-43 immunoreactivity is now considered to be an important biomarker in postomortem dignostics, it is not yet clear what the role of this post-translational modulation at TDP-43 level is. In fact, different research groups interpret phosphorylation as an incident that disadvantages aggregation instead of promoting it[202,203], thus assuming the existence of additional signalling mechanisms modulating the activity of TDP-43 and the co-generating aggregate

formation.

Recent studies by Cohen on TDP-43 acetylation, point to a new therapeutic openness closely linked to translational post modifications that are often associated with proteins involved in ALS. In fact, it has been shown as one single acetylation-mimic (K145Q) modulating the TDP-43 RNA regulatory function, lead to a robust aggregation, when it is targeted at cytoplasm, triggering many of pathological distinctive signs associated with TDP-43 proteinopathy included hyperphosphorylation, mitochondrial perturbations and an inflammatory response. However, it is possible to improve chaperone function relieved the burden of TDP-43 aggregates through a HSF-1 transcriptional waterfall, as acting with an endogenous reconstruction program, it is able to engage and remove the TDP-43 in vivo inclusions[251].

Another important experimental test that best clarifies the key role of acetylation has been obtained from recent in vitro studies that have shown that loss of nucleic acid binding converts TDP-43 into unstable insoluble agglomerates[247,248], implying physical association with nucleus that acids can act to stabilize the terminal C-terminal TDP-43 as a prion domain, bringing a more soluble functional protein that can thus act as a switch regulator to modify TDP-43 affinity for the target RNA. Indeed, treatment of cells with agents that increase oxidative stress and which promote loss of association with RNA led to TDP-43 acetylation and insoluble accumulation.

It is probable that acetylation of K145 residue, located in the site that allows TDP-43 DNA/RNA linkage, is facilitated, as this site is more accessible, thus allowing a more rapid alteration of TDP- 43[254].[198].

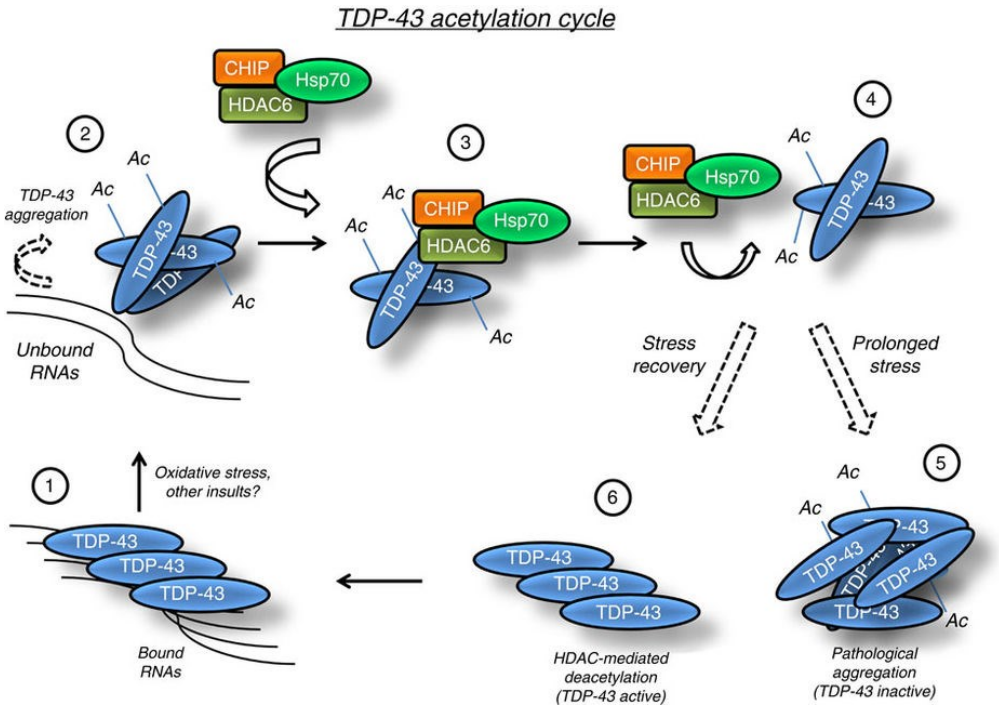

Figure 8 Acetylation and deacetylation cycle of TDP-43. *TDP-43 which normally has a high affinity for nuclei when it is subjected to excessive stress oxidizing, for example by external factors or genetic factors, undergoing modifications such as crosslinking of cysteine disulfide11 and lysine acetylation , going to alter the affinity of the bond with RNA but above all, leading to a buildup of protein in the cytokine and nucleus. These agglomerates are easily linked by the HDAC6 / CHIP / Hsp70 complex which is capable of deacetating these complexes and reporting TDP-43 under normal conditions, provided the stress is not too prolonged over time*[5].

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MATERIALS AND METHODS

Antibodies and reagents

The following primary antibodies were used in this study: Myc monoclonal antibody (M4439, Sigma-Aldrich), β-actin antibody (A5441, Sigma-Aldrich), Flag (F3165, Sigma-Aldrich), HDAC1 (10197-1-AP, Proteintech), TARDBP (190782-2-AP, Proteintech), Acetylated-Lysine antibody (9441, Cell Signaling), anti-rabbit peroxidase-conjugated secondary antibody (AP132P EMD Millipore and anti-mouse peroxidase-conjugated secondary antibody (AP124P EMD Millipore); anti-rabbit, anti-mouse Alexa 488 (A-11001, Life Technologies) or 647-conjugated secondary antibody (A-21244, Life Technologies). All antibodies were used at the dilution recommended by the manufacturer's instructions.
The following HDAC inhibitors were used in this study: Sodium phenil butyrate (SML0309, Sigma-Aldrich), Trichostatin A (T8552, Sigma-Aldrich), Sodium butyrate (B5887, Sigma-Aldrich), Valproic acid sodium salt (P4543, Sigma-Aldrich).

Mice tissue

Mice tissues were dissected from BALB31c mice housed at the Istituto Zooprofilattico della Sardegna (Sassari, Italy). All animal procedures have been performed according to the European Guidelines for the use of animals in research (86/609/CEE) and the requirements of Italian laws (D.L. 116/92, Directive 2010/63/EU). The ethical procedure has been approved by the Animal welfare office, Department of Public Health and Veterinary, Nutrition and Food Safety, General Management of Animal Care and Veterinary Drugs of the Italian Ministry of Health (Application number 32/08 of 7 July 2008; Approval number 744 of 9 January 2009). All the experiments were performed by authorized investigators. Dissected tissue were immediately freezed in liquid nitrongen and stored at -80°C.

Plasmids construction and oligonucleotides

Sequence coding for human TDP-43 (NM_007375.3) or human HDAC1 (NM_004964.2) were cloned in different expression vectors (pCS2-MTK, pCMV-3xFlag or pShuttle2) and used for site directed mutagenesis (QuickChange site-directed mutagenesis kit, Agilent). Mutants were obtained by mutagenesis starting from hTDP43, using the Quickchange site-directed mutagenesis kit (Stratagene) and primers indicated in the following table:

Mutation	Oligonucleotides
M337V	5'CAGTTGGGGTATGGTGGGCATGTTAGC 3'
A382T	5'AATTCTGGTGCAACAATTGGTTGGG3 3'
K145A-K192A	5' GATCTTAAGACTGGTCATTCAGCGGGGTTTGGCTTTGTTCG 3'
K145Q-K192Q	5' GCCTTTGAGAAGCAGACAAGTGTTTGTGGGGCGCTGTACAG 3'
K236E	5' TCCAATGCCGAACCTGAGCACAATAGCAATAGA 3'
D169G	5' TTCTCTCAGTAGGGGGTGTGAT 3'
ΔNterm	5' GAATTCAAGGCCTCTCGAGCCATAGTGTTGGGTCTCCCATGG 3'
ΔRRM1	5' CAGAAAACATCCGATTTAATAGTGCCTAATTCTAAGCAAAGC 3'
ΔRRM2	5' GAGCCTTTGAGAAGCAGAAAACACAATAGCAATAGACAG 3'
ΔG-rich	5' CAGTTAGAAAGAAGTGGAAGAGGAATGTAGCTCGAG 3'
ΔRRM1- ΔRRM2	5' CAGAAAACATCCGATTTAATAGTGCCTAATTCTAAGCAAAGC 3'

Table3: *Primer tablets used for mutagenesis from hTDP43.*

Site-directed mutagenesis was performed according to instruction manual (Agilent Technologies Inc, Stratagene#200518). PCR was performed using oligos indicated in table 3 and PFU DNA Polymerase (Promega, M7741).

PCR was performed as follows:

95 °C for 5‟

95° C for 3‟

54-56 °C for 30‟‟

72°C (1‟ for Kb)

} Repeated for 18 cycles

72° C for 10‟

PCR amplified DNA was DpnI digested (to disrupt non mutated DNA), and then transformed in competent DH5α cells. Positive clones were screened by sequencing.

Adenoviral vectors

All adenoviral vectors (*pAdenoX-hTDP43WT/Q331K/M337V/A382T/ΔRRM1-2/K145A-K192A/K145Q-K192Q and pAdenoX-hHDAC1*) were generated using the Adeno-X Expression System 1 (Clontech). Their production was completed in two stages. First, generation of mammalian expression cassette was done by cloning the gene of interest into pShuttle2. Second, excision of expression cassette from pShuttle2 and its insertion into I-Ceu I and PI-Sce I sites of BD Adeno-X Viral DNA was accomplished. All constructions were verified by automated sequencing.

Bacterial strain

Escherichia coli DH5-α were used as competent cells. Bacterial cells were defective for restriction and had mutations in relA1 and recA1 genes, to improve the stability and quality of recombinant plasmids.

Cell lines

SH-SY5Y cell line: SH-SY5Y cells (CRL-2266, ATCC, Rockville, MD) were human cells derived from neuroblastoma cell line.

Adeno-X 293 cell line: Adenovirus 5-transformed Human Embryonic Kidney 293 cell line (CRL 1573 HEK 293; ATCC, Rockville, MD,) was used to package and propagate the recombinant adenoviral- based vectors produced with the BD Adeno-X Expression System.

Cell culture

SH-SY5Y were cultured in Dulbecco MEM/F12 ground (Gibco BRL), *Adeno-X 293* in Dulbecco MEM (Gibco BRL), in presence of 10% fetal calf serum free of tetracycline contamination (Tet-free FCS, Clontech), previously inactivated at 56 °C for 30'minutes. Medium contained 100 units/ml of penicillin G and 100 µg/ml of streptomycin (Gibco

BRL). Cells were grown in an incubator at 37 °C, in (with) a humidified atmosphere containing 5% CO2. Trypsin (0.5 g/ml, 68 mM EDTA) was added to split cells and then diluted in fresh medium. Transient expression of each vector (2,5 µg DNA/1 × 10^6 cells) was obtained with Lipofectamine Plus reagent (Invitrogen) according to manufacturer's instructions. After an incubation of 4 h with transfection reagents, the cells were cultured in normal growth medium for 24 or 48 h. Trasduction with adenoviral particle with a MOI of 5 pfu/cell was performedaccording to[6]).

Standard techniques of molecular biology.

All standard techniques of molecular biology were performed according to *Molecular Cloning–A Laboratory Manual*[7].

Coimmunoprecipitation

Briefly, cultured cells were lysed with lysis buffer (120 mM NaCl, 50 mM Tris pH 7.5, 5 mM EDTA, 0.5% NP-40, and 1 mM freshly prepared PMSF), containing protease inhibitors (SIGMA P 8340). Cell lysates were immunoprecipitated, overnight at 4°C, with specific antibodies; immunocomplexes were then captured by incubating, for 16 hours at 4°C with continuous gentle shaking, with protein-A sepharose from *Staphylococcus aureus* (SIGMA P3391). Subsequently, immunocomplexes were analyzed by means of Western blotting, using specific antibodies.

SDS PAGE and Western immunoblotting

Protein content was determined using Bradford protein assay (27813 SIGMA). Equal amounts of protein extracts were resolved by standard SDS/PAGE. Samples were then electroblotted onto Protan nitrocellulose membranes (GE Healthcare Life Science). Afterwards, membranes were incubated in 3% low-fat milk, diluted in 1 X PBS-Tween 0.05% solution with the indicated antibody for 16 h at 4°C. Anti-Rabbit IgG (whole molecule)- and Anti-Mouse IgG (whole molecule)-Peroxidase antibody (EMD Millipore) were used to reveal immunocomplexes by enhanced chemioluminescence (Thermo

Fischer). The apparent molecular weight of proteins was determined by calibrating the blots with prestained molecular weight markers (Bio-Rad Laboratories).

2D electrophoresis analysis

Two-dimensional electrophoresis was been used to separate of proteins according to their isoelectric point (1st dimension) and, orthogonally, to their molecular weight (2nd dimension).

The samples were applied to 70 mm IPG strips (pH 4-7, Bio-Rad laboratories), by overnight rehydration loading at 20 ° C, and subsequently isoelectrofocused at 50 µA / IPG strip for 22 kVh at 20 ° C.

After focusing, proteins were in-gel reduced by incubating IPG strips with 50 mM Tris buffer containing 6 M Urea, 30% glycerol v / v, 3% SDS w / v and 1% DTT w / v, followed by in- gel alkylation with the same solution containing 2.5% iodoacetamide w / v, in place of DTT, under continuous shaking for 15 minutes before the second dimension. IPG strips were then sealed with 0.5% low melting point agarose w / v, in SDS running buffer, at top of second dimension gels (8 cm x 7 cm x 0.1 cm). SDS-PAGE was performed using 15% T, 3% C polyacrylamide gels at 50V for 15 minutes and subsequently at 150V for about 90 minutes. Later gels were subjected to western blot analysis, as described above.

Immunofluorescence

Cells were grown in 35 mm plates, fixed with 4% paraformaldehyde in 1 × PBS and permeabilized with 0.2% Triton X-100 in 1 × PBS. After a blocking step for 1 h in 5% BSA, diluted in 1 × PBS–0.05% Tween-20, cells were incubated with the primary antibody mouse anti-Myc (Sigma-Aldrich), diluted 1:10000 in blocking solution, overnight at 4°C, and then incubated with a secondary antibody Alexa Fluor®488 goat anti-mouse IgG (Life Technologies), diluted 1:1000 in blocking solution, for 1 h at room temperature. Cells were then analyzed with a Leica TCS SP5 confocal microscopy, with LAS lite 170 image software.

Chromatin Immunoprecipitation (ChIP)

SH-SY5Y cells (4 x 10^6) were plated 24 hours before transduction, and infected by using viruses encoding for TDP WT at a multiplicity of 30 pfu/cell. After 24 hours, cells were harvested and chromatin immunoprecipitation was performed using EZ-Magna ChIP™ (Millipore), according to the manufacturer's protocol.

Each immunoprecipitated (IP) reaction was performed using about 1x10^6 cells equivalents of chromatin. The antibodies used for immunoprecipitation were the following: TARDBP Polyclonal Antibody (Proteintech_10782-2-AP) and Normal Rabbit Ig (reagent supplied) as negative control. Purified chromatin was eluted and DNA fragments were used for qPCR.

Luciferase activity assay

The DDIT3 (DNA-damage-inducible transcript 3, gene-synonym CEBPZ, CHOP, GADD153[8]) promoter from -954 to +91 was cloned between the *XhoI* and *HindIII* sites in the pGLE-Basic Vector. All constructions were verified by automated sequencing. SH-SY5Y cells were seeded in 24-well plates and cultured for 16 h. Cells were then transfected by wild-type or mutant of TDP43, HDAC1 and luciferase constructs, in addition to a Renilla vector, used as an internal control for luciferase activity; transfected cells were further cultured for 48h. Luciferase assays were conducted using dual luciferase assay system (Promega). Each experiment was performed in triplicate.

Splicing assays

SH-SY5Y cells (8 x 10^5) were plated 24h before transduction and then infected by using viruses encoding for TDP WT or with A382T mutation and for HDAC1 at a multiplicity of 30 pfu/cell (plaque-forming unit/cell). After 48 hours, cells were harvested and total RNA was collected using Trizol Reagent (Invitrogen). Reverse transcription was performed using M-MLV Reverse Transcriptase (Promega), according to the manufacturer's protocol. PCR with DNA Polymerase (Promega) was carried out for 25 amplification cycles (95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds). To test the splicing pattern of endogenous gene POLDIP3/SKAR the following primers were used: forward (5'-GCTTAATGCCAGACCGGGAGTTGGA-3') and reverse (5'-

TCATCTTCATCCAGGTCATATAAATT-3'). To evaluate transduction efficiency and expression of exogenous TDP, both WT and mutant, and HADC1, PCR reaction was performed by using the following primers: TDP-forward (5'-AACAATCAAGGTAGTAATATGG-3'), TDP-reverse (5'-TGTAACCATTATAAGCTGCAA-3') and HDAC1-forward (5'-AAATGCAGGCGATTCCTGA-3'), HDAC1-reverse (5'-TGTAACCATTATAAGCTGCAA-3'). All PCR products were analyzed on 1.5% agarose gels.

Densitometric analyses for the quantification of agarose gels were performed using Quantity One software program (Bio Rad Laboratories).

qPCR

2µl aliquots of each sample was used in triplicate for qPCR analysis of the CHOP promoter. Thermal cycling was performed using a Rotor-Gene Q (Qiagen). SYBR Green (Invitrogen) was added at 1:10,000 dilution to each 20µl PCR reaction. qPCR was performed in triplicate for each input, control (IgG antibody) and immunoprecipitation sample (TDP antibody).

DNA in immunoprecipitated samples was quantified and relatively expressed by using control IgG samples set as 1.

Factor binding to the CHOP gene was determined to be significantly enriched using one-way ANOVA.

The primers used to perform the qPCR are the following: CHOP1-F (5'-GTGAAACGTAGTCTCGCTCTG -3') and CHOP1-R (5'- CCAGCTAATGGGCACATAGG-3'), CHOP2-F (5'- CCCAGTGGATGGATACCAAC-3') and CHOP2-R (5'- GTTTGGCAACCGGTGTCTG-3'), CHOP3-F (5'- CAGACACCGGTTGCCAAAC-3') and CHOP3-R (5'-GCCTTAGACTTAAGTCTCTGACC-3'), CHOP4-F (5'- CTCCAGGGTTCAAGCGATTCT-3') and CHOP4-R (5'- AGCGGATCACTTGAGGTCAG-3'), CHOP5-F (5'- AACGGCGGGTAAAGCTAGG-3') and CHOP5-R (5'- GTGGGGGAGAGGAGAGAGG-3'), CHOP6-F (5'-ACATTGCATCATCCCCGC-3') and CHOP6-R (5'- TCGCTCCCTCTCGCTAGG-3').

Silencing histone deacetylases1 (HDAC1) gene by RNA interference.

SH-SY5Y cells (1 x 10^5) were seeded 24 hours before the first transfection with the small interfering RNA (siRNA) oligonucleotide specific for HDAC1 gene. Lipofectamine 3000 reagent (Lipofectamine® 3000, Invitrogen by Life Technologies) was combined with Optimem medium (Promega) (reaction 1); meanwhile, in a different tube, 148 pmolof HDAC1 siRNA and 500 ng of TDP, WT or mutant, were mixed with Optimem (reaction 2). Both reactions were mixed and left for 5 minutes at room temperature. Afterwards, the mix was added to the cells and incubated at 37°C and 5% of CO_2. After 48 hours the transfection procedure was repeated only for HDAC1 siRNA, and cells were incubated at 37°C and 5% of CO_2 for other 24 houres. After 72 hours a MTS assay was performed, and cells were finally washed 2 times with PBS; subsequently, cell lysates were subjected to western blot analysis with anti-HDAC1 antibodies to evaluate expression level.

Design of targeting components and the use of the CRISPR Design Tool

The web interface of CRISPR Design Tool (http://tools.genome-engineering.org) was used to develop gRNAs, listed belowe.

gRNA1 HDAC1F	**caccgTGAGTCATGCGGATTCGGTG**
gRNA1 HDAC1R	aaacCACCGAATCCGCATGACTCAc
gRNA3 HDAC1F	caccgGATACCAGAGATGGCTTTTT
gRNA3 HDAC1R	aaacAAAAAGCCATCTCTGGTATCc
gRNA6 HDAC1F	caccgCCCAATGAAGCCTCACCGAA
gRNA6 HDAC1R	aaacTTCGGTGAGGCTTCATTGGGc

Table 4. *The oligo table used for creating the CRISP/Cas9 constructs.*

Off-target activity was evaluated additionally with Blastn (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

The pSpCas9(BB)-2A-Puro (Addgene # 48139) that expresses the Streptococcus pyogenes Cas9 (including an NLS and a FLAG tag) from a CAG promoter, and has a U6 promoter driven gRNA was used as cloning backbone according to (ran et al., 2013 Nature Protocols 8, 2281–2308). Briefly, phosphorylation and annealing was performed with the three

couple of oligos, mentioned above, harboring a BbsI overhang. Afterwards, BbsI (#FD1014, ThermoFisher) mediated digestion and T4 DNA ligase (#M0318L, NEB) directed ligation in the linearized pSpCas9(BB)-2A-Puro was performed. After the transformation, cloning has been verified with a control PCR with the primers in the table. Plasmids were purified and sequenced. After transfection of the indicated combinations of pSpCas9(BB)-2A-Puro-gRNAs (Fig. 1B), positive cells were selected using puromycin (2 μg mL−1) for five days prior to clonal expansion. Empty pSpCas9(BB)-2A-Puro was used as negative control.

HDAC inhibitors

To inhibit HDAC1 activity, we used : Sodium phenil butyrate (SML0309, Sigma-Aldrich), Trichostatin A (T8552, Sigma-Aldrich), Sodium butyrate (B5887, Sigma-Aldrich), Valproic acid sodium salt(P4543, Sigma-Aldrich).

Sodium Butyrate was weighted, solved in water at a stock concentration of 10 mM, and then diluted at the final concentration of 0,2 and 0,04 mM. Trichostatin A was solved in ethanol, at the stock concentration of 1 mg/ml, and then diluted at the final concentration of 10 and 25 nM. We plated target cells (SH-SY5Y or CRISP-Cas9 HDAC1 clones) in culture plates desired 12–24 hours before infection. The next day, we removed the growth medium and infected target cells diluting viruses at a multiplicity of between 5–30 pfu/cell in OPTIMEM (Thermo Fisher Scientific). We then incubated the cells in a humidified CO_2 (5%) incubator at 37°C for 1 hour, to allow virus to infect the cells. Then, we added fresh complete growth medium and incubated in a humidified 5% CO_2 at 37°C to allow the expression of the adenoviral construct. We analyzed gene expression at different time points, previously decided, and then we proceeded with further experimental manipulations.

Viability of both control SH-SY5Y cells or SH-SY5Y cells infected with adenoviruses encoding for TDP-43-causative gene, was calculated after 72 hours, through an MTS assay. Cell viability was assessed by a colorimetric assay using 3(4,5-dimethylthiazol-2yl)-5-(3-carboxymethoxyphenyl)-2-(4- sulfophenyl)-2H-tetrazolium (MTS) assay (Cell Titer 96 Aqueous One Solution Assay, Promega), according to manufacturer's instructions. Absorbance at 490 nm was measured in a multilabel counter (Victor X5, PerkinElmer).

DNA Damage

SH-SY5Y cells (1 x 10^5) were plated 24 hours before transduction, and infected by using viruses encoding for TDP WT, M337V, A382T, ΔRRm1-2, KK-AA and KK-QQ at a multiplicity of 40 pfu/cell .

48 hours after infection, for UV irradiation, cells were treated with UV-C (254 nm) using a lowpressure mercury lamp, and the cells were either subjected to global (5 J/m2).

After the microirradiation, cells were incubated for 4 hours at 37 °C, in a humidified atmosphere containing 5% CO_2. All slides are processed successfully by assaying the previously mentioned immunofluorescence protocol and analyzed by confocal microscopy.

Statistical analysis

The results are presented as means ± S.D. of n≥3 independent experiments. Statistical evaluation was conducted by one-way or two-way ANOVA and Bonferroni post test. Values significantly different from the relative control are indicated with an symbol: *p<0,05; **p<0,01; ***p<0,001 or § p<0,05; §§ p<0,01; §§§ p<0,001.

RESULTS

TDP-43 interacts with HDAC1 in vitro and in vivo, via RNA binding domains

Preliminary experiments performed in our laboratory (Masala A., PhD thesis 2016) have demonstrated that, in transfected SH-SY5Y cells, TDP-43 can interacts with HDAC1, in a similar way as it has been demonstrated for FUS[9]. To extend this result to more physiological conditions, co-immunoprecipation from mice neuronal tissues, using an anti-TDP-43 antibody, was performed. As shown in Figure 9A, a strong and specific interaction was observed between TDP-43 and HDAC1 in different neuronal tissues, but especially in the spinal cord.

In order to extend these results, we performed a battery of co-immunoprepitation experiments. HEK293 cells were transfected with HDAC1 fused with Flag-tag in association with WT or pathological mutant TDP-43 fused with Myc-tag and Myc-tagged FUS as positive control. We confirmed the interaction between FUS and HDAC1, and moreover TDP-43 interacts with HDAC1, independently from the pathogenic TDP-43 variants M337V and A382T (Figure 9B-C).

To characterize the domain(s) of TDP-43 responsible for the TDP-43/HDAC1 interaction, we generated a series of Myc-tagged TDP-43 fragments representing the various putative functional domains of the protein (N-terminal, RRM1, RRM2 and G-rich domain). Co-immunoprecipitation experiments, performed on cell lysates from transfect HEK293 cells, demonstrate that TDP-43 interacts with HDAC1 via both RRM1 and RRM2 domains, and that only in the double deletion mutant the interaction is abolished (Figure 9D).

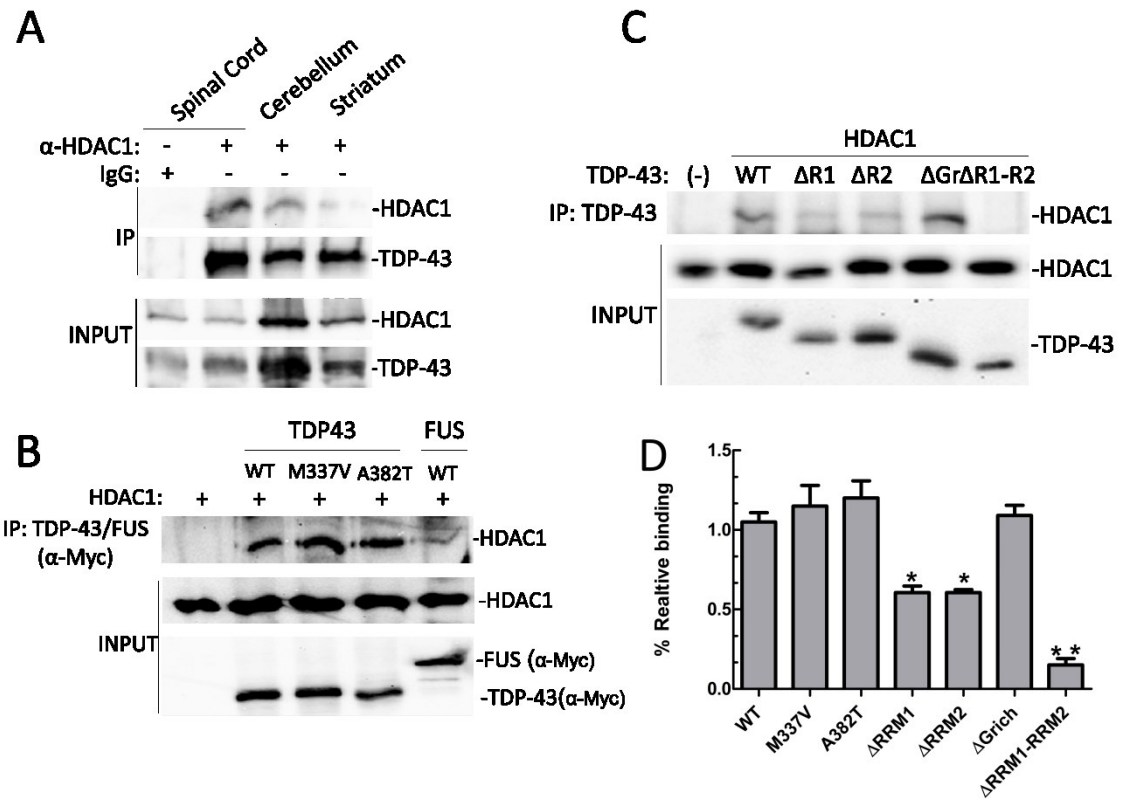


Figure 9. TDP43 interacts with TDP43 in vivo and in vitro via RRM1 and RRM2 domains.

*A) Spinal cord, striatum cerebellum of BALB31c mice were used for co-immunoprecipitation experimets using α-TDP-43; B)HEK 293T cells were transiently transfected with Myc-tagged TDP43 WT or deletion mutant expression constructs (ΔRRM1, ΔRRM2, ΔG-rich, ΔRRM1/RRM2) and FLAG-taggedHDCA1; C) HEK 293T cells were transiently transfected with myc-tagged TDP-43 WT or mutant expression constructs (M337V or A382T) and FLAG-tagged HDCA1; D) Bar graph shows the relative binding of HDAC1 to mutantTDP-43, normalized to TDP-43 WT. The data were obtained from four independent experiments; *indicates p > 0.05 and **indicates p > 0.01 versus WT binding, analyzed with one-way ANOVA.*

RMM1 and RRM2 domains have been shown to be crucial in TDP-43 physiopathology. They are necessary for regulation of alternative splicing and deletion of RRM-1 domain alters TDP-43 translocation in the nucleus[5,10]. Moreover the two major TDP-43 acetylation sites, K145 and K192[5], and two of the three TDP-43 pathological mutants that are out of the Grich domain, ALS linked mutation D169G and FTLD-TDP linked mutation K263E[11], are localized in this regions. Plasmids contructions coding for these mutants (D169G, K263E, RRM1-2, KK-AA, KK-QQ) were generated, transiently expressed in SH-SY5Y cells and analyzed by immunofluorescence (Figure 10), indicating that the localization of these

mutatedproteins is exclusively nuclear, with the exception of TDP-43 D169G which is also located partially in the cytoplasm. Moreover according to previous published results, Plasmids contructions coding for these mutants (D169G, K263E, RRM1-2, KK-AA, KK-QQ) were generated, transiently expressed in SH-SY5Y cells an

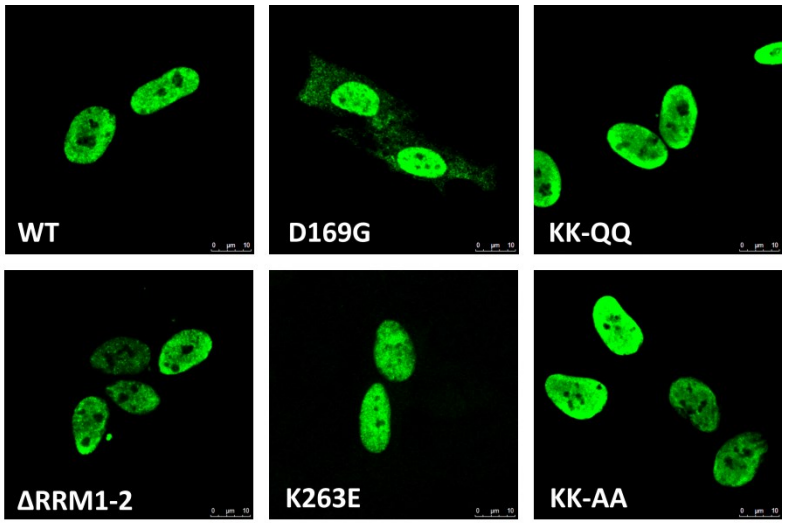


Figure 10. Evaluation of TDP-43 WT subcellular localization and some mutants*. (A) SH-SY5Y were transfected with the TDP-43 WT, D169G, K263E, RRM1-2, KK-AA,KK-QQ constructs and analyzed 48 hours later by immunofluorescence. The TDP-43 signal was revealed by primary anti-myc antibodies and anti-mouse ALEXA 488 secondary. The slides were analyzed by Leica confocal microscope.*

Co-immunoprecipitation experiments were performed, using the indicated mutants as described in Figure 9 and only the acetylation mimic mutant (KK-QQ) displays a significant decrease in HDAC1 binding (Figure 11A-11B).

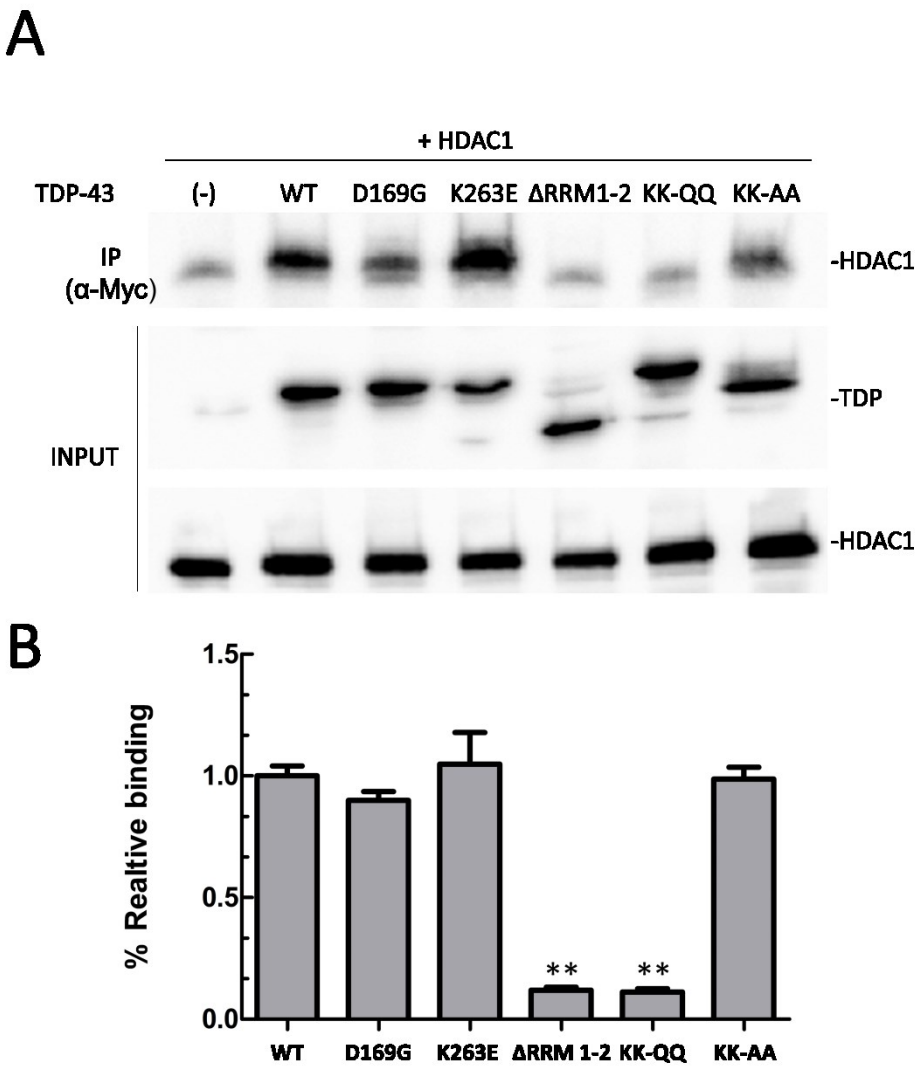


Figure 11. Coimmunoprecipitation between TDP-43 mutants and HDAC1. *A) HEK293T cells were transiently transfected with myc-tagged TDP-43 WT or mutant expression constructs (D169G, K263E, ΔRRM1-2, KK-AA or KK-QQ) and FLAG-tagged HDCA1; B) Bar graph shows the relative binding of HDAC1 to mutant TDP-43, normalized to TDP-43 WT. The data were obtained from four independent experiments; **indicates p > 0.01 versus WT binding, analyzed with one-way ANOVA.*

HDCA1 modulates TDP-43 acetylation

Acetylated-TDP-43 was been demonstrated to be an HDAC6 substrate[5]. In order to investigate if also HDAC1 overexpression can alter TDP-43 acetylation, we performed a 2D

gel-analysis of immunoprecipitated TDP-43. SH-SY5Y cells were transfected with TDP-43 alone or in combination with HDAC1 and 48h after transfection, TDP-43 was immunoprecipitated, separated by isoelectro-focusing (pH range comprised between 3 and 6) followed by SDS/PAGE. The level of TDP-43 acetylation was evaluated using anti-acetyl Lysine antibody (Figure 12).

The co-transfection with HDCA1, as well as the one with HDAC6, that it has been shown to remove acetyl group(s) from acetylated targets, shifts the isoelectric point (pI) of immunoprecipitated TDP-43. This effect is partially prevented by deletion of RRM1-RRM2 domains, confirming, as previously demonstrated, that K145 and K192 are not the only acetylated residues on TDP-43 and demonstrating that the TDP-43 interaction domain with HDAC1 is necessary for the deacetylation to occur.

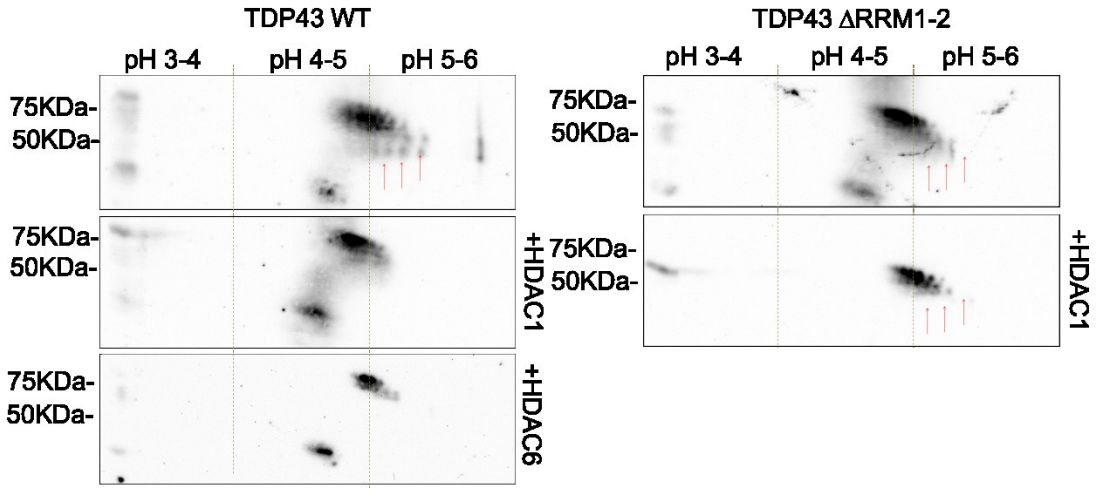


Figure 12. 2-DE maps reporting TDP-43 acetylation level after immunoprecipitation. *Representative 2-DE maps showing the change in TDP-43 acetylation status, revealed by using an anti-acetyl lysine antibody, after immunoprecipitation with anti-Myc from HEK293 transfected with TDP-43, HDAC1 or HDAC6.*

Adenoviral delivery of TDP-43 and HDAC1

In order to study TDP-43 physiopathology, in relationship with HDAC1, we decided to use an adenovirus-based transient expression system, since recombinant adenoviral

expression vectors exploit the high nuclear transfer efficiency and the low pathogenicity of the virus to deliver genes to the host cell (*Adeno-X* Expression *System,* Clontech). The assembly of recombinant adenoviruses has been completed in few stages: first, the cDNA encoding for HDCA1, TDP-43 WT, pathological mutants M337V and A382T, mutants K145A-K192A (KK-AA), K192Q-K192Q (KK-QQ) or ΔRRM1-2 were cloned into pShuttle2 plasmid DNA. Second, the expression cassette was excised from recombinant pShuttle2 vector by digestion with I-*Ceu* I and PI-*Sce* I, ligated into pAdeno-X and selected. Third, the recombinant adenoviral constructs were cleaved by *Pac*I to expose the inverted terminal repeats (ITR) and transfected into HEK-293 AdenoX cell lines. After 7–10 days, viruses were harvested and amplified by infecting packaging cells for three times to obtain high titer virus stocks. The final yields were evaluated performing an end-point dilution assay. To characterize TDP-43 adenoviral expression vectors, recombinant adenoviruses were used to transduce SH-SY5Y cells with a scalar multiplicity of infection (M.O.I.): 5-40 pfu/cell. Forty-eight hours after transduction, cells were lysed and protein extracts analysed by Western Blot. As shown in Figure 5 all adenoviral preparations are able to produce a high level of expression of TDP-43 (Figure A and B) and HDAC1 (Figure C) in SH-SY5Y cell lines. Interestingly only the pathological mutants, and at a less extend WT TDP-43, induce reduction in cell vitality at low MOI. The decrease in cell viability observed with the other mutants and with HDAC1 can be observed only with a MOI of 40 pfu/cell and it is likely due to adenoviral toxicity itself.

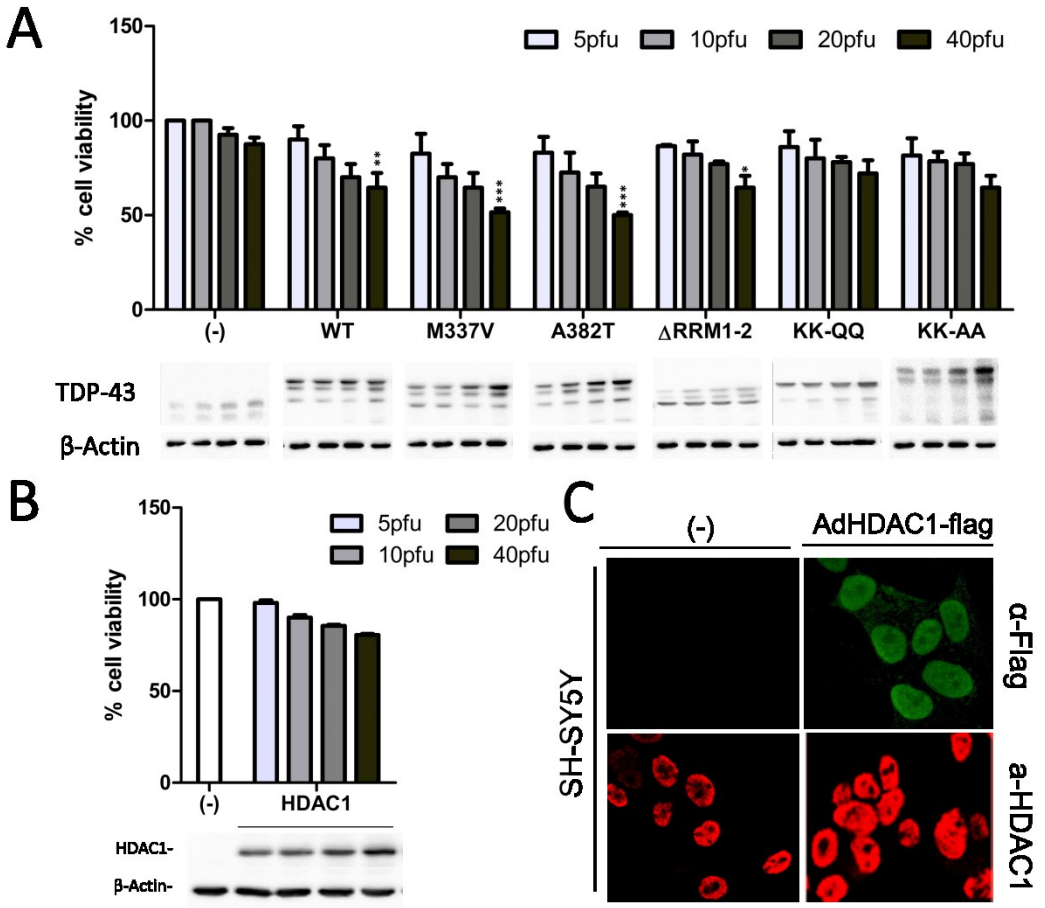


Figure 13. Effect of adenoviral delivery of WT or mutant TDP43 and HDAC1 on SH-SY5Y cell viability. *A) MTS assay on SH-SY5Y cells transduced by TDP-43 WT, M337V, A382T, ΔRRM1-2, KK-AA and KK-QQ adenoviruses, at a final concentration of 5, 10, 20 and 40 PFU/cell, and analyzed 48 hours after transduction. Cell lysates were subjected to reducing SDS-PAGE and western blot. The anti-TDP-43 antibody was used to visualize TDP-43 expression level and β-actin serves as controls for equal loading of samples. B) MTS assay on SH-SY5Y cells transduced by HDAC1 adenoviruses, at a final concentration of 5, 10, 20 and 40 PFU/cell and analyzed 48 hours after transduction. Cell lysates were subjected to reducing SDS-PAGE and western blot. The anti-HDAC1 antibody was used to visualize HDAC1 expression level and β-actin serves as controls for equal loading of samples. C) The HDAC1 was detected by anti-HDAC1 antigen and secondary anti-rabbit ALEXA 647 or anti-Flag antigen and secondary anti-mouse ALEXA 488. The slides were analyzed by Leica confocal microscope. The data were obtained from four independent experiments; ** indicates p > 0.01 versus WT binding, analyzed with one-way ANOVA.*

HDAC1 is not delocalized in the cytoplasm upon TDP-43 expression

HDAC1, like most HDCAs, is a nuclear enzyme that can be rilocalised in the cytosol in damaged brain axons with multiple sclerosis, exemplary demyelinating models and in cultured neurons exposed to glutamate and TNF-α. More recently, during development of the Xenopus brain, HDAC1 was observed in both neurons and mitochondria of developing neurons[12]. TDP-43 instead is predominantly nuclear, although in fibrillary conditions it may move to the cytoplasm, causing a loss of function that results in detrimental effects on RNA metabolism. In pathological conditions, where TDP-43 is varied (hyperphosphorylated, ubiquitined, acetylated) and prompted to generate C-terminal fragments in the brain and spinal cord of frontotemporal lobo degeneration with ubiquitinated inclusions[13].

We therefore decided to test in our experimental models the subcellular localization of HDAC1 in the presence of different TDP-43 mutants. As shown in Figure 14, TDP-43, regardless of the excessive expression of HDAC1, still shows a nuclear location.

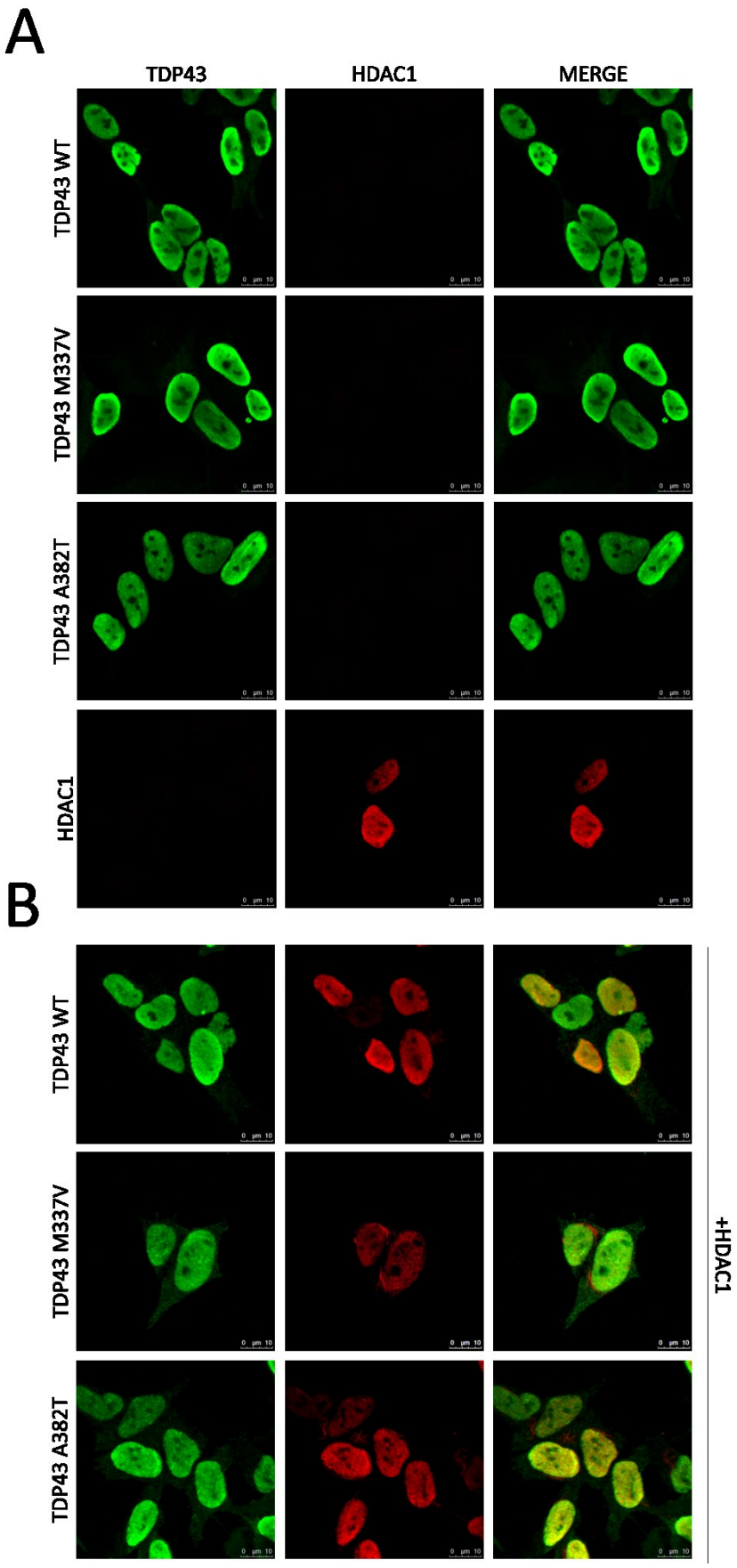

A
TDP43
HDAC1
MERGE
TDP43 WT
TDP43 M337V
TDP43 A382T
HDAC1
B
TDP43 WT
TDP43 M337V
TDP43 A382T
+HDAC1

Figure 14. Immunofluorescence analysis of TDP43 WT and deletion mutants localization and HDAC1 in SH-SY5Y cells: *A) SH-SY5Y cells were transduced with TDP-43 WT, M337V or A382T, without or B) with HDAC1. The TDP-43 signal was revealed by primary anti-myc antibodies and anti-mouse ALEXA 488 secondary, HDAC1 was detected by anti-HDAC1 antigen and secondary anti-rabbit ALEXA 647. The slides were analyzed by Leica confocal microscope.*

Physiological relevance of TDP-43/HDCA1 interaction: HDCA1 modulates TDP-43 transcriptional activity

TDP-43 have been originally described as transcription factor for TAR DNA of HIV1[14], but at present the only other target on DNA is the testis specific mouse acrv1 (SP10) promoter[15]. TDP-43 has been also shown to induce a transcriptional up regulation of C/EBP-homologous protein (CHOP) promoter and the genetic disruption of the CHOP gene markedly attenuated TDP-43-induced cell death[16]. Moreover, the TDP-43-induced up regulation of CHOP expression is mediated by both reduction of CHOP degradation and by up regulation of CHOP mRNA level. Thus, we decided to use CHOP promoter (from -954 to +91) to drive the expression of luciferase reporter and to test the ability of WT and TDP-43 pathological mutants of regulating this promoter.

48h after transfection with the indicated plasmid (Figure 15), SH-SY5Y cells were assayed for luciferase activity in a multiplate reader using the Dual-GlowTM Luciferase Assay System (Promega, USA). Firefly luciferase activity was then normalized to the Renilla luciferase activity to control the transfection efficiency. Data were then normalized to luciferase activity in cells transfected with empty vector, which was given a value of 100%. As show in Figure 15 TDP-43 can act as robust activator of CHOP promoter. Transcriptional activation on this promoter was reduced when pathological mutants (A382T) are expressed and abolished by RRM1-RRM2 deletion.

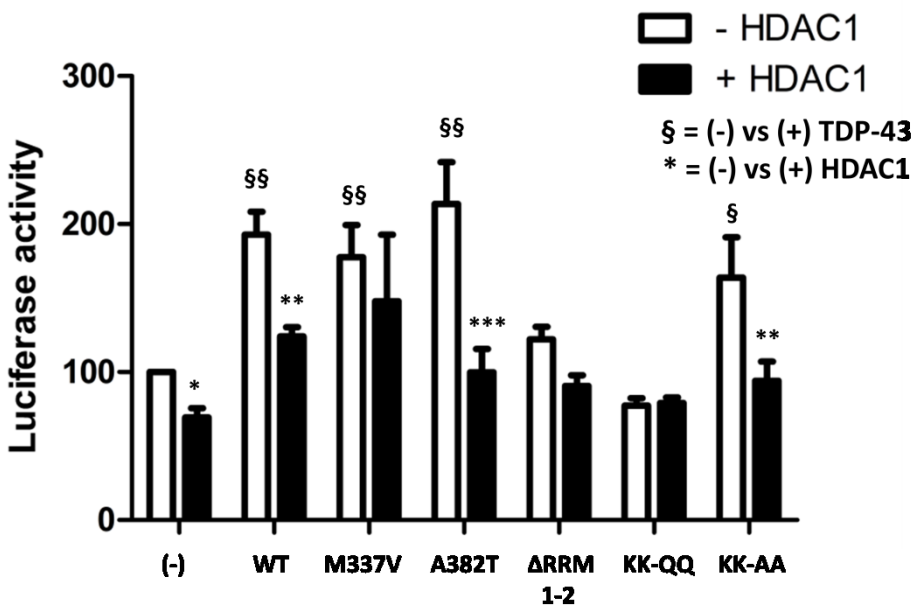


Figure 15. HDAC1 overpression interferes with transcriptional activity TDP 43 on CHOP*: The graph shows the data obtained from the luciferase assay performed on SH-SY5Y transfected with TDP-43 WT and different mutations, with or without HDAC1, showing that the presence of HDCA1 determines a reduction in CHOP transcriptional activation due to WT or mutated TDP43 expression, while this effect is no longer visible in the presence of the deletion mutant ΔRRM1/2. The data were obtained from four independent experiments; **indicates p> 0.01 and *** p > 0.001 versus HDAC1 expression, and indicates § > 0.05 and *** , § § > 0.01 versus TDP-43 expression; analyzed with two-way ANOVA.*

Since HDAC1 is a key component of an intricate network of acetylated substrates involved in transcription, not surprising, its overexpression induces a reduction of TDP-43 induced luciferase activity. Most importantly, also TDP-43 acetylation itself seems to be relevant for CHOP transcriptional activity since overexpression of the acetylation mimicking variant (KK-QQ), but not the non-acetylable mutant (KK-AA) impairs luciferases activity independently from HDAC1 overexpression.

These experimental evidence indicate a possible direct interaction between TDP-43 and CHOP promoter, which we confirm by ChIP approach. In a first attempt we tested the CHOP promoter activation in response to sodium arsenite, using as marker of transcriptional activation the dual modification of histone H3 phosphorylated at serine 10 and acetylated at lysine 14 (H3-PS10/AcK14). As expected CHOP transcriptional activation is accomplished by histone H3 phosho-acetylation (Figure 16).

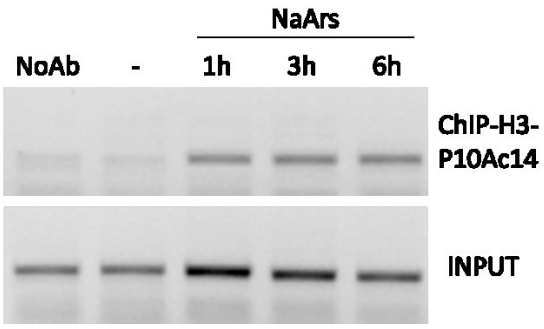


Figure 16. Setting the ChIP on SH-SY5Y:*The Figure shows the result of the ChIP performed on SH-SY5Y cells treated with Sodium Arsenite (10μM) for 1, 3 or 6 hours to evaluate the activation of the CHOP promoter in response to the treatment using an activation marker transcriptional such as the double modification of Hydroxy H3 phosphorylated to serine 10 and lysine 14 acetylated (H3-PS10 / AcK14).*

We, thus decided to transduce SH-SY5Y cells with adenoviral particle coding for 5xMyc-TDP-43 and After 24 hours chromatin was extracted and immunoprecipitated using anti-TDP-43 or anti-IgG as a negative control. As shown in Figure 17, we have been able to demonstrate that TDP-43 binds directly to the CHOP promoter by first amplifying a larger portion by using the pair indicated in materials and methods such as CHOP3-R and CHOP3-F, thus generating an amplifier of about 600 pb, then switch to the primer pair CHOP6-R and CHOP6-F, for qPCR, which amplify the reduced portion of the putative binding site of TDP-43 to CHOP.

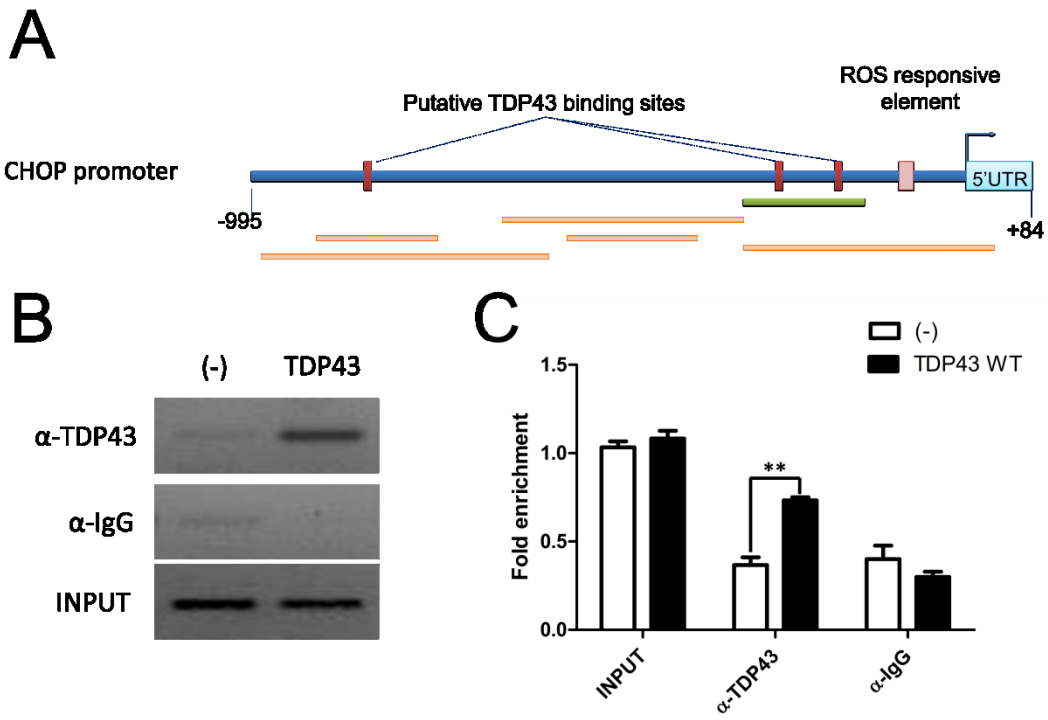


Figure 17. Identification of TDP43 binding sites on CHOP promoter. *A) To test the TDP-43 binding, we designed 6 pairs of primers that amplify several portions of the putative TDP-43 binding region on the CHOP promoter. Among these, the primer pair, CHOP 3 (indicated in the materials and methods), was selected, thus obtaining the amplified green region indicated in the figure used. B) The image shows the result of the agarose gel electrophoresis, of the analyzed samples qPCR after chromatin immunoassay. C) Actual banding was evaluated with data obtained from the qPCR and collected in the graph. The data were obtained from four independent experiments; ** indicates p> 0.015 for the fold enrichment analyzed with one-way ANOVA.*

TDP-43 splicing activity on POLIDP3 mRNA is modulated by acetylation levels, but not by HDCA1 overexpression

Since it has been demonstated that the acetylation-mimetic TDP-43 mutant shows a reduction in the RNA binding and splicing functionality on CFTR transcripts[17], we have examined in depth if HDCA1 overexpression may alter the TDP-43 splicing activity. As a target of TDP-43, we decide to monitor the inclusion of exon 3 of the interrelated polymerase delta [POLDIP3] mRNA, that has been demonstrated to be excluded by depletion of TDP-43[18,19]. RT-PCR experiments performed on SH-SY5Y cells demonstrate that, in our experimental systems, overexpression of TDP-43 WT or bearing the

pathological mutation A382T and the non-acetylable KK-AA, stimulates POLDIP3 exon 3 exclusion (increase of variant 2), effect that is exacerbated by the lack of RRM1-2 domains. Surprisingly both the acetylation mimic KK-QQ induce a comparable exon 3 exclusion, favouring a scenario in which the TDP-43 overexpression is altering the splicing pattern independently from any acetylation status. HDCA1 overexpression do not alter this phenomena in all the experimental conditions, althought we observed a genereal downregulation of exon3 exclusion in presence of HDAC1.

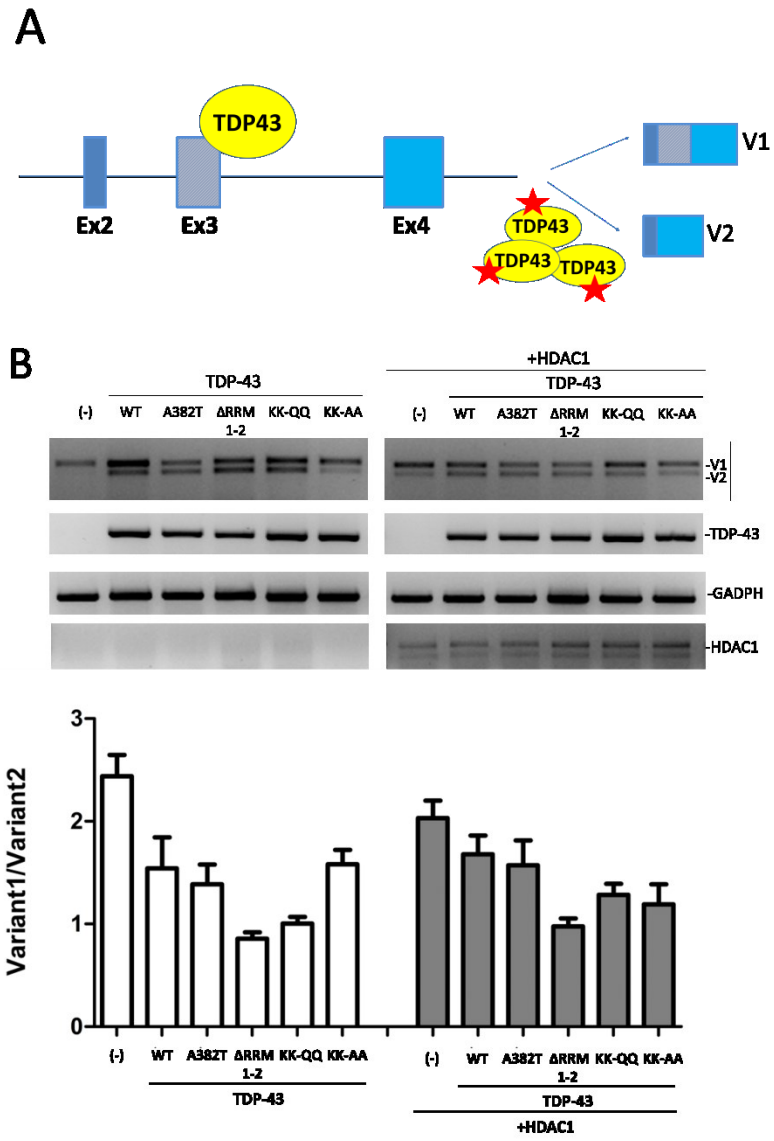


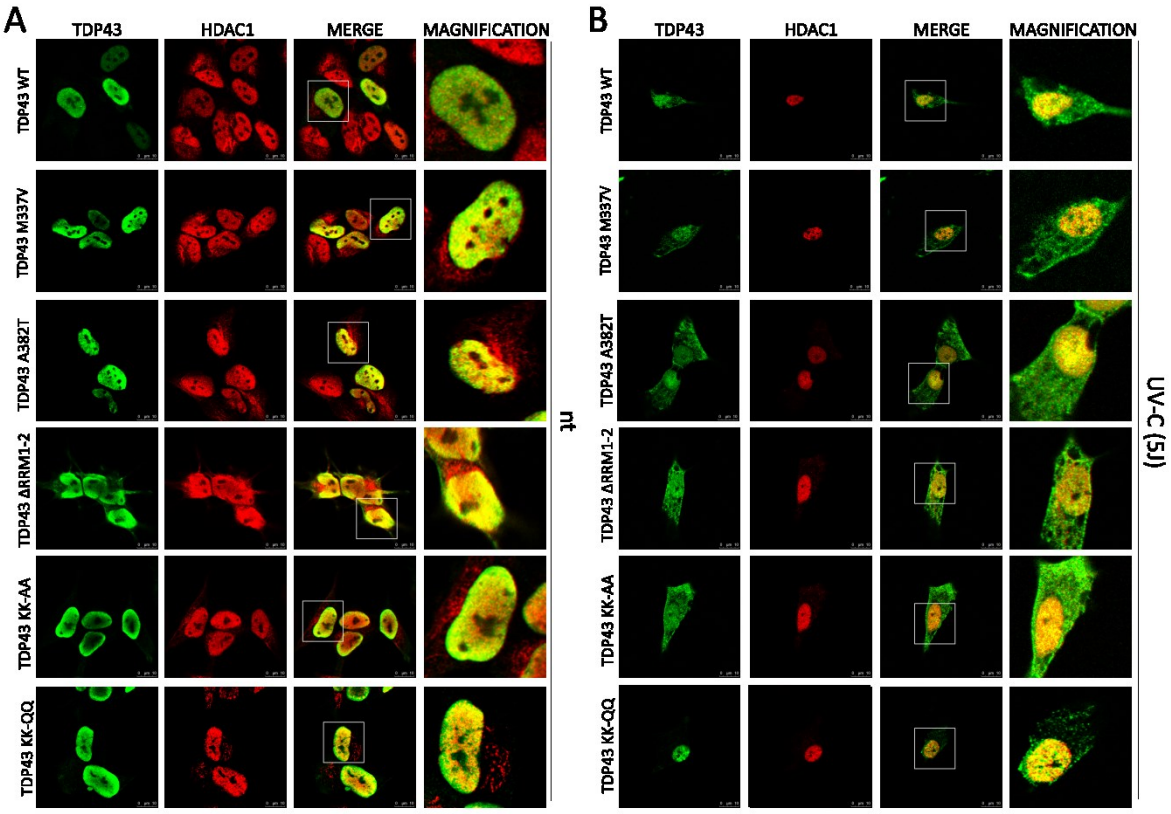
Figure 18. TDP-43 splicing activity on POLIDP3 mRNA: *A) Splicing diagram following the over-expression of TDP-43. B) SH-SY5Y cells transfected with TDP-43 WT and different mutants, with or without HDAc1 (C) Quantification of data obtained in B.*

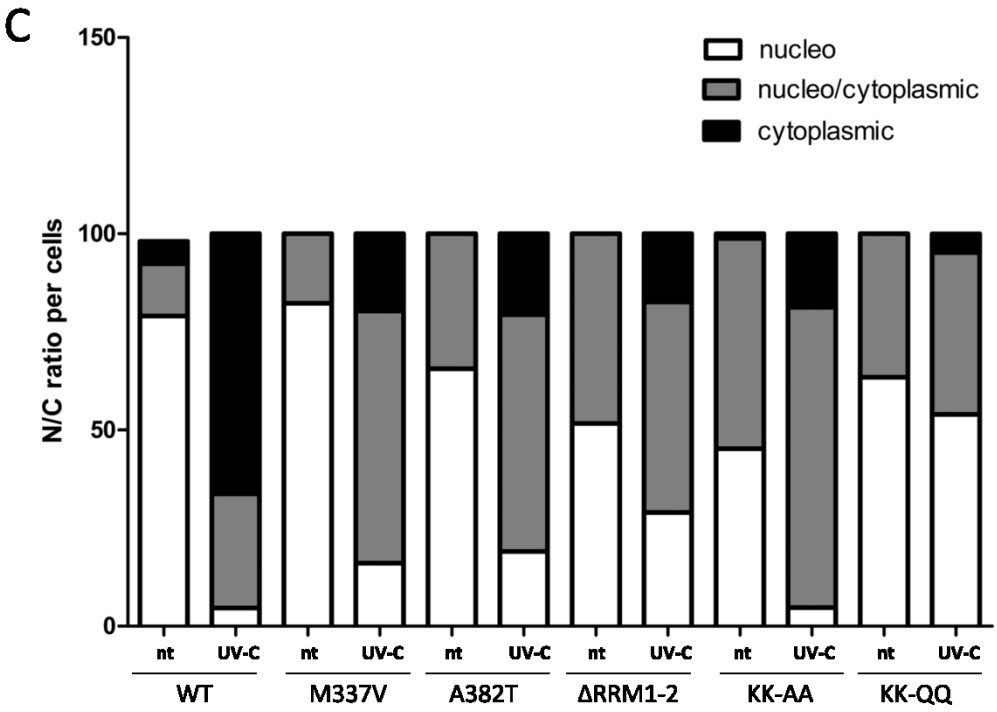
TDP 43 and HDCA1, their role in responding to DNA damage

FUS and TDP-43 have been shown to play a key role in the response to DNA damage, since the loss of function of one of these two proteins results in a faulty repair of DNA damage associated with stopping in transcription[20]. Based on these data, we assessed whether the presence of HDAC1 could interfere with this specific TDP43 activity. To determine whether TDP-43 together with HDAC1 is localized at the DNA damage foci, we induce double strand brecks using UV-C rays on SH-SY5Y previously transduced with TDP-43 WT or bearing different mutations. Immunofluorescence analysis demonstrates that, in irradiated cells, TDP-43 and HDCA1 colocalized in small spots, where, perhaps jointly, are involved in repair of DNA damage.

In addition, by cell count we quantified the localization of TDP-43 following treatment with UV-C. TDP-43 WT as well as the pathological mutants, relocalized in the cytoplasm (figure 19 B). Only the acetylation mimicking mutant is retaine in the nucleus at the same extend in both treated and untreated cells KK-QQ.

These data are also supported by the evaluation of cell viability following treatment (Figure 19 D), in fact, only with pathogenic mutants M337V and A382T there is a slight survival due to the loss of function due to mutated TDP-43 subtraction , which accumulates in the inclusion bodies.


A
TDP43
HDAC1
MERGE
MAGNIFICATION
TDP43 WT
TDP43 M337V
TDP43 A382T
TDP43 ΔRRM1-2
TDP43 KK-AA
TDP43 KK-QQ
nt
B
TDP43
HDAC1
MERGE
MAGNIFICATION
TDP43 WT
TDP43 M337V
TDP43 A382T
TDP43 ΔRRM1-2
TDP43 KK-AA
TDP43 KK-QQ
UV-C (5J)


C
nucleo
nucleo/cytoplasmic
cytoplasmic
N/C ratio per cells
150
100
50
0
nt
UV-C
WT
M337V
A382T
ΔRRM1-2
KK-AA
KK-QQ

D

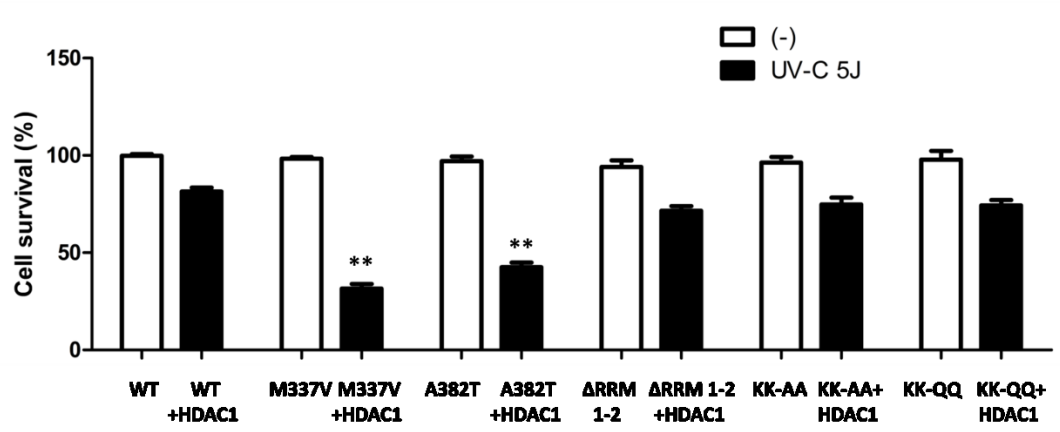


Figure 19. WT and mutant TDP-43 colocalize with HDCA1 at DNA damaged foci. *A) and B) SH-SY5Y transduced cells with TDP-43 WT and mutants treated with UV-C and analysed for TDP-43 and HDAC1 localization. The TDP-43 signal was revealed by primary anti-TDP-43 antibodies and anti-mouse ALEXA 488 secondary, HDAC1 was detected by anti-HDAC1 antigen and secondary anti-rabbit ALEXA 647. The slides were analyzed by Leica confocal microscope. C) The graph summarizes the image analysis, which reveals how TDP-43 becomes more cytoplasmic after DNA damage caused by UV-C exposure. D) The graph shows that as a result of UV-C exposure, there is less cellular survival in the specimens expressing the mutant M337V and A382T points. The data were obtained from four independent experiments; ** indicates p> 0.01 for the cell survival analyzed with one-way ANOVA.*

TDP-43 and HDAC1 have a synergistic effect in decreasing cell vitality

As described in the previous paragraph, WT or pathological mutant TDP-43 overexpression is neurotoxic, inducing a reduction in cell survival, according to what it has been described in many other cellular and animal systems[21-23]. We thus decided to evaluate the effect of HDAC1 expression level or activity perturbation on TDP-43 induced cell toxicity.

Data obtained in our laboratory have shown that HDAC1 stable overexpression in SH-SY5Y cells has a detrimental effect on TDP-43 mutated cell damage (Masala A. PhD thesis 2016). Then we decided to manipulate the expression or the activity of HDAC1 to study whether this could have any effect on cellular mortality induced by the overpression of TDP-43.

In order to knockdown HDAC1 we decided to use CRISPR/Cas9 genome editing technique in SH-SY5Y cells. To generate stable cell lines in which the expression of HDAC1 was blocked, SH-SY5Y were transfected with a plasmid coding for the humanized Cas9 and different gRNA targeting the second exon of HDAC1 gene. After the selection by puromycin, single stable clones were isolated and analyzed for HDAC1 expression (Figure 20A and B), leading to the identification of two clones in which HDAC1 protein expression was absent (Figure 20B). The two clones were then further analyzed by immunofluorescence (Figure 20 C) and sequencing.

A

Type of clone	Number of clones analyzed	Number of positive clones
HDAC1 gRNA 1	26	1
HDAC1 gRNA 3	23	0
HDAC1 gRNA 8	22	1
HDAC1 gRNA 1-3	19	0
HDAC1 gRNA 3-8	23	0

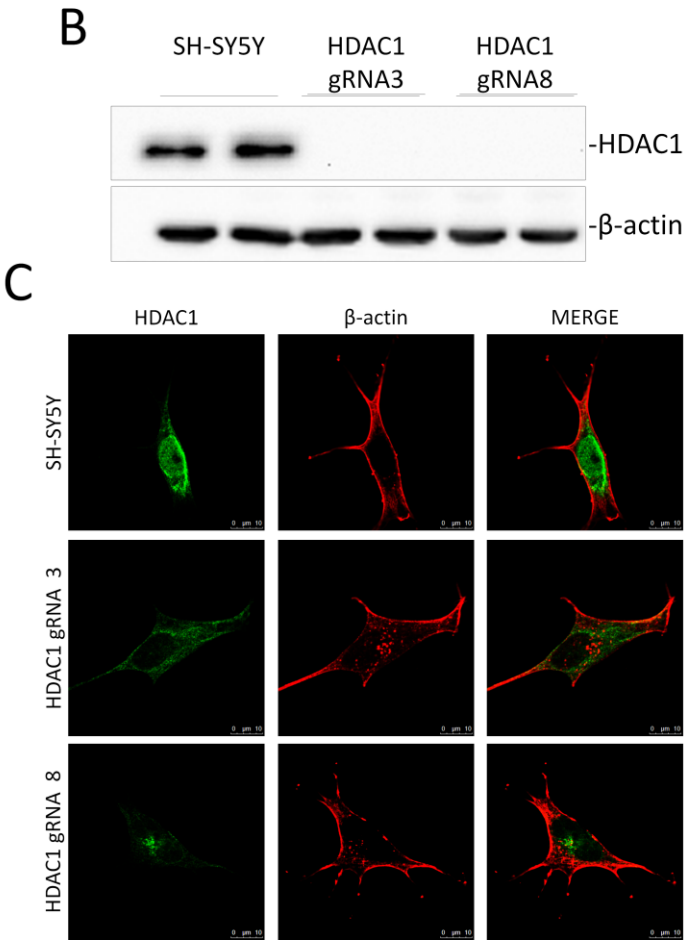


Figure 20. CRISPR/Cas9 genome modification of HDAC1 gene in SH-SY5Y cells: *A) Summary of monoclonal lines generated by CRISPR-Cas9; B) Stable CRISP-Cas9 HDAC1 clones were analyzed to evaluate HDAC1 expression levels by SDS-PAGE and Western blot using an anti-HDAC1 and (D) The HDAC1 signal was revealed by primary anti-HDAC1 antibodies and anti-rabbit ALEXA 488 secondary, β-actin was detected by anti-β-actin antigen and secondary anti-mouse ALEXA 647. The slides were analyzed by Leica confocal microscope.*

The two HDAC1-KO lines isolated were used in cellular vitality test, after being transduced with WT or pathological mutant TDP-43, as shown in Figure 21.

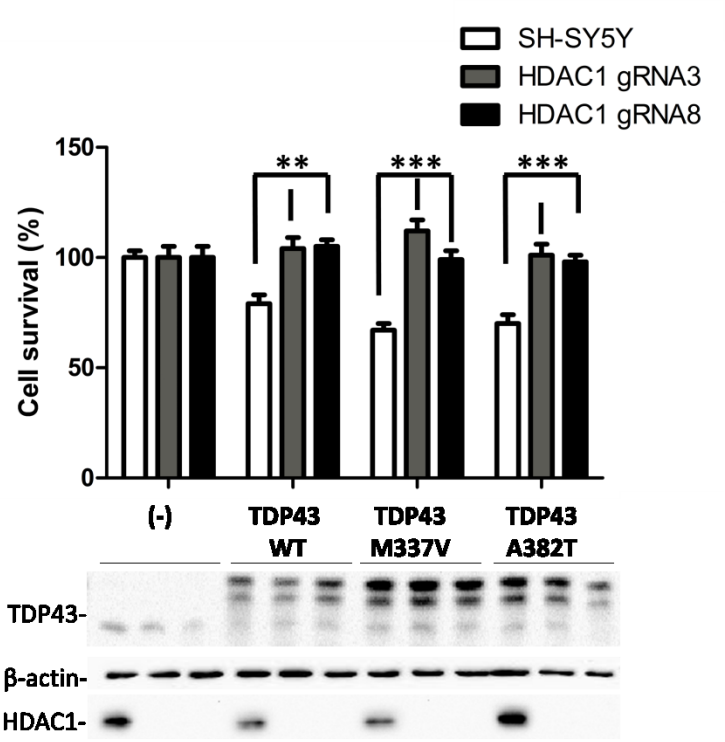


Figure 21. HDAC1-KO cell lines display reduction in cell toxicity induced by TDP-43*: A) Cell viability test in cell line SH-SY5YHDAC1 gRNA3 and HDCA1 gRNA8 (obtained using CRISP-Cas9 technology) after transduction with adenovirus coding for TDP43 WT, M337V, A382T. Western blotting using anti-TDP43, anti-*

*HDAC1 and anti-β-actin. The data were obtained from four independent experiments; ** indicates p > 0.01 versus SH-SY5Y, analyzed with one-way ANOVA.*

To extend this result, using a commercial HDAC1-siRNA, we induced a reduction of HDAC1 protein level of about 70% that resulted in a statistically significant decrease of TDP-43 induced cell toxicity, compared to the random sequence control (Figure 22), further confirming the results obtained by genetic ablation of HDCA1.

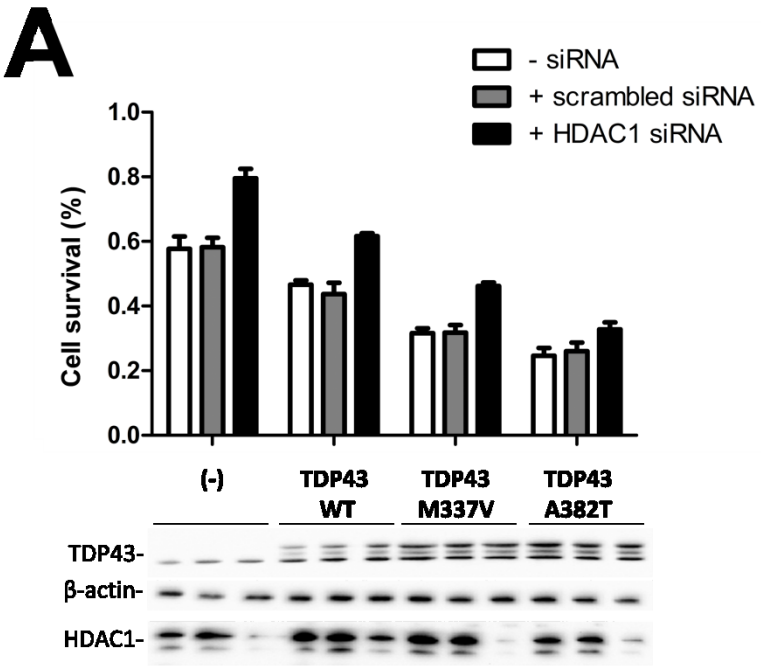


Figure 22. HDAC1 silencing whit CRISP/Cas9 reduces cell toxicity induced by mutant TDP43*: A) Cell viability test in SH-SY5Y cell line after transduction by adenovirus coding for TDP43 WT, M337V, A382T containing the 5xMyc epitope and following treatment with siRNA or scrambled. Western blotting using anti-TDP43, anti-HDAC1 and anti-β-actin. ** indicates p > 0.01 and *** p > 0.001 versus untreated with HDAC1 siRNA, analyzed with one-way ANOVA.*

HDAC inhibition has been shown to be protective in wide range of pathological conditions[24,25], including ALS. The HDACi sodium butyrate (NaB), 4-phenylbutyrate (SPB) and Trichostatin A (TSA), fatty acid derivatives that inhibit most class I and II HDACs, have have been tested used in SOD1-G93A mouse model. Moreover the therapeutic potential

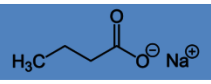
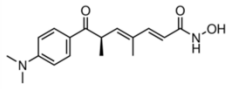
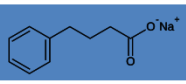
of NaB it has been demonstrated in other neurodegenerative diseases such as Alzheimer disease[26,27], Parkinson's[28] and Huntington[29] as well as with SPB treatment extends survival and motor performance in SOD1-G93A mice models and in, a phase 2 clinical trial, it has been demonstrated to be safe and well tolerated[30,31]; TSA induces a modest improvement in motor function and survival as well as protection against motor neuron death[32](Figure 15); VPA, currently used for treatment of epilepsy, even if it did not improve survival of SOD1-G86R mice, it did improve the acetylation status on the spinal cord[33]. Moreover butyrates and valproic acid are also known to readily cross the BBB[34]. We thus decided to test the effects of these four HDACis on TDP-43 induced cell toxicity.

In a preliminary experiment, we evaluated the cellular toxicity of the different HDAC inhibitors, using different HDACi concentrations, in a dose-response test that evaluated cell viability, as is shown in the figure 23.

Using this test, it was possible to identify which of the concentrations we used, were not toxic after 48h of treatment, and for each of these inhibitors we have chosen two different concentrations: 0.04mM and 0.2mM for NaB, 10nM and 25nM for TSA, 0.1mM and 0.5mM for SPB.

Afterwards, SH-SY5Y cells were transduced whit recombinant adenovirus coding for WT or mutant TDP-43 (M337V, A382T, K145A-K192A or K145Q-K192Q) and increasing concentration of the class I HDAC inhibitor NaB (0,04/0,2mM, Figure 24), TSA (25/10nM, Figure 25) and SPB (0,1/0,5mM, Figure 26), VPA (0,05/0,1mM, data not shown).

A

HDACi	Strutture	Class	HDAC	IC50	Clinical Trials
NaB		I	HDAC1	0,3mM	Parkinson Disease, HIV Infection
TSA		I	HDAC1	0,7mM	Myocardial Infarction, Benign Prostatic Hyperplasia
SPB		I	HDAC1	0,4mM	Schizophrenic Disorder, Shigellosis

B

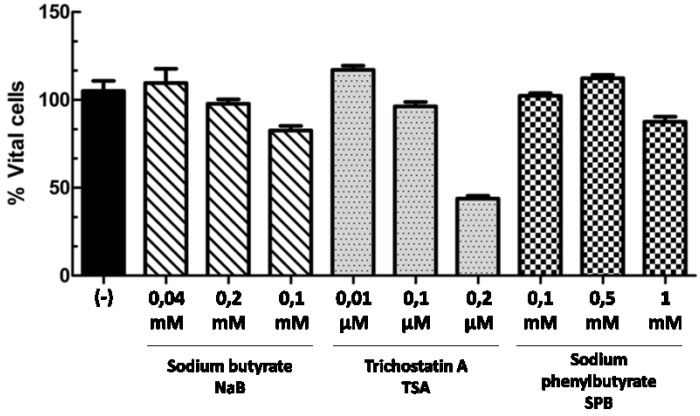


Figure 23. HDAC inhibitors used to evaluate the effect on TDP-43 overexpression induced toxicity: *A)Summary table with the characteristics of the HDCAi used. The table shows the concentrations required to inhibit 50% of the enzyme (IC50) and some of the clinical trials currently in progress. B) Dose-response curves, data arise from the analysis of values obtained from the cellular vitality assay after NaB, TSA, SPB treatment.*

Cell viability assays performed 48h after transduction with adenoviral particles coding for WT or mutant TDP43 demonstrate a dose dependent increase in survival, upon NaB and SPB and at less extend also for TSA treatment (Figure 24, 25, 26). VPA on the contrary appears to be ineffective in this experimental paradigm (data not shown).

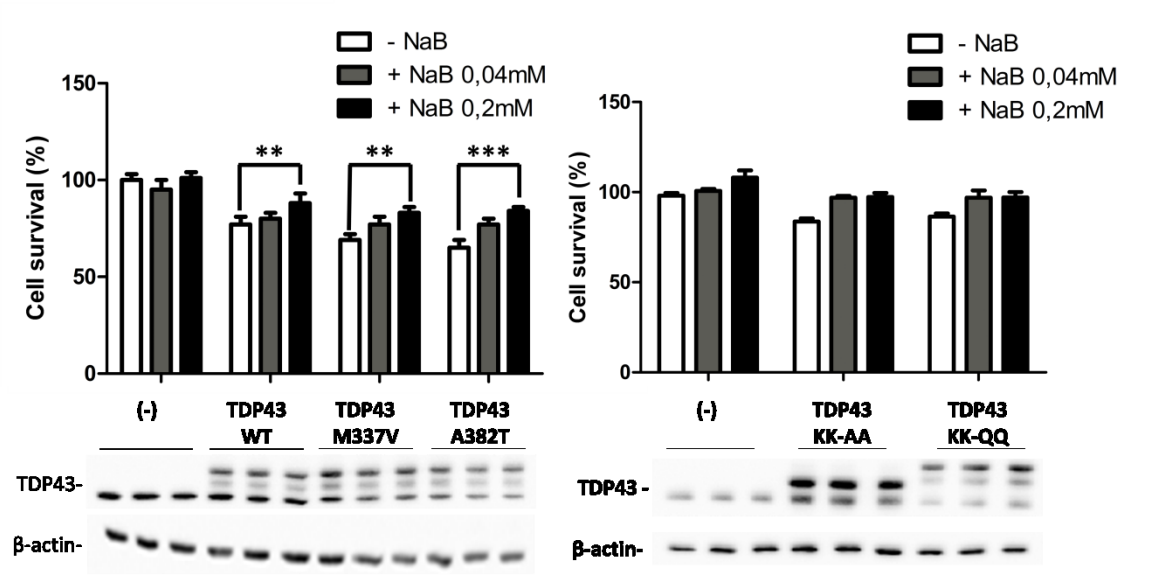


Figure 24. Effect of HDAC inhibitor Sodium butyrate on SH-SY5Y cell viability overexpressing TDP43 pathological mutant forms: *Cell viability test in SH-SY5Y cell line after transduction with adenovirus coding for TDP43 WT, M337V, A382T and for TDP43 KK-AA, KK-QQ containing the 5xMyc epitope and NaB concentration at indicated concentration. Western blotting using anti-TDP43 and anti-β- actin.** indicates p > 0.01 and ***p > 0,001 versus untreated whit NaB, analyzed with one-way ANOVA.*

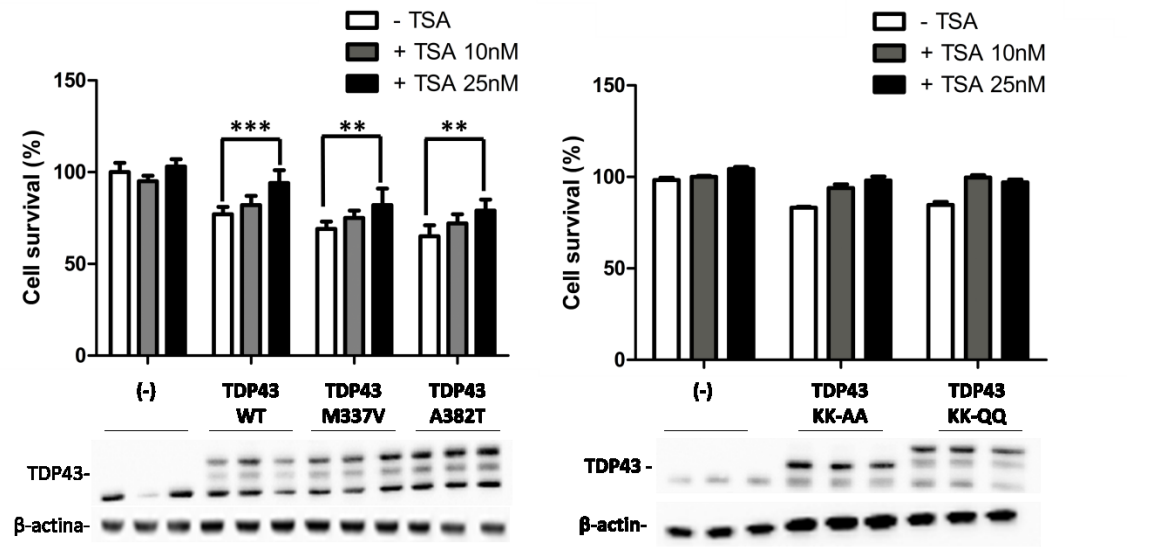


Figure 25. Effect of HDAC inhibitor Trichostatin A on SH-SY5Y cells viability overexpressing TDP43 pathological mutant forms: *A) Cellular vitality assay in SH-SY5Y cell line after transduction with adenovirus coding for TDP43 WT, M337V, A382T and B) for TDP43 KK-AA, KK-QQ containing the 5xMyc epitope and TSA treatment at indicated concentration. Western blotting using anti-TDP43 and anti-β actin. ** indicates p > 0.01 and ***p > 0,001 versus untreated whit TSA, analyzed with one-way ANOVA.*

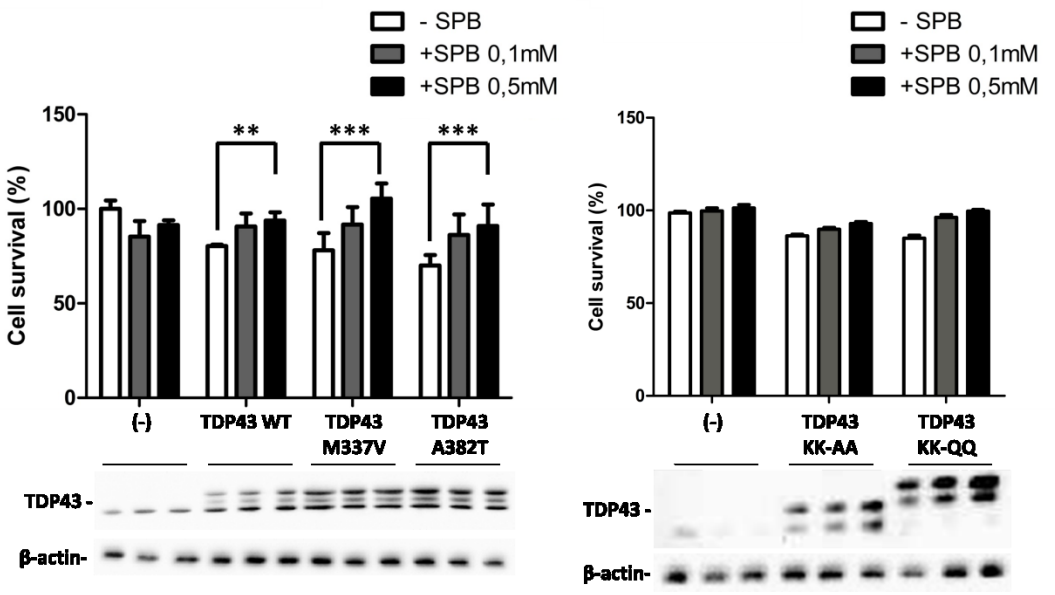


Figure 26. Effect of HDAC inhibitor 4-phenylbutyrate on SH-SY5Y cell viability overexpressing TDP43 pathological mutant forms: *A) Cellular vitality assay in SH-SY5Y cell line after transduction with adenovirus coding for TDP43 WT, M337V, A382T and B) for TDP43 KK-AA, KK-QQ containing the 5xMyc epitope and SPB treatment at indicated concentration. Western blotting using anti-TDP43 and anti-β actin. ** indicates p > 0.01 and ***p > 0,001 versus untreated whit SPB, analyzed with one-way ANOVA.*

When the KK-AA and the KK-QQ mutants were expressed, the three inhibitors display a mild effect on cell viability, that do not reach statistical significance.

The positive effect of -HDAC inhibition on TDP-43-induced cell toxicity was also confirmed in immunofluorescence experiments (Figure 27). HDCAi treated cells display a diffuse staining of TDP-43 in the nuclei, which appear more spherical in respect to the one of untreated cells.

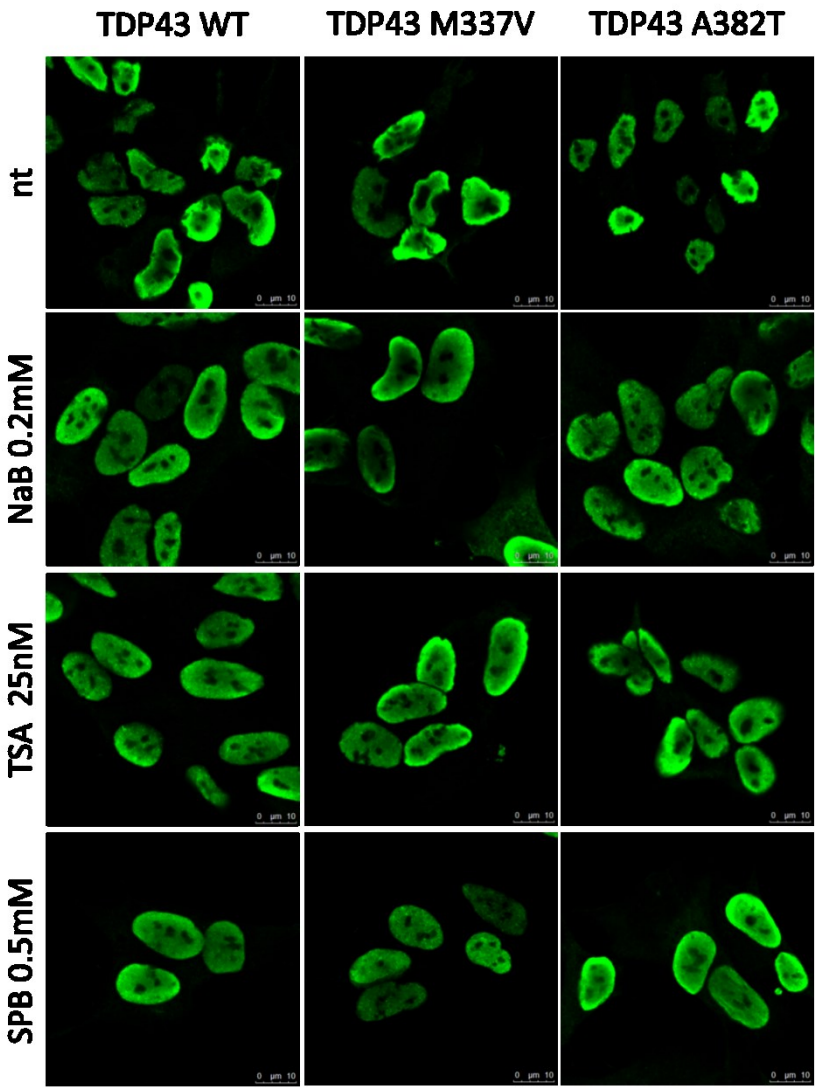


Figure 27. Immunofluorescence in SH-SY5Y expressing different TDP43 isoforms treated with NaB 0.2mM, TSA 25nM and SPB 0.5mM. *The cells were labeled with an anti-Myc antibody and detected with a secondary conjugate to Alexa 488 anti-mouse fluorophore as a secondary antibody. The slides were analyzed by Leica confocal microscope.*

Expression of hTDP43 in fly eyes leads to progressive eye defects, that is reduced by HDAC1 silecing

We have demonstrated in three different experimental paradigms (gemomic inactivation, siRNA and HDACi treatment) that in SH-SY5Y cells the inhibition or the decrease in HDAC1 level reduce the toxicity induced by WT or mutant TDP43. To confirm our results using an in vivo system, in collaboration with the group of Dr. Gianluca Cestra (Institute of

Molecular Biology and Pathology, CNR, Rome Italy), we decided to use Drosophila, a powerful genetic model widely used to study neurodegeneration including ALS.

Expression of hTDP-43 in the Drosophila eye leads to a well described retinal degeneration, as in Figure 28B, where large apoptotic areas, identifiable in as black portions are visible. But when the flies were crossed with a line in which Rpd3, the only HDAC1 and HDAC2 ortholog in Drosophila, is inactivated by expression of an RNAi, we clearly observe a reduction in retinal degeneration, which exhibits a much more control-like phenotype with reduced or absent apoptotic areas (Figure 28C).

This data demostrate that the silencing of HDAC1 in Drosophila, as well in SH-SY5Y cells, is sufficient to reduce the toxic effect induced by the over-expression of TDP-43.

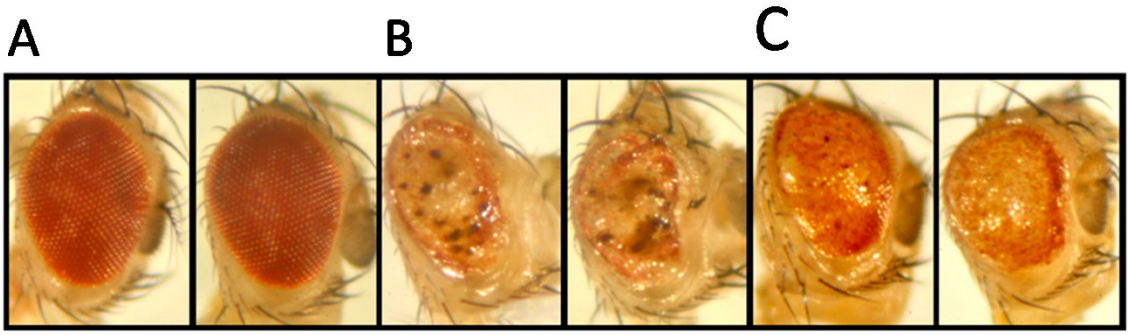


Figure 28. Expression of hTDP43 in fly eyes leads to progressive eye defects, that is reduced by HDAC1 silencing: *A) Drosophila line WT, B) Fly line, overexpressing human TDP-43, has a phenotype with obvious degeneration of the eye with wide apoptotic areas; C) the intersection of the line overexpressing human TDP-43 and with a line in which HDAC1 is silenced, shows a reduction in TDP-43 dependent degeneration.*

DISCUSSION AND CONCLUSION

ALS is predominantly a sporadic disease and environmental triggers may be involved in the onset of this disease, probably through epigenetic modifications (including histone post-translational modifications, DNA methylation, and RNA editing) that can be acquired during life and can contribute to explain pathogenesis, onset and progression. A better understanding of the genetic-epigenetic interaction can be crucial to fully comprehend the molecular mechanisms underlying motoneuronal death and, due to the reversible nature of epigenetic modifications, can be a key point for the design of new and more effective treatment (therapies).

In this respect, the present PhD Thesis adds a new piece to this complicated puzzle, demonstrating that TDP-43 can physically interact with deacetylase-1 histone (HDAC1) both in vivo and in vitro (figure 9) and, since acetylated TDP-43 would impair its RNA binding activity and enhance the formation of aggregates[17], targeting TDP-43 acetylation can be beneficial.

We demonstrated that the interaction between TDP-43 and HDAC1 involves both RMM1 and RRM2 domains, independently from the pathological mutations falling in these domains (D169G and K263E) or in the G-rich (M337V and A382T) (figure 11). Interestingly, TDP-43 has been demonstrated to undergo to an acetylation cycle that regulates not only the nucleo-cytoplasm shuttling, but also its binding activity to RNA[17]. The major, but not the exclusive, sites of acetylation have been identified on lysine 145 and 192, both comprised in the RMM1-RMM2 domains[17]. In our experimental paradigm, the mutant mimicking acetylation (referred to as KK-QQ, in whick K145 and K192 have been mutate in Q) loses HDAC1 binding activity, that is normal in the non-acetylable mutant (KK-AA).

The presence of additional and not yet identified acetylation sites, was further demonstrated by immunoprecipitation assays followed by 2D analysis, in which we observed that, in a similar way to HDAC6, the presence of HDAC1 changes (modifies) TDP-43 isoelectric point, and that when TDP-43 lacks the ΔRRM1 and ΔRRM2 domains it is partially acetylated (figure 12).

The RRM1 and RMM2 domains have been highlighted to have a fundamental role regarding the tight and highly specific binding of TDP-43 to UG repeats in RNA, and deletion of RMM1 alters not only TDP-43 role in splicing regulation[10,19], but also its dynamics in the nucleus[10]. In fact mutants lacking functional RRM-1 display a strong nuclear-matrix–scaffold association, implying a role for RRM domains in chromatin organization[10]. In this respect we investigated the biological consequences of TDP-43/HDAC1 interaction on some of the nuclear TDP-43 functions by monitoring transcription, splicing and DNA damage recovery.

TDP-43/HDAC1 interaction and transcription

Considering both that HDAC1 is a key component of an intricate network of acetylated substrates involved in transcription and that TDP-43 was found in human brain and in cell culture systems to associate with euchromatin, we first evaluated the functional relevance of TDP-43/HDAC1 by transcriptional assays. Despite TDP-43 has originally been described as a transcription factor for TAR DNA of HIV1[14], few target genes have been identified up to now. TDP-43 acts as a transcriptional repressor on both testis specific mouse acrv1 (SP10) promoter[15] and on VPS4B gene (vacuolar protein sorting 4 homolog B)[35], but can also act as a transcriptional activator on C/EBP-homologous protein (CHOP) promoter[16], although no direct interaction has been demonstrated so far. By ChIP analysis and luciferase assays we demonstrated that TDP-43 binds and stimulates CHOP promoter activity (Figure 15, 17). This effect can be a key point for cellular response to the stress generated by the overexpression of mutant TDP-43. In fact CHOP gene, initially identified as responsive gene induced by genotoxic stress such as UV irradiation, under chronic or irreversible ER stress conditions, is activated by unfolded protein response (UPR) system, and contributes to cell cycle arrest and apoptosis[36,37]. In Alzheimer disease animal

models, increase in CHOP protein was measured in presenilin-1 mutant knock-in mice[38] and β-Amyloid induces CHOP expression both in cells and animal brains, whereas treatment of cells with CHOP antisense RNA improved neuronal survival after exposure to β-amyloid[39]. A similar beneficial effect due to the reduction in CHOP expression by siRNA was observed also in 6-hydroxydopamine model of Parkinson diseases[40]. In ALS context, CHOP overexpression was observed in spinal cord tissues from patients with sporadic ALS[41] and in experimental models characterized by overexpression of either related or causing gene such as SOD1[41], OPTN[42], VAPB[43], as well as after treatment with β-methylamino-L-alanine (BMAA)[44]. Here we demonstrate for the first time, by ChIP analysis, that TDP-43 WT is a direct activator of CHOP (figure 17). The pathological mutants A382T and M337V retain this ability and concomitant overexpression of HDAC1 contributes to CHOP transcriptional down regulation. This effect is partially due to the general effect of HDCA1 on transcription, but TDP43 acetylation has a prominent role since KK-QQ TDP43 mutant does not activate CHOP transcription, despite its nuclear localization (figure 15 and 17).

TDP-43/HDAC1 interaction and splicing

Among the different TDP-43 splicing targets, POLIDP3 pre-mRNA has been widely used as a reporter gene in animal and cellular models, including SH-SH5Y cells[45,46]. RNA binding ability of TDP-43 is necessary to include POLDIP3 exon 3 and this can be relevant for the disease, since an increment of POLDIP3 variant-2 mRNA, in which exon 3 is excluded, has been described in motor cortex, spinal cord and spinal motor neurons collected by laser capture microdissection from ALS patients[19].

In SH-SY5Y cells the expression of a pro-aggregative TDP-43 mutant favours the synthesis of the POLDIP3 β isoform over the main α isoform, mirroring a protein level that was observed at mRNA level[47]. In our experimental paradigm we observed that WT or A382T TDP-43 overexpression stimulates exon 3 exclusion (decrease in V1/V2 ratio) and the concomitant HDAC1 overexpression do not significantly reduce this effect. When KK-QQ mutant is expressed the V2 isoform increases and the presence of HDAC1 has no effects on V1/V2 ratio. The overexpression of deletion mutant ΔRRM1-2 also exhibits a similar

phenotype, with a V1/V2 ratio reduction that is even more evident. This last effect can be due to an unrelated TDP-43 interaction with the POLIDP3 RNA, since TDP43 lacks RNA interaction domains, or via recruitment of endogenous TDP-43[48,49]. Globally these data indicate that the concomitant overexpression of HDAC1 with the different TDP-43 mutants analysed do not alter the splicing profile of POLDIP3 (figure 18).

TDP-43/HDAC1 interaction and DNA Damage Recovery

The role of severe DNA damage and endogenous DNA repair strategies in ALS initiation and progression are well defined. TDP-43 and FUS are localized at sites of transcription associated to DNA damage, given their colocalization with γH2AX and phosphorylated RPA in an increased percentage of UV-damaged cells[20]. Moreover, like TDP43, FUS can interact with HDCA1 and this interaction is crucial for successful DNA repair after laser micro-irradiation[9]. By measuring the effect of WT or mutant TDP-43 overexpression in a cell damage paradigm (UV-C treatment) we show, for the first time, that pathological mutants M337V and A382T, as well as the acetylation mimicking mutant KK-QQ, reduce cell recovery (figure 19) whilst with a different mechanisms. In fact, while TDP-43 relocalizes in cytoplasm upon (after) UV-C insult, KK-QQ mutant is retained in the nucleus. Although these data are preliminary and more experimental evidences should be provided, they may suggest that TDP-43, like FUS, colocalizes with HDCA1 on UV-damaged DNA and the presence of pathological mutations worsens cell survival.

TDP-43/HDAC1: the reduction of HDCA1 level or activity rescues TDP-43 induced cell toxicity

We provided evidence that TDP-43/HDAC1 interaction can have a deep impact on TDP-43 molecular function at different levels and experimental evidence obtained with the acetylation mimicking mutant KK-QQ clearly indicates that the modulation of TDP-43 acetylation status can be considered a new pharmacological target. Using different and complementary strategies, spanning from genetic inactivation by CRISPR/Cas9 technology or siRNA, to chemical inhibition using three different HDAC pan-inhibitors, we

demonstrated in vitro, in SH-SY5Y cells, and in vivo, in Drosophila eye, that HDCA1 decrease/ablation can mitigate TDP-43 toxicity (figure 21, 22, 24, 25, 26, 27, 28). These results, although counterintuitive, can be explained considering that moderate TDP-43 overexpression in mice results in downregulation of endogenous TDP-43 and causes TDP-43 aggregation, axonal degeneration, reactive gliosis, gait abnormalities and early lethality[50,51]. In this context HDCA1 decrease/ablation, or in general treatment with HDAC pan-inhibitors, can stabilize TDP-43 acetylation and consequently protects cells from damage associated to TDP-43 translocation in the cytoplasm that most likely is acquiring a new toxic function(s). In this respect we are currently evaluating if HDACi treatment can prevent CHOP promoter activation, explaining the positive effect on cell viability, according to the positive effect of CHOP reduction by siRNA on SOD1 G93A toxicity[52].

Conclusion

The data presented in this PhD thesis clearly indicate that TDP-43 acetylation/deaceteylation status, mediated also by HDAC1, affects its biological activity and, most importantly, suggest that specific reduction of HDAC1, or the use of pan-HDAC inhibitors, reduce TDP-43 induced cell-toxicity. Although we can only speculate on the molecular mechanisms underlying these phenomena, the evidence obtained in vivo and in vitro certainly create a good substrate to hypothesize future pharmacological tests. HDAC inhibitors were originally applied to cancer therapy and some of them, such as Panobinostat, have been approved from FDA for multiple myeloma treatment, while others, like VPA and SPB, are in clinical trials of phase III and II, respectively, for solid and hematological malignancies. At present more than 350 clinical trials have been carried out or are on-going involving HDACi not only as single therapeutic but also in combination with other targeted agents against various human diseases, including neurodegenerative diseases. VPA was approved by FDA in 1978 as an anticonvulsant drug for the treatment of seizure disorders, even if the molecular targets of these drugs were not known yet. The possibility of using HDACi for neurodegenerative treatment originates in 2008, when Hahnen identified two major HDACi neuroprotective mechanisms, including the transcriptional activation of disease-modifying genes and the rectification of

destabilization in histone acetylation homeostasis[53]. Several pan-HDAC inhibitors reduced ALS development in mice[54], but, even if they were safe, tolerable and efficient in improving histone acetylation levels, they failed to ameliorate clinical parameters in ALS patients[31]. The translational failure can be due to lack of selectivity for different HDACs and to unwanted off-target effects and, for sure, more specific drugs should be developed. But it is worth to keep in mind the "edaravone story": only after a post-hoc analysis of a previous phase III study significant differences compared with placebo were highlighted in subset of ALS patients[55]. Therefore, considering that HDAC inhibition can be a precious therapeutic option, molecular characterization of TDP-43/HDAC1 functional interaction can, most likely, open new scenario in ALS therapy.

BIBLIOGRAPHY

1 Wijesekera, L. C. & Leigh, P. N. Amyotrophic lateral sclerosis. *Orphanet journal of rare diseases* **4**, 3, doi:10.1186/1750-1172-4-3 (2009).

2 Bendotti, C. & Carri, M. T. Lessons from models of SOD1-linked familial ALS. *Trends in molecular medicine* **10**, 393-400, doi:10.1016/j.molmed.2004.06.009 (2004).

3 Traynor, B. J. *et al.* Incidence and prevalence of ALS in Ireland, 1995-1997: a population-based study. *Neurology* **52**, 504-509 (1999).

4 Annegers, J. F., Appel, S., Lee, J. R. & Perkins, P. Incidence and prevalence of amyotrophic lateral sclerosis in Harris County, Texas, 1985-1988. *Archives of neurology* **48**, 589-593 (1991).

5 McGuire, V., Longstreth, W. T., Jr., Koepsell, T. D. & van Belle, G. Incidence of amyotrophic lateral sclerosis in three counties in western Washington state. *Neurology* **47**, 571-573 (1996).

6 Chio, A. *et al.* ALS clinical trials: do enrolled patients accurately represent the ALS population? *Neurology* **77**, 1432-1437, doi:10.1212/WNL.0b013e318232ab9b (2011).

7 Chio, A. *et al.* ALS/FTD phenotype in two Sardinian families carrying both C9ORF72 and TARDBP mutations. *Journal of neurology, neurosurgery, and psychiatry* **83**, 730-733, doi:10.1136/jnnp-2012-302219 (2012).

8 Strong, M. J. & Garruto, R. M. Potentiation in the neurotoxic induction of experimental chronic neurodegenerative disorders: N-butyl benzenesulfonamide and aluminum chloride. *Neurotoxicology* **12**, 415-425 (1991).

9 Andersen, P. M. & Al-Chalabi, A. Clinical genetics of amyotrophic lateral sclerosis: what do we really know? *Nature reviews. Neurology* **7**, 603-615, doi:10.1038/nrneurol.2011.150 (2011).

10 Laferriere, F. & Polymenidou, M. Advances and challenges in understanding the multifaceted pathogenesis of amyotrophic lateral sclerosis. *Swiss medical weekly* **145**, w14054, doi:10.4414/smw.2015.14054 (2015).

11 Couthouis, J., Raphael, A. R., Daneshjou, R. & Gitler, A. D. Targeted exon capture and sequencing in sporadic amyotrophic lateral sclerosis. *PLoS genetics* **10**, e1004704, doi:10.1371/journal.pgen.1004704 (2014).

12 Chen, S., Sayana, P., Zhang, X. & Le, W. Genetics of amyotrophic lateral sclerosis: an update. *Molecular neurodegeneration* **8**, 28, doi:10.1186/1750-1326-8-28 (2013).

13 van Doormaal, P. T. C. *et al.* The role of de novo mutations in the development of amyotrophic lateral sclerosis. *Human mutation* **38**, 1534-1541, doi:10.1002/humu.23295 (2017).

14 Rosen, D. R. Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. *Nature* **364**, 362, doi:10.1038/364362c0 (1993).

15 Pasinelli, P. & Brown, R. H. Molecular biology of amyotrophic lateral sclerosis: insights from genetics. *Nature reviews. Neuroscience* **7**, 710-723, doi:10.1038/nrn1971 (2006).

16 Ilieva, H., Polymenidou, M. & Cleveland, D. W. Non-cell autonomous toxicity in neurodegenerative disorders: ALS and beyond. *The Journal of cell biology* **187**, 761-772, doi:10.1083/jcb.200908164 (2009).

17 Alderman, E. M. *et al.* Angiogenic activity of human tumor plasma membrane

components. *Biochemistry* **24**, 7866-7871 (1985).

18 Chio, A. *et al.* A de novo missense mutation of the FUS gene in a "true" sporadic ALS case. *Neurobiology of aging* **32**, 553 e523-556, doi:10.1016/j.neurobiolaging.2010.05.016 (2011).

19 DeJesus-Hernandez, M. *et al.* De novo truncating FUS gene mutation as a cause of sporadic amyotrophic lateral sclerosis. *Human mutation* **31**, E1377-1389, doi:10.1002/humu.21241 (2010).

20 Lattante, S. *et al.* Contribution of major amyotrophic lateral sclerosis genes to the etiology of sporadic disease. *Neurology* **79**, 66-72, doi:10.1212/WNL.0b013e31825dceca (2012).

21 Pamphlett, R., Cheong, P. L., Trent, R. J. & Yu, B. Transmission of C9orf72 hexanucleotide repeat expansions in sporadic amyotrophic lateral sclerosis: an Australian trio study. *Neuroreport* **23**, 556-559, doi:10.1097/WNR.0b013e3283544718 (2012).

22 van der Zee, J. *et al.* A pan-European study of the C9orf72 repeat associated with FTLD: geographic prevalence, genomic instability, and intermediate repeats. *Human mutation* **34**, 363-373, doi:10.1002/humu.22244 (2013).

23 Neumann, M. *et al.* Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Science* **314**, 130-133, doi:10.1126/science.1134108 (2006).

24 Arai, T. *et al.* TDP-43 is a component of ubiquitin-positive tau-negative inclusions in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Biochemical and biophysical research communications* **351**, 602-611, doi:10.1016/j.bbrc.2006.10.093 (2006).

25 Tan, C. F. *et al.* TDP-43 immunoreactivity in neuronal inclusions in familial amyotrophic lateral sclerosis with or without SOD1 gene mutation. *Acta neuropathologica* **113**, 535-542, doi:10.1007/s00401-007-0206-9 (2007).

26 Mackenzie, I. R. *et al.* Pathological TDP-43 distinguishes sporadic amyotrophic lateral sclerosis from amyotrophic lateral sclerosis with SOD1 mutations. *Annals of neurology* **61**, 427-434, doi:10.1002/ana.21147 (2007).

27 Vance, C. *et al.* Mutations in FUS, an RNA processing protein, cause familial amyotrophic lateral sclerosis type 6. *Science* **323**, 1208-1211, doi:10.1126/science.1165942 (2009).

28 Kwiatkowski, T. J., Jr. *et al.* Mutations in the FUS/TLS gene on chromosome 16 cause familial amyotrophic lateral sclerosis. *Science* **323**, 1205-1208, doi:10.1126/science.1166066 (2009).

29 DeJesus-Hernandez, M. *et al.* Expanded GGGGCC hexanucleotide repeat in noncoding region of C9ORF72 causes chromosome 9p-linked FTD and ALS. *Neuron* **72**, 245-256, doi:10.1016/j.neuron.2011.09.011 (2011).

30 Al-Sarraj, S. *et al.* p62 positive, TDP-43 negative, neuronal cytoplasmic and intranuclear inclusions in the cerebellum and hippocampus define the pathology of C9orf72-linked FTLD and MND/ALS. *Acta neuropathologica* **122**, 691-702, doi:10.1007/s00401-011-0911-2 (2011).

31 Troakes, C. *et al.* An MND/ALS phenotype associated with C9orf72 repeat expansion: abundant p62-positive, TDP-43-negative inclusions in cerebral cortex, hippocampus and cerebellum but without associated cognitive decline. *Neuropathology : official journal of the Japanese Society of Neuropathology* **32**, 505-514, doi:10.1111/j.1440-1789.2011.01286.x (2012).

32 Ash, P. E. *et al.* Unconventional translation of C9ORF72 GGGGCC expansion generates insoluble polypeptides specific to c9FTD/ALS. *Neuron* **77**, 639-646, doi:10.1016/j.neuron.2013.02.004 (2013).

33 Zu, T. *et al.* RAN proteins and RNA foci from antisense transcripts in C9ORF72 ALS and frontotemporal dementia. *Proceedings of the National Academy of Sciences of the United*

States of America **110**, E4968-4977, doi:10.1073/pnas.1315438110 (2013).

34 Lagier-Tourenne, C. *et al.* Targeted degradation of sense and antisense C9orf72 RNA foci as therapy for ALS and frontotemporal degeneration. *Proceedings of the National Academy of Sciences of the United States of America* **110**, E4530-4539, doi:10.1073/pnas.1318835110 (2013).

35 Renton, A. E., Chio, A. & Traynor, B. J. State of play in amyotrophic lateral sclerosis genetics. *Nature neuroscience* **17**, 17-23, doi:10.1038/nn.3584 (2014).

36 Bonduelle, V. M. [The familial forms of amyotrophic lateral sclerosis]. *Wien Med Wochenschr* **125**, 330-331 (1975).

37 Baltazar, M. T. *et al.* Pesticides exposure as etiological factors of Parkinson's disease and other neurodegenerative diseases--a mechanistic approach. *Toxicology letters* **230**, 85-103, doi:10.1016/j.toxlet.2014.01.039 (2014).

38 Satta, C. T. *et al.* Multiannual Chattonella subsalsa Biecheler (Raphidophyceae) blooms in a Mediterranean lagoon (Santa Giusta Lagoon, Sardinia Island, Italy). *Harmful algae* **67**, 61-73, doi:10.1016/j.hal.2017.06.002 (2017).

39 Popova, A. A. & Koksharova, O. A. Neurotoxic Non-proteinogenic Amino Acid beta-N-Methylamino-L-alanine and Its Role in Biological Systems. *Biochemistry. Biokhimiia* **81**, 794-805, doi:10.1134/S0006297916080022 (2016).

40 Wang, H. *et al.* Smoking and risk of amyotrophic lateral sclerosis: a pooled analysis of 5 prospective cohorts. *Archives of neurology* **68**, 207-213, doi:10.1001/archneurol.2010.367 (2011).

41 Trojsi, F., Monsurro, M. R. & Tedeschi, G. Exposure to environmental toxicants and pathogenesis of amyotrophic lateral sclerosis: state of the art and research perspectives. *International journal of molecular sciences* **14**, 15286-15311, doi:10.3390/ijms140815286 (2013).

42 de Jong, S. W. *et al.* Smoking, alcohol consumption, and the risk of amyotrophic lateral sclerosis: a population-based study. *American journal of epidemiology* **176**, 233-239, doi:10.1093/aje/kws015 (2012).

43 Veldink, J. H. *et al.* SMN genotypes producing less SMN protein increase susceptibility to and severity of sporadic ALS. *Neurology* **65**, 820-825, doi:10.1212/01.wnl.0000174472.03292.dd (2005).

44 Alfahad, T. & Nath, A. Retroviruses and amyotrophic lateral sclerosis. *Antiviral research* **99**, 180-187, doi:10.1016/j.antiviral.2013.05.006 (2013).

45 Ingre, C., Roos, P. M., Piehl, F., Kamel, F. & Fang, F. Risk factors for amyotrophic lateral sclerosis. *Clinical epidemiology* **7**, 181-193, doi:10.2147/CLEP.S37505 (2015).

46 Ferraiuolo, L., Kirby, J., Grierson, A. J., Sendtner, M. & Shaw, P. J. Molecular pathways of motor neuron injury in amyotrophic lateral sclerosis. *Nature reviews. Neurology* **7**, 616-630, doi:10.1038/nrneurol.2011.152 (2011).

47 Hardiman, O. *et al.* Amyotrophic lateral sclerosis. *Nature reviews. Disease primers* **3**, 17071, doi:10.1038/nrdp.2017.71 (2017).

48 Ince, P. G., Lowe, J. & Shaw, P. J. Amyotrophic lateral sclerosis: current issues in classification, pathogenesis and molecular pathology. *Neuropathology and applied neurobiology* **24**, 104-117 (1998).

49 Ince, P. G., Tomkins, J., Slade, J. Y., Thatcher, N. M. & Shaw, P. J. Amyotrophic lateral sclerosis associated with genetic abnormalities in the gene encoding Cu/Zn superoxide dismutase: molecular pathology of five new cases, and comparison with previous reports and 73 sporadic cases of ALS. *Journal of neuropathology and experimental neurology* **57**, 895-904 (1998).

50 Piao, Y. S. *et al.* Neuropathology with clinical correlations of sporadic amyotrophic lateral

sclerosis: 102 autopsy cases examined between 1962 and 2000. *Brain Pathol* **13**, 10-22 (2003).

51 Sreedharan, J. *et al.* TDP-43 mutations in familial and sporadic amyotrophic lateral sclerosis. *Science* **319**, 1668-1672, doi:10.1126/science.1154584 (2008).

52 Shibata, N. *et al.* Cu/Zn superoxide dismutase-like immunoreactivity in Lewy body-like inclusions of sporadic amyotrophic lateral sclerosis. *Neuroscience letters* **179**, 149-152 (1994).

53 Rakhit, R. *et al.* An immunological epitope selective for pathological monomer-misfolded SOD1 in ALS. *Nature medicine* **13**, 754-759, doi:10.1038/nm1559 (2007).

54 Bosco, D. A. *et al.* Wild-type and mutant SOD1 share an aberrant conformation and a common pathogenic pathway in ALS. *Nature neuroscience* **13**, 1396-1403, doi:10.1038/nn.2660 (2010).

55 Groen, E. J. *et al.* FUS mutations in familial amyotrophic lateral sclerosis in the Netherlands. *Archives of neurology* **67**, 224-230, doi:10.1001/archneurol.2009.329 (2010).

56 Hewitt, C. *et al.* Novel FUS/TLS mutations and pathology in familial and sporadic amyotrophic lateral sclerosis. *Archives of neurology* **67**, 455-461, doi:10.1001/archneurol.2010.52 (2010).

57 Onesto, E. *et al.* Gene-specific mitochondria dysfunctions in human TARDBP and C9ORF72 fibroblasts. *Acta neuropathologica communications* **4**, 47, doi:10.1186/s40478-016-0316-5 (2016).

58 Atsumi, T. The ultrastructure of intramuscular nerves in amyotrophic lateral sclerosis. *Acta neuropathologica* **55**, 193-198 (1981).

59 Afifi, A. K., Aleu, F. P., Goodgold, J. & MacKay, B. Ultrastructure of atrophic muscle in amyotrophic lateral sclerosis. *Neurology* **16**, 475-481 (1966).

60 Wiedemann, F. R. *et al.* Impairment of mitochondrial function in skeletal muscle of patients with amyotrophic lateral sclerosis. *Journal of the neurological sciences* **156**, 65-72 (1998).

61 Siklos, L. *et al.* Ultrastructural evidence for altered calcium in motor nerve terminals in amyotropic lateral sclerosis. *Annals of neurology* **39**, 203-216, doi:10.1002/ana.410390210 (1996).

62 Higgins, C. M., Jung, C. & Xu, Z. ALS-associated mutant SOD1G93A causes mitochondrial vacuolation by expansion of the intermembrane space and by involvement of SOD1 aggregation and peroxisomes. *BMC neuroscience* **4**, 16, doi:10.1186/1471-2202-4-16 (2003).

63 Bendotti, C. *et al.* Early vacuolization and mitochondrial damage in motor neurons of FALS mice are not associated with apoptosis or with changes in cytochrome oxidase histochemical reactivity. *Journal of the neurological sciences* **191**, 25-33 (2001).

64 Sasaki, S. *et al.* Immunohistochemical and ultrastructural study of basophilic inclusions in adult-onset motor neuron disease. *Acta neuropathologica* **102**, 200-206 (2001).

65 Ferri, A. *et al.* Familial ALS-superoxide dismutases associate with mitochondria and shift their redox potentials. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 13860-13865, doi:10.1073/pnas.0605814103 (2006).

66 Rizzardini, M. *et al.* Neurodegeneration induced by complex I inhibition in a cellular model of familial amyotrophic lateral sclerosis. *Brain research bulletin* **69**, 465-474, doi:10.1016/j.brainresbull.2006.02.013 (2006).

67 Damiano, M. *et al.* Neural mitochondrial Ca2+ capacity impairment precedes the onset of motor symptoms in G93A Cu/Zn-superoxide dismutase mutant mice. *Journal of neurochemistry* **96**, 1349-1361, doi:10.1111/j.1471-4159.2006.03619.x (2006).

68 Mattiazzi, M. *et al.* Mutated human SOD1 causes dysfunction of oxidative

phosphorylation in mitochondria of transgenic mice. *The Journal of biological chemistry* **277**, 29626-29633, doi:10.1074/jbc.M203065200 (2002).

69 Friedlander, R. M. Apoptosis and caspases in neurodegenerative diseases. *The New England journal of medicine* **348**, 1365-1375, doi:10.1056/NEJMra022366 (2003).

70 Andersen, J. K. Oxidative stress in neurodegeneration: cause or consequence? *Nature medicine* **10 Suppl**, S18-25, doi:10.1038/nrn1434 (2004).

71 Barber, S. C., Mead, R. J. & Shaw, P. J. Oxidative stress in ALS: a mechanism of neurodegeneration and a therapeutic target. *Biochimica et biophysica acta* **1762**, 1051-1067, doi:10.1016/j.bbadis.2006.03.008 (2006).

72 Beckman, J. S., Carson, M., Smith, C. D. & Koppenol, W. H. ALS, SOD and peroxynitrite. *Nature* **364**, 584, doi:10.1038/364584a0 (1993).

73 Cozzolino, M., Ferri, A., Valle, C. & Carri, M. T. Mitochondria and ALS: implications from novel genes and pathways. *Molecular and cellular neurosciences* **55**, 44-49, doi:10.1016/j.mcn.2012.06.001 (2013).

74 Cestra, G., Rossi, S., Di Salvio, M. & Cozzolino, M. Control of mRNA Translation in ALS Proteinopathy. *Frontiers in molecular neuroscience* **10**, 85, doi:10.3389/fnmol.2017.00085 (2017).

75 Cercignani, M. *et al.* Investigation of quantitative magnetisation transfer parameters of lesions and normal appearing white matter in multiple sclerosis. *NMR in biomedicine* **22**, 646-653, doi:10.1002/nbm.1379 (2009).

76 Suzuki, N. *et al.* FUS/TLS-immunoreactive neuronal and glial cell inclusions increase with disease duration in familial amyotrophic lateral sclerosis with an R521C FUS/TLS mutation. *Journal of neuropathology and experimental neurology* **71**, 779-788, doi:10.1097/NEN.0b013e318264f164 (2012).

77 Jang, J. H. *et al.* Analysis of the C9orf72 hexanucleotide repeat expansion in Korean patients with familial and sporadic amyotrophic lateral sclerosis. *Neurobiology of aging* **34**, 1311 e1317-1319, doi:10.1016/j.neurobiolaging.2012.09.004 (2013).

78 Schwartz, J. C. *et al.* FUS binds the CTD of RNA polymerase II and regulates its phosphorylation at Ser2. *Genes & development* **26**, 2690-2695, doi:10.1101/gad.204602.112 (2012).

79 Mirra, A. *et al.* Functional interaction between FUS and SMN underlies SMA-like splicing changes in wild-type hFUS mice. *Scientific reports* **7**, 2033, doi:10.1038/s41598-017-02195-0 (2017).

80 Wegorzewska, I. & Baloh, R. H. TDP-43-based animal models of neurodegeneration: new insights into ALS pathology and pathophysiology. *Neuro-degenerative diseases* **8**, 262-274, doi:10.1159/000321547 (2011).

81 Joyce, P. I., Fratta, P., Fisher, E. M. & Acevedo-Arozena, A. SOD1 and TDP-43 animal models of amyotrophic lateral sclerosis: recent advances in understanding disease toward the development of clinical treatments. *Mammalian genome : official journal of the International Mammalian Genome Society* **22**, 420-448, doi:10.1007/s00335-011-9339-1 (2011).

82 Xiao, S. *et al.* RNA targets of TDP-43 identified by UV-CLIP are deregulated in ALS. *Molecular and cellular neurosciences* **47**, 167-180, doi:10.1016/j.mcn.2011.02.013 (2011).

83 Ratti, A. & Buratti, E. Physiological functions and pathobiology of TDP-43 and FUS/TLS proteins. *Journal of neurochemistry* **138** Suppl 1, 95-111, doi:10.1111/jnc.13625 (2016).

84 Wang, W. Y. *et al.* Interaction of FUS and HDAC1 regulates DNA damage response and repair in neurons. *Nature neuroscience* **16**, 1383-1391, doi:10.1038/nn.3514 (2013).

85 Hill, S. J. *et al.* Two familial ALS proteins function in prevention/repair of transcription-associated DNA damage. *Proceedings of the National Academy of Sciences of the United*

States of America **113**, E7701-E7709, doi:10.1073/pnas.1611673113 (2016).

86 van Rheenen, W. *et al.* Genome-wide association analyses identify new risk variants and the genetic architecture of amyotrophic lateral sclerosis. *Nature genetics* **48**, 1043-1048, doi:10.1038/ng.3622 (2016).

87 Kenna, K. P. *et al.* NEK1 variants confer susceptibility to amyotrophic lateral sclerosis. *Nature genetics* **48**, 1037-1042, doi:10.1038/ng.3626 (2016).

88 Fang, X. *et al.* The NEK1 interactor, C21ORF2, is required for efficient DNA damage repair. *Acta biochimica et biophysica Sinica* **47**, 834-841, doi:10.1093/abbs/gmv076 (2015).

89 Kim, H. J. & Taylor, J. P. Lost in Transportation: Nucleocytoplasmic Transport Defects in ALS and Other Neurodegenerative Diseases. *Neuron* **96**, 285-297, doi:10.1016/j.neuron.2017.07.029 (2017).

90 Henkel, J. S. *et al.* Presence of dendritic cells, MCP-1, and activated microglia/macrophages in amyotrophic lateral sclerosis spinal cord tissue. *Annals of neurology* **55**, 221-235, doi:10.1002/ana.10805 (2004).

91 Kuhle, J. *et al.* Increased levels of inflammatory chemokines in amyotrophic lateral sclerosis. *European journal of neurology* **16**, 771-774, doi:10.1111/j.1468-1331.2009.02560.x (2009).

92 Mantovani, S. *et al.* Immune system alterations in sporadic amyotrophic lateral sclerosis patients suggest an ongoing neuroinflammatory process. *Journal of neuroimmunology* **210**, 73-79, doi:10.1016/j.jneuroim.2009.02.012 (2009).

93 Kipnis, J., Avidan, H., Caspi, R. R. & Schwartz, M. Dual effect of CD4(+)CD25(+) regulatory T cells in neurodegeneration: A dialogue with microglia. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 14663-14669, doi:10.1073/pnas.0404842101 (2004).

94 Beers, D. R., Henkel, J. S., Zhao, W. H., Wang, J. H. & Appel, S. H. CD4+T cells support glial neuroprotection, slow disease progression, and modify glial morphology in an animal model of inherited ALS. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 15558-15563, doi:10.1073/pnas.0807419105 (2008).

95 Lincecum, J. M. *et al.* From transcriptome analysis to therapeutic anti-CD40L treatment in the SOD1 model of amyotrophic lateral sclerosis. *Nature genetics* **42**, 392-U345, doi:10.1038/ng.557 (2010).

96 Shaw, P. J., Forrest, V., Ince, P. G., Richardson, J. P. & Wastell, H. J. Csf and Plasma Amino-Acid Levels in Motor-Neuron Disease - Elevation of Csf Glutamate in a Subset of Patients. *Neurodegeneration* **4**, 209-216, doi:DOI 10.1006/neur.1995.0026 (1995).

97 Rothstein, J. D., Martin, L. J. & Kuncl, R. W. Decreased Glutamate Transport by the Brain and Spinal-Cord in Amyotrophic-Lateral-Sclerosis. *New Engl J Med* **326**, 1464-1468, doi:Doi 10.1056/Nejm199205283262204 (1992).

98 Rothstein, J. D., Vankammen, M., Levey, A. I., Martin, L. J. & Kuncl, R. W. Selective Loss of Glial Glutamate Transporter Glt-1 in Amyotrophic-Lateral-Sclerosis. *Annals of neurology* **38**, 73-84, doi:DOI 10.1002/ana.410380114 (1995).

99 Fray, A. E. *et al.* The expression of the glial glutamate transporter protein EAAT2 in motor neuron disease: an immunohistochemical study. *European Journal of Neuroscience* **10**, 2481-2489, doi:DOI 10.1046/j.1460-9568.1998.00273.x (1998).

100 Foran, E. & Trotti, D. Glutamate Transporters and the Excitotoxic Path to Motor Neuron Degeneration in Amyotrophic Lateral Sclerosis. *Antioxidants & redox signaling* **11**, 1587-U1586, doi:10.1089/ars.2009.2444 (2009).

101 Vucic, S., Nicholson, G. A. & Kiernan, M. C. Cortical hyperexcitability may precede the onset of familial amyotrophic lateral sclerosis (vol 131, pg 1540, 2008). *Brain : a journal of neurology* **131**, 2234-2234, doi:10.1093/brain/awn141 (2008).

102 Vucic, S. & Kiernan, M. C. Novel threshold tracking techniques suggest that cortical hyperexcitability is an early feature of motor neuron disease. *Brain : a journal of neurology* **129**, 2436-2446, doi:10.1093/brain/awl172 (2006).

103 Kwak, S., Hideyama, T., Yamashita, T. & Aizawa, H. AMPA receptor-mediated neuronal death in sporadic ALS. *Neuropathology : official journal of the Japanese Society of Neuropathology* **30**, 182-188, doi:10.1111/j.1440-1789.2009.01090.x (2010).

104 Mitchell, J. *et al.* Familial amyotrophic lateral sclerosis is associated with a mutation in D-amino acid oxidase. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 7556-7561, doi:10.1073/pnas.0914128107 (2010).

105 Meehan, C. F. *et al.* Intrinsic properties of lumbar motor neurones in the adult G127insTGGG superoxide dismutase-1 mutant mouse in vivo: evidence for increased persistent inward currents. *Acta Physiol* **200**, 361-376, doi:10.1111/j.1748-1716.2010.02188.x (2010).

106 Boston-Howes, W. *et al.* Caspase-3 cleaves and inactivates the glutamate transporter EAAT2. *Journal of Biological Chemistry* **281**, 14076-14084, doi:10.1074/jbc.M600653200 (2006).

107 Milanese, M. *et al.* Abnormal exocytotic release of glutamate in a mouse model of amyotrophic lateral sclerosis. *Journal of neurochemistry* **116**, 1028-1042, doi:10.1111/j.1471-4159.2010.07155.x (2011).

108 Sunico, C. R. *et al.* Reduction in the Motoneuron Inhibitory/Excitatory Synaptic Ratio in an Early-Symptomatic Mouse Model of Amyotrophic Lateral Sclerosis. *Brain Pathology* **21**, 1-15, doi:10.1111/j.1750-3639.2010.00417.x (2011).

109 Van Damme, P. *et al.* Astrocytes regulate GluR2 expression in motor neurons and their vulnerability to excitotoxicity. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 14825-14830, doi:10.1073/pnas.0705046104 (2007).

110 Cheah, B. C., Vucic, S., Krishnan, A. V. & Kiernan, M. C. Riluzole, Neuroprotection and Amyotrophic Lateral Sclerosis. *Curr Med Chem* **17**, 1942-1959 (2010).

111 Lacomblez, L., Bensimon, G., Leigh, P. N., Guillet, P. & Meininger, V. Dose-ranging study of riluzole in amyotrophic lateral sclerosis. *Lancet* **347**, 1425-1431 (1996).

112 Da Cruz, S. & Cleveland, D. W. Understanding the role of TDP-43 and FUS/TLS in ALS and beyond. *Current opinion in neurobiology* **21**, 904-919, doi:10.1016/j.conb.2011.05.029 (2011).

113 Ling, S. C. *et al.* ALS-associated mutations in TDP-43 increase its stability and promote TDP-43 complexes with FUS/TLS. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 13318-13323, doi:10.1073/pnas.1008227107 (2010).

114 Cohen, T. J., Lee, V. M. & Trojanowski, J. Q. TDP-43 functions and pathogenic mechanisms implicated in TDP-43 proteinopathies. *Trends in molecular medicine* **17**, 659-667, doi:10.1016/j.molmed.2011.06.004 (2011).

115 Lagier-Tourenne, C., Polymenidou, M. & Cleveland, D. W. TDP-43 and FUS/TLS: emerging roles in RNA processing and neurodegeneration. *Human molecular genetics* **19**, R46-64, doi:10.1093/hmg/ddq137 (2010).

116 Tripathi, V. B., Baskaran, P., Shaw, C. E. & Guthrie, S. Tar DNA-binding protein-43 (TDP-43) regulates axon growth in vitro and in vivo. *Neurobiology of disease* **65**, 25-34, doi:10.1016/j.nbd.2014.01.004 (2014).

117 Gitcho, M. A. *et al.* TDP-43 A315T mutation in familial motor neuron disease. *Annals of neurology* **63**, 535-538, doi:10.1002/ana.21344 (2008).

118 Gijselinck, I. *et al.* Neuronal inclusion protein TDP-43 has no primary genetic role in FTD and ALS. *Neurobiology of aging* **30**, 1329-1331, doi:10.1016/j.neurobiolaging.2007.11.002 (2009).

119 Guerreiro, R. J. *et al.* TDP-43 is not a common cause of sporadic amyotrophic lateral sclerosis. *PloS one* **3**, e2450, doi:10.1371/journal.pone.0002450 (2008).

120 Kabashi, E. *et al.* TARDBP mutations in individuals with sporadic and familial amyotrophic lateral sclerosis. *Nature genetics* **40**, 572-574, doi:10.1038/ng.132 (2008).

121 Chio, A. *et al.* Large proportion of amyotrophic lateral sclerosis cases in Sardinia due to a single founder mutation of the TARDBP gene. *Archives of neurology* **68**, 594-598, doi:10.1001/archneurol.2010.352 (2011).

122 Quadri, M. *et al.* Broadening the phenotype of TARDBP mutations: the TARDBP Ala382Thr mutation and Parkinson's disease in Sardinia. *Neurogenetics* **12**, 203-209, doi:10.1007/s10048-011-0288-3 (2011).

123 Ticozzi, N. *et al.* Genetics of familial Amyotrophic lateral sclerosis. *Archives italiennes de biologie* **149**, 65-82, doi:10.4449/aib.v149i1.1262 (2011).

124 Ou, S. H., Wu, F., Harrich, D., Garcia-Martinez, L. F. & Gaynor, R. B. Cloning and characterization of a novel cellular protein, TDP-43, that binds to human immunodeficiency virus type 1 TAR DNA sequence motifs. *Journal of virology* **69**, 3584-3596 (1995).

125 Lalmansingh, A. S., Urekar, C. J. & Reddi, P. P. TDP-43 Is a Transcriptional Repressor THE TESTIS-SPECIFIC MOUSE acrv1 GENE IS A TDP-43 TARGET IN VIVO. *Journal of Biological Chemistry* **286**, 10970-10982, doi:10.1074/jbc.M110.166587 (2011).

126 Xiao, Y. *et al.* Identification of cell-specific targets of sumoylation during mouse spermatogenesis. *Reproduction* **151**, 149-166, doi:10.1530/REP-15-0239 (2016).

127 Schwenk, B. M. *et al.* TDP-43 loss of function inhibits endosomal trafficking and alters trophic signaling in neurons. *The EMBO journal* **35**, 2350-2370, doi:10.15252/embj.201694221 (2016).

128 Ling, S. C., Polymenidou, M. & Cleveland, D. W. Converging mechanisms in ALS and FTD: disrupted RNA and protein homeostasis. *Neuron* **79**, 416-438, doi:10.1016/j.neuron.2013.07.033 (2013).

129 Suzuki, H., Lee, K. & Matsuoka, M. TDP-43-induced death is associated with altered regulation of BIM and Bcl-xL and attenuated by caspase-mediated TDP-43 cleavage. *The Journal of biological chemistry* **286**, 13171-13183, doi:10.1074/jbc.M110.197483 (2011).

130 Swarup, V. & Julien, J. P. ALS pathogenesis: recent insights from genetics and mouse models. *Progress in neuro-psychopharmacology & biological psychiatry* **35**, 363-369, doi:10.1016/j.pnpbp.2010.08.006 (2011).

131 Lee, Y. *et al.* Oligodendroglia metabolically support axons and contribute to neurodegeneration. *Nature* **487**, 443-448, doi:10.1038/nature11314 (2012).

132 Suzuki, H. & Matsuoka, M. TDP-43 toxicity is mediated by the unfolded protein response-unrelated induction of C/EBP homologous protein expression. *Journal of neuroscience research* **90**, 641-647, doi:10.1002/jnr.22777 (2012).

133 Borrelli, E., Nestler, E. J., Allis, C. D. & Sassone-Corsi, P. Decoding the Epigenetic Language of Neuronal Plasticity. *Neuron* **60**, 961-974, doi:10.1016/j.neuron.2008.10.012 (2008).

134 Strahl, B. D. & Allis, C. D. The language of covalent histone modifications. *Nature* **403**, 41-45 (2000).

135 Jeong, H. *et al.* Acetylation targets mutant huntingtin to autophagosomes for degradation. *Cell* **137**, 60-72, doi:10.1016/j.cell.2009.03.018 (2009).

136 Bhardwaj, A., Myers, M. P., Buratti, E. & Baralle, F. E. Characterizing TDP-43 interaction with its RNA targets. *Nucleic acids research* **41**, 5062-5074, doi:10.1093/nar/gkt189 (2013).

137 Chambon, P. Summary: the molecular biology of the eukaryotic genome is coming of age. *Cold Spring Harbor symposia on quantitative biology* **42 Pt 2**, 1209-1234 (1978).

138 Jaenisch, R. & Bird, A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nature genetics* **33**, 245-254, doi:10.1038/ng1089 (2003).

139 Hasegawa, M. *et al.* Analysis of RNA metabolism in peripheral WBCs of TDP-43 KI mice identifies novel biomarkers of ALS. *Neuroscience research* **106**, 12-22, doi:10.1016/j.neures.2015.11.009 (2016).

140 Lander, E. S. *et al.* Initial sequencing and analysis of the human genome. *Nature* **409**, 860-921, doi:10.1038/35057062 (2001).

141 Hua-Van, A., Le Rouzic, A., Boutin, T. S., Filee, J. & Capy, P. The struggle for life of the genome's selfish architects. *Biology direct* **6**, 19, doi:10.1186/1745-6150-6-19 (2011).

142 Hancks, D. C. & Kazazian, H. H., Jr. Active human retrotransposons: variation and disease. *Current opinion in genetics & development* **22**, 191-203, doi:10.1016/j.gde.2012.02.006 (2012).

143 Saito, K. & Siomi, M. C. Small RNA-mediated quiescence of transposable elements in animals. *Developmental cell* **19**, 687-697, doi:10.1016/j.devcel.2010.10.011 (2010).

144 Baillie, J. K. *et al.* Somatic retrotransposition alters the genetic landscape of the human brain. *Nature* **479**, 534-537, doi:10.1038/nature10531 (2011).

145 Coufal, N. G. *et al.* L1 retrotransposition in human neural progenitor cells. *Nature* **460**, 1127-1131, doi:10.1038/nature08248 (2009).

146 Muotri, A. R. *et al.* Somatic mosaicism in neuronal precursor cells mediated by L1 retrotransposition. *Nature* **435**, 903-910, doi:10.1038/nature03663 (2005).

147 Muotri, A. R., Zhao, C., Marchetto, M. C. & Gage, F. H. Environmental influence on L1 retrotransposons in the adult hippocampus. *Hippocampus* **19**, 1002-1007, doi:10.1002/hipo.20564 (2009).

148 Greenwood, A. D. *et al.* Bovine spongiform encephalopathy infection alters endogenous retrovirus expression in distinct brain regions of cynomolgus macaques (Macaca fascicularis). *Molecular neurodegeneration* **6**, 44, doi:10.1186/1750-1326-6-44 (2011).

149 Muotri, A. R. *et al.* L1 retrotransposition in neurons is modulated by MeCP2. *Nature* **468**, 443-446, doi:10.1038/nature09544 (2010).

150 Douville, R., Liu, J., Rothstein, J. & Nath, A. Identification of active loci of a human endogenous retrovirus in neurons of patients with amyotrophic lateral sclerosis. *Annals of neurology* **69**, 141-151, doi:10.1002/ana.22149 (2011).

151 Lathe, R. & Harris, A. Differential display detects host nucleic acid motifs altered in scrapie-infected brain. *Journal of molecular biology* **392**, 813-822, doi:10.1016/j.jmb.2009.07.045 (2009).

152 Kaneko, H. *et al.* DICER1 deficit induces Alu RNA toxicity in age-related macular degeneration. *Nature* **471**, 325-330, doi:10.1038/nature09830 (2011).

153 Tan, H. *et al.* Retrotransposon activation contributes to fragile X premutation rCGG-mediated neurodegeneration. *Human molecular genetics* **21**, 57-65, doi:10.1093/hmg/ddr437 (2012).

154 Jeong, B. H., Lee, Y. J., Carp, R. I. & Kim, Y. S. The prevalence of human endogenous retroviruses in cerebrospinal fluids from patients with sporadic Creutzfeldt-Jakob disease. *Journal of clinical virology : the official publication of the Pan American Society for Clinical Virology* **47**, 136-142, doi:10.1016/j.jcv.2009.11.016 (2010).

155 Wang, H. Y., Wang, I. F., Bose, J. & Shen, C. K. Structural diversity and functional implications of the eukaryotic TDP gene family. *Genomics* **83**, 130-139 (2004).

156 Sephton, C. F. *et al.* Identification of neuronal RNA targets of TDP-43-containing ribonucleoprotein complexes. *The Journal of biological chemistry* **286**, 1204-1215, doi:10.1074/jbc.M110.190884 (2011).

157 Tollervey, J. R. *et al.* Characterizing the RNA targets and position-dependent splicing regulation by TDP-43. *Nature neuroscience* **14**, 452-458, doi:10.1038/nn.2778 (2011).
158 Polymenidou, M. *et al.* Long pre-mRNA depletion and RNA missplicing contribute to neuronal vulnerability from loss of TDP-43. *Nature neuroscience* **14**, 459-468, doi:10.1038/nn.2779 (2011).
159 Shan, X., Chiang, P. M., Price, D. L. & Wong, P. C. Altered distributions of Gemini of coiled bodies and mitochondria in motor neurons of TDP-43 transgenic mice. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 16325-16330, doi:10.1073/pnas.1003459107 (2010).
160 Wang, W. *et al.* The inhibition of TDP-43 mitochondrial localization blocks its neuronal toxicity. *Nature medicine* **22**, 869-878, doi:10.1038/nm.4130 (2016).
161 Kroemer, G., Dallaporta, B. & Resche-Rigon, M. The mitochondrial death/life regulator in apoptosis and necrosis. *Annual review of physiology* **60**, 619-642, doi:10.1146/annurev.physiol.60.1.619 (1998).
162 Galluzzi, L. & Kroemer, G. Necroptosis: a specialized pathway of programmed necrosis. *Cell* **135**, 1161-1163, doi:10.1016/j.cell.2008.12.004 (2008).
163 Jaenisch, R. & Bird, A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nature genetics* **33 Suppl**, 245-254, doi:10.1038/ng1089 (2003).
164 Borrelli, E., Nestler, E. J., Allis, C. D. & Sassone-Corsi, P. Decoding the epigenetic language of neuronal plasticity. *Neuron* **60**, 961-974, doi:10.1016/j.neuron.2008.10.012 (2008).
165 Grozinger, C. M. & Schreiber, S. L. Deacetylase enzymes: biological functions and the use of small-molecule inhibitors. *Chemistry & biology* **9**, 3-16 (2002).
166 Ruthenburg, A. J., Li, H., Patel, D. J. & Allis, C. D. Multivalent engagement of chromatin modifications by linked binding modules. *Nature reviews. Molecular cell biology* **8**, 983-994, doi:10.1038/nrm2298 (2007).
167 Cheung, P., Allis, C. D. & Sassone-Corsi, P. Signaling to chromatin through histone modifications. *Cell* **103**, 263-271 (2000).
168 Strahl, B. D. & Allis, C. D. The language of covalent histone modifications. *Nature* **403**, 41-45, doi:10.1038/47412 (2000).
169 Berger, S. L. The complex language of chromatin regulation during transcription. *Nature* **447**, 407-412, doi:10.1038/nature05915 (2007).
170 Ito, K., Barnes, P. J. & Adcock, I. M. Glucocorticoid receptor recruitment of histone deacetylase 2 inhibits interleukin-1beta-induced histone H4 acetylation on lysines 8 and 12. *Molecular and cellular biology* **20**, 6891-6903 (2000).
171 Trojer, P. & Reinberg, D. Histone lysine demethylases and their impact on epigenetics. *Cell* **125**, 213-217, doi:10.1016/j.cell.2006.04.003 (2006).
172 Turner, B. M. Histone acetylation and an epigenetic code. *BioEssays : news and reviews in molecular, cellular and developmental biology* **22**, 836-845, doi:10.1002/1521-1878(200009)22:9<836::AID-BIES9>3.0.CO;2-X (2000).
173 Dhalluin, C. *et al.* Structure and ligand of a histone acetyltransferase bromodomain. *Nature* **399**, 491-496, doi:10.1038/20974 (1999).
174 Jacobson, R. H., Ladurner, A. G., King, D. S. & Tjian, R. Structure and function of a human TAFII250 double bromodomain module. *Science* **288**, 1422-1425 (2000).
175 Richards, E. J. & Elgin, S. C. Epigenetic codes for heterochromatin formation and silencing: rounding up the usual suspects. *Cell* **108**, 489-500 (2002).
176 Levenson, J. M. & Sweatt, J. D. Epigenetic mechanisms in memory formation. *Nature reviews. Neuroscience* **6**, 108-118, doi:10.1038/nrn1604 (2005).
177 Saha, R. N. & Pahan, K. HATs and HDACs in neurodegeneration: a tale of disconcerted

acetylation homeostasis. *Cell death and differentiation* **13**, 539-550, doi:10.1038/sj.cdd.4401769 (2006).

178 Bjerling, P. *et al.* Functional divergence between histone deacetylases in fission yeast by distinct cellular localization and in vivo specificity. *Molecular and cellular biology* **22**, 2170-2181 (2002).

179 Wade, P. A. Transcriptional control at regulatory checkpoints by histone deacetylases: molecular connections between cancer and chromatin. *Human molecular genetics* **10**, 693-698 (2001).

180 Mai, A. *et al.* Histone deacetylation in epigenetics: an attractive target for anticancer therapy. *Medicinal research reviews* **25**, 261-309, doi:10.1002/med.20024 (2005).

181 Whittle, J. R. *et al.* Sirtuins, nuclear hormone receptor acetylation and transcriptional regulation. *Trends in endocrinology and metabolism: TEM* **18**, 356-364, doi:10.1016/j.tem.2007.07.007 (2007).

182 Shakespear, M. R., Halili, M. A., Irvine, K. M., Fairlie, D. P. & Sweet, M. J. Histone deacetylases as regulators of inflammation and immunity. *Trends in immunology* **32**, 335-343, doi:10.1016/j.it.2011.04.001 (2011).

183 Graff, J. & Tsai, L. H. The potential of HDAC inhibitors as cognitive enhancers. *Annual review of pharmacology and toxicology* **53**, 311-330, doi:10.1146/annurev-pharmtox-011112-140216 (2013).

184 Finnin, M. S. *et al.* Structures of a histone deacetylase homologue bound to the TSA and SAHA inhibitors. *Nature* **401**, 188-193, doi:10.1038/43710 (1999).

185 Lahm, A. *et al.* Unraveling the hidden catalytic activity of vertebrate class IIa histone deacetylases. *Proceedings of the National Academy of Sciences of the United States of America* **104**, 17335-17340, doi:10.1073/pnas.0706487104 (2007).

186 Didonna, A. & Opal, P. The promise and perils of HDAC inhibitors in neurodegeneration. *Annals of clinical and translational neurology* **2**, 79-101, doi:10.1002/acn3.147 (2015).

187 Yang, X. J. & Seto, E. Collaborative spirit of histone deacetylases in regulating chromatin structure and gene expression. *Current opinion in genetics & development* **13**, 143-153 (2003).

188 Huang, Y., Myers, S. J. & Dingledine, R. Transcriptional repression by REST: recruitment of Sin3A and histone deacetylase to neuronal genes. *Nature neuroscience* **2**, 867-872, doi:10.1038/13165 (1999).

189 Hayakawa, T. & Nakayama, J. Physiological roles of class I HDAC complex and histone demethylase. *Journal of biomedicine & biotechnology* **2011**, 129383, doi:10.1155/2011/129383 (2011).

190 Galasinski, S. C., Resing, K. A., Goodrich, J. A. & Ahn, N. G. Phosphatase inhibition leads to histone deacetylases 1 and 2 phosphorylation and disruption of corepressor interactions. *The Journal of biological chemistry* **277**, 19618-19626, doi:10.1074/jbc.M201174200 (2002).

191 Pflum, M. K., Tong, J. K., Lane, W. S. & Schreiber, S. L. Histone deacetylase 1 phosphorylation promotes enzymatic activity and complex formation. *The Journal of biological chemistry* **276**, 47733-47741, doi:10.1074/jbc.M105590200 (2001).

192 Cho, Y. & Cavalli, V. HDAC signaling in neuronal development and axon regeneration. *Current opinion in neurobiology* **27**, 118-126, doi:10.1016/j.conb.2014.03.008 (2014).

193 de Ruijter, A. J., van Gennip, A. H., Caron, H. N., Kemp, S. & van Kuilenburg, A. B. Histone deacetylases (HDACs): characterization of the classical HDAC family. *The Biochemical journal* **370**, 737-749, doi:10.1042/BJ20021321 (2003).

194 Guedes-Dias, P. & Oliveira, J. M. Lysine deacetylases and mitochondrial dynamics in neurodegeneration. *Biochimica et biophysica acta* **1832**, 1345-1359,

doi:10.1016/j.bbadis.2013.04.005 (2013).
195 Ellis, J. J. *et al.* CaM kinase IIdeltaC phosphorylation of 14-3-3beta in vascular smooth muscle cells: activation of class II HDAC repression. *Molecular and cellular biochemistry* **242**, 153-161 (2003).
196 Verdel, A. *et al.* Active maintenance of mHDA2/mHDAC6 histone-deacetylase in the cytoplasm. *Current biology : CB* **10**, 747-749 (2000).
197 Hubbert, C. *et al.* HDAC6 is a microtubule-associated deacetylase. *Nature* **417**, 455-458, doi:10.1038/417455a (2002).
198 Cohen, T. J. *et al.* An acetylation switch controls TDP-43 function and aggregation propensity. *Nature communications* **6**, 5845, doi:10.1038/ncomms6845 (2015).
199 Bertos, N. R., Wang, A. H. & Yang, X. J. Class II histone deacetylases: structure, function, and regulation. *Biochemistry and cell biology = Biochimie et biologie cellulaire* **79**, 243-252 (2001).
200 Pandey, U. B. *et al.* HDAC6 rescues neurodegeneration and provides an essential link between autophagy and the UPS. *Nature* **447**, 859-863, doi:10.1038/nature05853 (2007).
201 Blander, G. & Guarente, L. The Sir2 family of protein deacetylases. *Annual review of biochemistry* **73**, 417-435, doi:10.1146/annurev.biochem.73.011303.073651 (2004).
202 Michishita, E., Park, J. Y., Burneskis, J. M., Barrett, J. C. & Horikawa, I. Evolutionarily conserved and nonconserved cellular localizations and functions of human SIRT proteins. *Molecular biology of the cell* **16**, 4623-4635, doi:10.1091/mbc.E05-01-0033 (2005).
203 Ramadori, G. *et al.* Brain SIRT1: anatomical distribution and regulation by energy availability. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **28**, 9989-9996, doi:10.1523/JNEUROSCI.3257-08.2008 (2008).
204 North, B. J., Marshall, B. L., Borra, M. T., Denu, J. M. & Verdin, E. The human Sir2 ortholog, SIRT2, is an NAD+-dependent tubulin deacetylase. *Molecular cell* **11**, 437-444 (2003).
205 Finkel, T., Deng, C. X. & Mostoslavsky, R. Recent progress in the biology and physiology of sirtuins. *Nature* **460**, 587-591, doi:10.1038/nature08197 (2009).
206 Liu, D., Gharavi, R., Pitta, M., Gleichmann, M. & Mattson, M. P. Nicotinamide prevents NAD+ depletion and protects neurons against excitotoxicity and cerebral ischemia: NAD+ consumption by SIRT1 may endanger energetically compromised neurons. *Neuromolecular medicine* **11**, 28-42, doi:10.1007/s12017-009-8058-1 (2009).
207 Liu, H., Hu, Q., Kaufman, A., D'Ercole, A. J. & Ye, P. Developmental expression of histone deacetylase 11 in the murine brain. *Journal of neuroscience research* **86**, 537-543, doi:10.1002/jnr.21521 (2008).
208 Wang, H. *et al.* Histone deacetylase inhibitor LAQ824 augments inflammatory responses in macrophages through transcriptional regulation of IL-10. *J Immunol* **186**, 3986-3996, doi:10.4049/jimmunol.1001101 (2011).
209 Janssen, C. *et al.* Differential histone deacetylase mRNA expression patterns in amyotrophic lateral sclerosis. *Journal of neuropathology and experimental neurology* **69**, 573-581, doi:10.1097/NEN.0b013e3181ddd404 (2010).
210 Kim, J. Y. *et al.* HDAC1 nuclear export induced by pathological conditions is essential for the onset of axonal damage. *Nature neuroscience* **13**, 180-189, doi:10.1038/nn.2471 (2010).
211 Hammond, J. W., Cai, D. & Verhey, K. J. Tubulin modifications and their cellular functions. *Current opinion in cell biology* **20**, 71-76, doi:10.1016/j.ceb.2007.11.010 (2008).
212 Chen, S., Owens, G. C., Makarenkova, H. & Edelman, D. B. HDAC6 regulates mitochondrial transport in hippocampal neurons. *PloS one* **5**, e10848, doi:10.1371/journal.pone.0010848 (2010).
213 Suzuki, K. & Koike, T. Mammalian Sir2-related protein (SIRT) 2-mediated modulation of

resistance to axonal degeneration in slow Wallerian degeneration mice: a crucial role of tubulin deacetylation. *Neuroscience* **147**, 599-612, doi:10.1016/j.neuroscience.2007.04.059 (2007).

214 Bilsland, L. G. *et al.* Deficits in axonal transport precede ALS symptoms in vivo. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 20523-20528, doi:10.1073/pnas.1006869107 (2010).

215 Taes, I. *et al.* Hdac6 deletion delays disease progression in the SOD1G93A mouse model of ALS. *Human molecular genetics* **22**, 1783-1790, doi:10.1093/hmg/ddt028 (2013).

216 Bardai, F. H., Price, V., Zaayman, M., Wang, L. & D'Mello, S. R. Histone deacetylase-1 (HDAC1) is a molecular switch between neuronal survival and death. *The Journal of biological chemistry* **287**, 35444-35453, doi:10.1074/jbc.M112.394544 (2012).

217 Chuang, D. M., Leng, Y., Marinova, Z., Kim, H. J. & Chiu, C. T. Multiple roles of HDAC inhibition in neurodegenerative conditions. *Trends in neurosciences* **32**, 591-601, doi:10.1016/j.tins.2009.06.002 (2009).

218 Yabe, J. T., Pimenta, A. & Shea, T. B. Kinesin-mediated transport of neurofilament protein oligomers in growing axons. *Journal of cell science* **112 (Pt 21)**, 3799-3814 (1999).

219 Pant, H. C. Dephosphorylation of neurofilament proteins enhances their susceptibility to degradation by calpain. *The Biochemical journal* **256**, 665-668 (1988).

220 Williamson, T. L. & Cleveland, D. W. Slowing of axonal transport is a very early event in the toxicity of ALS-linked SOD1 mutants to motor neurons. *Nature neuroscience* **2**, 50-56, doi:10.1038/4553 (1999).

221 Kazantsev, A. G. & Thompson, L. M. Therapeutic application of histone deacetylase inhibitors for central nervous system disorders. *Nature reviews. Drug discovery* **7**, 854-868, doi:10.1038/nrd2681 (2008).

222 Kramer, O. H., Gottlicher, M. & Heinzel, T. Histone deacetylase as a therapeutic target. *Trends in endocrinology and metabolism: TEM* **12**, 294-300 (2001).

223 Lazo-Gomez, R., Ramirez-Jarquin, U. N., Tovar, Y. R. L. B. & Tapia, R. Histone deacetylases and their role in motor neuron degeneration. *Frontiers in cellular neuroscience* **7**, 243, doi:10.3389/fncel.2013.00243 (2013).

224 Valle, C. *et al.* Tissue-specific deregulation of selected HDACs characterizes ALS progression in mouse models: pharmacological characterization of SIRT1 and SIRT2 pathways. *Cell death & disease* **5**, e1296, doi:10.1038/cddis.2014.247 (2014).

225 Montgomery, R. L., Hsieh, J., Barbosa, A. C., Richardson, J. A. & Olson, E. N. Histone deacetylases 1 and 2 control the progression of neural precursors to neurons during brain development. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 7876-7881, doi:10.1073/pnas.0902750106 (2009).

226 Harrison, M. R., Georgiou, A. S., Spaink, H. P. & Cunliffe, V. T. The epigenetic regulator Histone Deacetylase 1 promotes transcription of a core neurogenic programme in zebrafish embryos. *BMC genomics* **12**, 24, doi:10.1186/1471-2164-12-24 (2011).

227 Tahirovic, S. & Bradke, F. Neuronal polarity. *Cold Spring Harbor perspectives in biology* **1**, a001644, doi:10.1101/cshperspect.a001644 (2009).

228 Zhang, X. *et al.* HDAC6 modulates cell motility by altering the acetylation level of cortactin. *Molecular cell* **27**, 197-213, doi:10.1016/j.molcel.2007.05.033 (2007).

229 Ageta-Ishihara, N. *et al.* Septins promote dendrite and axon development by negatively regulating microtubule stability via HDAC6-mediated deacetylation. *Nature communications* **4**, 2532, doi:10.1038/ncomms3532 (2013).

230 Kim, A. H. *et al.* A centrosomal Cdc20-APC pathway controls dendrite morphogenesis in postmitotic neurons. *Cell* **136**, 322-336, doi:10.1016/j.cell.2008.11.050 (2009).

231 Erturk, A., Hellal, F., Enes, J. & Bradke, F. Disorganized microtubules underlie the

formation of retraction bulbs and the failure of axonal regeneration. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **27**, 9169-9180, doi:10.1523/JNEUROSCI.0612-07.2007 (2007).

232 Bradke, F., Fawcett, J. W. & Spira, M. E. Assembly of a new growth cone after axotomy: the precursor to axon regeneration. *Nature reviews. Neuroscience* **13**, 183-193, doi:10.1038/nrn3176 (2012).

233 Cho, Y. & Cavalli, V. HDAC5 is a novel injury-regulated tubulin deacetylase controlling axon regeneration. *The EMBO journal* **31**, 3063-3078, doi:10.1038/emboj.2012.160 (2012).

234 Zhang, Y. *et al.* Deacetylation of cortactin by SIRT1 promotes cell migration. *Oncogene* **28**, 445-460, doi:10.1038/onc.2008.388 (2009).

235 Robberecht, W. & Philips, T. The changing scene of amyotrophic lateral sclerosis. *Nature reviews. Neuroscience* **14**, 248-264, doi:10.1038/nrn3430 (2013).

236 Corcoran, L. J., Mitchison, T. J. & Liu, Q. A novel action of histone deacetylase inhibitors in a protein aggresome disease model. *Current biology : CB* **14**, 488-492, doi:10.1016/j.cub.2004.03.003 (2004).

237 Kanai, H., Sawa, A., Chen, R. W., Leeds, P. & Chuang, D. M. Valproic acid inhibits histone deacetylase activity and suppresses excitotoxicity-induced GAPDH nuclear accumulation and apoptotic death in neurons. *The pharmacogenomics journal* **4**, 336-344, doi:10.1038/sj.tpj.6500269 (2004).

238 Bertrand, P. Inside HDAC with HDAC inhibitors. *European journal of medicinal chemistry* **45**, 2095-2116, doi:10.1016/j.ejmech.2010.02.030 (2010).

239 Yoo, Y. E. & Ko, C. P. Treatment with trichostatin A initiated after disease onset delays disease progression and increases survival in a mouse model of amyotrophic lateral sclerosis. *Experimental neurology* **231**, 147-159, doi:10.1016/j.expneurol.2011.06.003 (2011).

240 Rouaux, C. *et al.* Sodium valproate exerts neuroprotective effects in vivo through CREB-binding protein-dependent mechanisms but does not improve survival in an amyotrophic lateral sclerosis mouse model. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **27**, 5535-5545, doi:10.1523/JNEUROSCI.1139-07.2007 (2007).

241 Ryu, H. *et al.* Sodium phenylbutyrate prolongs survival and regulates expression of anti-apoptotic genes in transgenic amyotrophic lateral sclerosis mice. *Journal of neurochemistry* **93**, 1087-1098, doi:10.1111/j.1471-4159.2005.03077.x (2005).

242 Batty, N., Malouf, G. G. & Issa, J. P. Histone deacetylase inhibitors as anti-neoplastic agents. *Cancer letters* **280**, 192-200, doi:10.1016/j.canlet.2009.03.013 (2009).

243 Yoshida, M. *et al.* Histone deacetylase as a new target for cancer chemotherapy. *Cancer chemotherapy and pharmacology* **48 Suppl 1**, S20-26 (2001).

244 Cohen, T. J. *et al.* The acetylation of tau inhibits its function and promotes pathological tau aggregation. *Nature communications* **2**, 252, doi:10.1038/ncomms1255 (2011).

245 Cook, C. *et al.* Acetylation of the KXGS motifs in tau is a critical determinant in modulation of tau aggregation and clearance. *Human molecular genetics* **23**, 104-116, doi:10.1093/hmg/ddt402 (2014).

246 Irwin, D. J. *et al.* Acetylated tau neuropathology in sporadic and hereditary tauopathies. *The American journal of pathology* **183**, 344-351, doi:10.1016/j.ajpath.2013.04.025 (2013).

247 Irwin, D. J. *et al.* Acetylated tau, a novel pathological signature in Alzheimer's disease and other tauopathies. *Brain : a journal of neurology* **135**, 807-818, doi:10.1093/brain/aws013 (2012).

248 Min, S. W. *et al.* Acetylation of tau inhibits its degradation and contributes to tauopathy. *Neuron* **67**, 953-966, doi:10.1016/j.neuron.2010.08.044 (2010).

249 Bhardwaj, A., Myers, M. P., Buratti, E. & Baralle, F. E. Characterizing TDP-43 interaction with its RNA targets. *Nucleic acids research* **41**, 5062-5074, doi:10.1093/nar/gkt189 (2013).

250 Choksi, D. K. *et al.* TDP-43 Phosphorylation by casein kinase Iepsilon promotes oligomerization and enhances toxicity in vivo. *Human molecular genetics* **23**, 1025-1035, doi:10.1093/hmg/ddt498 (2014).

251 Hasegawa, M. *et al.* Phosphorylated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis. *Annals of neurology* **64**, 60-70, doi:10.1002/ana.21425 (2008).

252 Kametani, F. *et al.* Identification of casein kinase-1 phosphorylation sites on TDP-43. *Biochemical and biophysical research communications* **382**, 405-409, doi:10.1016/j.bbrc.2009.03.038 (2009).

253 Liachko, N. F. *et al.* CDC7 inhibition blocks pathological TDP-43 phosphorylation and neurodegeneration. *Annals of neurology* **74**, 39-52, doi:10.1002/ana.23870 (2013).

254 Wang, P., Wander, C. M., Yuan, C. X., Bereman, M. S. & Cohen, T. J. Acetylation-induced TDP-43 pathology is suppressed by an HSF1-dependent chaperone program. *Nature communications* **8**, 82, doi:10.1038/s41467-017-00088-4 (2017).

255 Esposito, S. *et al.* Redox-sensitive GFP to monitor oxidative stress in neurodegenerative diseases. *Reviews in the neurosciences* **28**, 133-144, doi:10.1515/revneuro-2016-0041 (2017).

256 Sambrook, J. & Russell, D. W. S. *The condensed protocols from molecular cloning: a laboratory manual*. (2006).

257 Bruhat, A. *et al.* Amino acids control mammalian gene transcription: activating transcription factor 2 is essential for the amino acid responsiveness of the CHOP promoter. *Molecular and cellular biology* **20**, 7192-7204 (2000).

258 Ayala, Y. M. *et al.* Structural determinants of the cellular localization and shuttling of TDP-43. *Journal of cell science* **121**, 3778-3785, doi:10.1242/jcs.038950 (2008).

259 Kovacs, G. *et al.* TARDBP variation associated with frontotemporal dementia, supranuclear gaze palsy, and chorea. *Mov Disord* **24**, 1843-1847, doi:10.1002/mds.22697 (2009).

260 Guo, X. *et al.* Subcellular Localization of Class I Histone Deacetylases in the Developing Xenopus tectum. *Frontiers in cellular neuroscience* **9**, 510, doi:10.3389/fncel.2015.00510 (2015).

261 Sasaki, S., Takeda, T., Shibata, N. & Kobayashi, M. Alterations in subcellular localization of TDP-43 immunoreactivity in the anterior horns in sporadic amyotrophic lateral sclerosis. *Neuroscience letters* **478**, 72-76, doi:10.1016/j.neulet.2010.04.068 (2010).

262 Lalmansingh, A. S., Urekar, C. J. & Reddi, P. P. TDP-43 is a transcriptional repressor: the testis-specific mouse acrv1 gene is a TDP-43 target in vivo. *The Journal of biological chemistry* **286**, 10970-10982, doi:10.1074/jbc.M110.166587 (2011).

263 Fiesel, F. C., Weber, S. S., Supper, J., Zell, A. & Kahle, P. J. TDP-43 regulates global translational yield by splicing of exon junction complex component SKAR. *Nucleic acids research* **40**, 2668-2682, doi:10.1093/nar/gkr1082 (2012).

264 Shiga, A. *et al.* Alteration of POLDIP3 splicing associated with loss of function of TDP-43 in tissues affected with ALS. *PloS one* **7**, e43120, doi:10.1371/journal.pone.0043120 (2012).

265 Ash, P. E. *et al.* Neurotoxic effects of TDP-43 overexpression in C. elegans. *Human molecular genetics* **19**, 3206-3218, doi:10.1093/hmg/ddq230 (2010).

266 Estes, P. S. *et al.* Wild-type and A315T mutant TDP-43 exert differential neurotoxicity in a Drosophila model of ALS. *Human molecular genetics* **20**, 2308-2321, doi:10.1093/hmg/ddr124 (2011).

267 Miguel, L., Frebourg, T., Campion, D. & Lecourtois, M. Both cytoplasmic and nuclear accumulations of the protein are neurotoxic in Drosophila models of TDP-43 proteinopathies. *Neurobiology of disease* **41**, 398-406, doi:10.1016/j.nbd.2010.10.007 (2011).

268 Petri, S. *et al.* Additive neuroprotective effects of a histone deacetylase inhibitor and a catalytic antioxidant in a transgenic mouse model of amyotrophic lateral sclerosis. *Neurobiology of disease* **22**, 40-49, doi:10.1016/j.nbd.2005.09.013 (2006).

269 Falkenberg, K. J. & Johnstone, R. W. Histone deacetylases and their inhibitors in cancer, neurological diseases and immune disorders. *Nature reviews. Drug discovery* **13**, 673-691, doi:10.1038/nrd4360 (2014).

270 Kim, D. *et al.* SIRT1 deacetylase protects against neurodegeneration in models for Alzheimer's disease and amyotrophic lateral sclerosis. *The EMBO journal* **26**, 3169-3179, doi:10.1038/sj.emboj.7601758 (2007).

271 Govindarajan, N., Agis-Balboa, R. C., Walter, J., Sananbenesi, F. & Fischer, A. Sodium butyrate improves memory function in an Alzheimer's disease mouse model when administered at an advanced stage of disease progression. *Journal of Alzheimer's disease : JAD* **26**, 187-197, doi:10.3233/JAD-2011-110080 (2011).

272 Kontopoulos, E., Parvin, J. D. & Feany, M. B. Alpha-synuclein acts in the nucleus to inhibit histone acetylation and promote neurotoxicity. *Human molecular genetics* **15**, 3012-3023, doi:10.1093/hmg/ddl243 (2006).

273 Ferrante, R. J. *et al.* Histone deacetylase inhibition by sodium butyrate chemotherapy ameliorates the neurodegenerative phenotype in Huntington's disease mice. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **23**, 9418-9427 (2003).

274 Cudkowicz, M. E. *et al.* Phase 2 study of sodium phenylbutyrate in ALS. *Amyotrophic lateral sclerosis : official publication of the World Federation of Neurology Research Group on Motor Neuron Diseases* **10**, 99-106, doi:10.1080/17482960802320487 (2009).

275 Roux, J. C., Dura, E., Moncla, A., Mancini, J. & Villard, L. Treatment with desipramine improves breathing and survival in a mouse model for Rett syndrome. *The European journal of neuroscience* **25**, 1915-1922, doi:10.1111/j.1460-9568.2007.05466.x (2007).

276 Butler, R. & Bates, G. P. Histone deacetylase inhibitors as therapeutics for polyglutamine disorders. *Nature reviews. Neuroscience* **7**, 784-796, doi:10.1038/nrn1989 (2006).

277 Li, Y., Guo, Y., Tang, J., Jiang, J. & Chen, Z. New insights into the roles of CHOP-induced apoptosis in ER stress. *Acta biochimica et biophysica Sinica* **47**, 146-147, doi:10.1093/abbs/gmu128 (2015).

278 Hetz, C. & Mollereau, B. Disturbance of endoplasmic reticulum proteostasis in neurodegenerative diseases. *Nature reviews. Neuroscience* **15**, 233-249, doi:10.1038/nrn3689 (2014).

279 Prasanthi, J. R., Larson, T., Schommer, J. & Ghribi, O. Silencing GADD153/CHOP gene expression protects against Alzheimer's disease-like pathology induced by 27-hydroxycholesterol in rabbit hippocampus. *PloS one* **6**, e26420, doi:10.1371/journal.pone.0026420 (2011).

280 Marciniak, S. J. *et al.* CHOP induces death by promoting protein synthesis and oxidation in the stressed endoplasmic reticulum. *Genes & development* **18**, 3066-3077, doi:10.1101/gad.1250704 (2004).

281 Takeda, A., Sugeno, N., Hasegawa, T., Kobayashi, M. & Kikuchi, A. [Cellular pathophysiology of Parkinson's disease]. *Rinsho shinkeigaku = Clinical neurology* **48**, 984-985 (2008).

282 Ito, Y. *et al.* Involvement of CHOP, an ER-stress apoptotic mediator, in both human sporadic ALS and ALS model mice. *Neurobiology of disease* **36**, 470-476,

doi:10.1016/j.nbd.2009.08.013 (2009).

283 Fifita, J. A. *et al.* A novel amyotrophic lateral sclerosis mutation in OPTN induces ER stress and Golgi fragmentation in vitro. *Amyotrophic lateral sclerosis & frontotemporal degeneration* **18**, 126-133, doi:10.1080/21678421.2016.1218517 (2017).

284 Tokutake, Y., Gushima, K., Miyazaki, H., Shimosato, T. & Yonekura, S. ALS-associated P56S-VAPB mutation restrains 3T3-L1 preadipocyte differentiation. *Biochemical and biophysical research communications* **460**, 831-837, doi:10.1016/j.bbrc.2015.03.118 (2015).

285 Main, B. J., Dunlop, R. A. & Rodgers, K. J. The use of L-serine to prevent beta-methylamino-L-alanine (BMAA)-induced proteotoxic stress in vitro. *Toxicon : official journal of the International Society on Toxinology* **109**, 7-12, doi:10.1016/j.toxicon.2015.11.003 (2016).

286 Prpar Mihevc, S., Baralle, M., Buratti, E. & Rogelj, B. TDP-43 aggregation mirrors TDP-43 knockdown, affecting the expression levels of a common set of proteins. *Scientific reports* **6**, 33996, doi:10.1038/srep33996 (2016).

287 Mompean, M., Baralle, M., Buratti, E. & Laurents, D. V. An Amyloid-Like Pathological Conformation of TDP-43 Is Stabilized by Hypercooperative Hydrogen Bonds. *Frontiers in molecular neuroscience* **9**, 125, doi:10.3389/fnmol.2016.00125 (2016).

288 Smethurst, P., Sidle, K. C. & Hardy, J. Review: Prion-like mechanisms of transactive response DNA binding protein of 43 kDa (TDP-43) in amyotrophic lateral sclerosis (ALS). *Neuropathology and applied neurobiology* **41**, 578-597, doi:10.1111/nan.12206 (2015).

289 Xu, Y. F. *et al.* Wild-type human TDP-43 expression causes TDP-43 phosphorylation, mitochondrial aggregation, motor deficits, and early mortality in transgenic mice. *The Journal of neuroscience : the official journal of the Society for Neuroscience* **30**, 10851-10859, doi:10.1523/JNEUROSCI.1630-10.2010 (2010).

290 Wils, H. *et al.* TDP-43 transgenic mice develop spastic paralysis and neuronal inclusions characteristic of ALS and frontotemporal lobar degeneration. *Proceedings of the National Academy of Sciences of the United States of America* **107**, 3858-3863, doi:10.1073/pnas.0912417107 (2010).

291 Soo, K. Y. *et al.* Bim links ER stress and apoptosis in cells expressing mutant SOD1 associated with amyotrophic lateral sclerosis. *PloS one* **7**, e35413, doi:10.1371/journal.pone.0035413 (2012).

292 Hahnen, E. *et al.* Histone deacetylase inhibitors: possible implications for neurodegenerative disorders. *Expert opinion on investigational drugs* **17**, 169-184, doi:10.1517/13543784.17.2.169 (2008).

293 Iannitti, T. & Palmieri, B. Clinical and experimental applications of sodium phenylbutyrate. *Drugs in R&D* **11**, 227-249, doi:10.2165/11591280-000000000-00000 (2011).

294 Safety and efficacy of edaravone in well defined patients with amyotrophic lateral sclerosis: a randomised, double-blind, placebo-controlled trial. *The Lancet. Neurology* **16**, 505-512, doi:10.1016/S1474-4422(17)30115-1 (2017).