UNIVERSITY OF SASSARI

PhD School in Biomolecular and Biotechnological Sciences

Curriculum in Physiology, Biochemistry and Molecular Biology

XXVIII cycle

Epigenetic modifications associated with Amyotrophic Lateral Sclerosis (ALS) onset and progression

***Supervisor*: Prof. Claudia Crosio**

***PhD School Director*: Prof. Leonardo Sechi**

***PhD Student*: Alessandra Masala**

Academic year 2016/2017

ABSTRACT

In the recent years a growing amount of evidence indicates the functional significance of epigenetics in various aspects of neural function and dysfunction. Alterations in chromatin structure resulting in long lasting changes in gene expression have been associated to many different aspects of neuronal biology, including neurodegenerative disorders such Amyotrophic Lateral Sclerosis (ALS). ALS is predominantly sporadic and environmental triggers may be involved in disease initiation. In this respect, the epigenome can provide the key to transform the genetic information into phenotype. Alterations in the epigenetic machinery and/or epigenetic modifications can be considered a readout of disease onset and progression and an ideal target for therapeutic interventions. My PhD thesis is, indeed, focused on different aspect of epigenetic modifications in ALS cellular and animal models. By in vitro and in vivo assays I demonstrate that some epigenetic markers, linked to transcriptional activation or repression, are selectively altered in cellular and animal model of ALS. Moreover I observed a physical interaction between the ALS-causing gene TDP43 and the histone deacetylases 1 (HDAC1). Finally, I demonstrate that perturbation of HDAC1 level or activity affects TDP-43-induced cell damage. Although preliminary, these data can be extended by testing more specific HDAC1 inhibitors which can be chemically optimized by computational biology approaches.

1. INTRODUCTION

1.1 AMYOTHOPHIC LATERAL SCLEROSIS (ALS)

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disorder characterized by the death of motor neurons in the brain, brainstem, and spinal cord, resulting in fatal paralysis. ALS usually begins with asymmetric involvement of the muscles of one or more limbs, speech or deglutition in middle adult life. The involvement appears to spread in an anatomically contiguous manner and eventually becomes bilateral and symmetrical, progressing to paralysis and death. Mean survival is 3 years after the onset of symptoms (Chio, Logroscino et al. 2009). Classical clinical classification proposed three phenotypic forms of familial ALS, each inherited as an autosomal dominant disorder. The first form they delineated is characterized by rapidly progressive loss of motor function with predominantly lower motor neuron manifestations and a course of less than 5 years. Pathologic changes are limited to the anterior horn cells and pyramidal tracts. The second form is clinically identical to the first, but at autopsy additional changes are found in the posterior columns, Clarke column, and spinocerebellar tracts. The third form is similar to the second except for a much longer survival, usually beyond 10 and often 20 years (Meyer, Schwan et al. 2005).

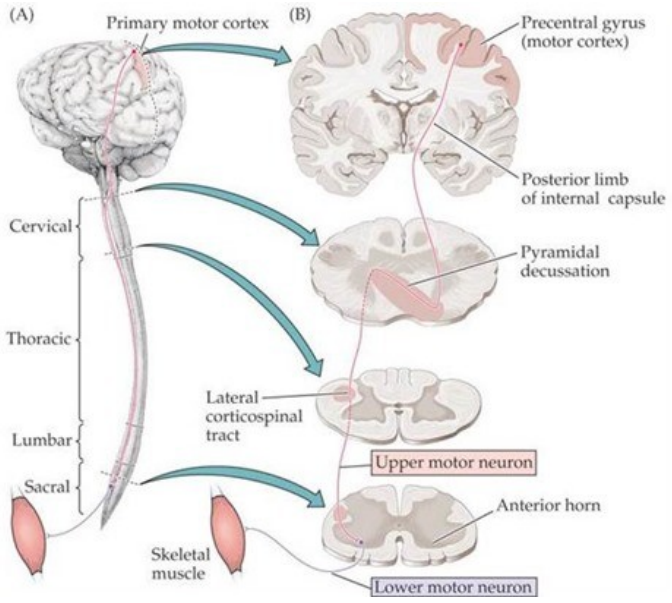


Figure 1. Motor neurons selectively affected in ALS. Degeneration of motor neurons in the motor cortex leads to clinically apparent signs of upper motor neuron abnormalities. Degeneration of motor neurons in the brain stem and spinal cord causes muscle atrophy, weakness and fasciculation.

Anyway, more recently, clinicians prefer to analyze the complete phenotypic criteria and to formulate a diagnosis basing on continuous variations on the severity of the symptoms, without distinguishing in ALS forms (Rutter-Locher, Turner et al. 2016).

Al Chalabi and colleagues proposed a new classification method that combines the classical diagnostic approach based on El Escorial categories with the rich variety of phenotypic descriptions (Al-Chalabi, Hardiman et al. 2016).

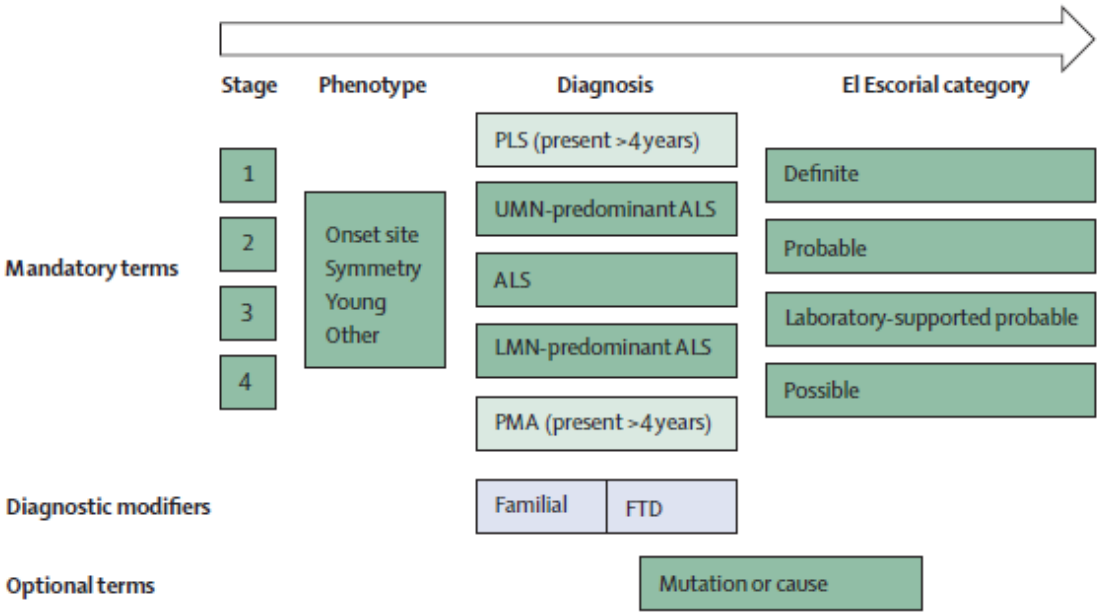


Figure 2. Classification system for amyotrophic lateral sclerosis (Al-Chalabi, Hardiman et al. 2016)

Population based studies have established that the incidence of ALS in Europe is fairly uniform at 2,16 per 100 000 person-years (Logroscino, Traynor et al. 2010). Although ALS affects people worldwide, an exact incidence of this disease is not yet known. Men have a higher incidence of disease (3,0 per 100 000 person-years; 95% CI 2,8–3,3) than do women (2,4 per 100 000 person-years; 95% CI 2,2–2,6), although the incidence between men and women is about the same in familial disease.

1.1.1 ALS etiology: a multifactorial perspective

ALS etiology has been recognized to be multifactorial (Vucic and Kiernan 2009). It occurs as an apparently sporadic disease (sALS) in 90% of cases; while the residual10% of ALS cases are familial (fALS) and linked to a specific gene mutation (Beleza-

Meireles and Al-Chalabi 2009). In sALS, environmental factors may contribute to the pathology, i.e. lesions in frontotemporal lobes or exposition to some pesticides like organochlorine insecticides aldrin or toxaphene have been linked to the development of the disease (Kamel, Umbach et al. 2012).

1.1.2 Genetic factors

Approximately 10% of ALS is classified as familial, whereas the remaining 90% of the cases are considered sporadic, as they appear to occur randomly throughout the community.

Since now, 22 locus have been linked to the development of ALS. Among them, the most studied are those displayed in table 1.

Genes known to carry ALS-causing mutations					
			Percentage explained		Putative protein function
Gene	Location	Inheritance	Familial ALS	Sporadic ALS	
TARDBP	1p36	AD	4	1	RNA metabolism
SQSTM1	5q35	AD	1	<1	Ubiquitination; autophagy
C9ORF72	9p21	AD	40	7	DENN protein
VCP	9p13	AD	1	1	Proteasome;vesicle trafficking
OPTN	10p13	AR and AD	<1	<1	Vesicle trafficking
FUS	16p11	AD and AR	4	1	RNA metabolism
PFN1	17p13	AD	<1	<1	Cytoskeletal dynamics
SOD1	21q22	AD and AR	12	1–2	Superoxide metabolism
UBQLN2	Xp11	XD	<1	<1	Proteasome
Others<1					

Table1.Principal gene locus involved in ALS and relative mutations.

SOD1 is the first historically discovered ALS causing gene (Rosen, Siddique et al. 1993). Over than 200 point mutations have been reported on SOD1 gene. Nevertheless, SOD1 mutations account for the 12% of fALS and for the 1% of sALS.

Locus 16p11 and locus 1p36 encode respectively for FUS and TDP43 protein, both involved in RNA metabolism. Together they are linked with the 8% of all fALS.

In 2009 two research groups reported that a massive hexanucleotide repeat expansion in *C9ORF72* is the cause of chromosome 9p21–linked ALS and FTD (DeJesus-Hernandez, Mackenzie et al. 2011, Renton, Majounie et al. 2011). This pathogenic expansion accounts for a remarkable percentage of both familial ALS (~40%) and

familial FTD (~25%). In addition, the repeat expansion has been found to account ~7% of apparently sporadic ALS cases in people of European ancestry, marking the first time that a genetic etiology has been identified for more than just the occasional sporadic case (Renton, Chio et al. 2014).

1.1.2.1 ALS1: Superoxide dismutase 1 (SOD1)

21q22 gene locus encodes for a 32 kD homodimeric protein called Cu/Zn SOD1. It is the major cytoplasmic antioxidant enzyme that metabolizes superoxide radicals to molecular oxygen and hydrogen peroxide, thus providing a defense against oxygen toxicity. It forms a β-barrel and contains an intramolecular disulfide bond and a binuclear Cu/Zn site in each subunit. This Cu/Zn site holds the copper and a zinc ion and is responsible for catalyzing the disproportion of superoxide to hydrogen peroxide and dioxygen (Niwa, Yamada et al. 2007).

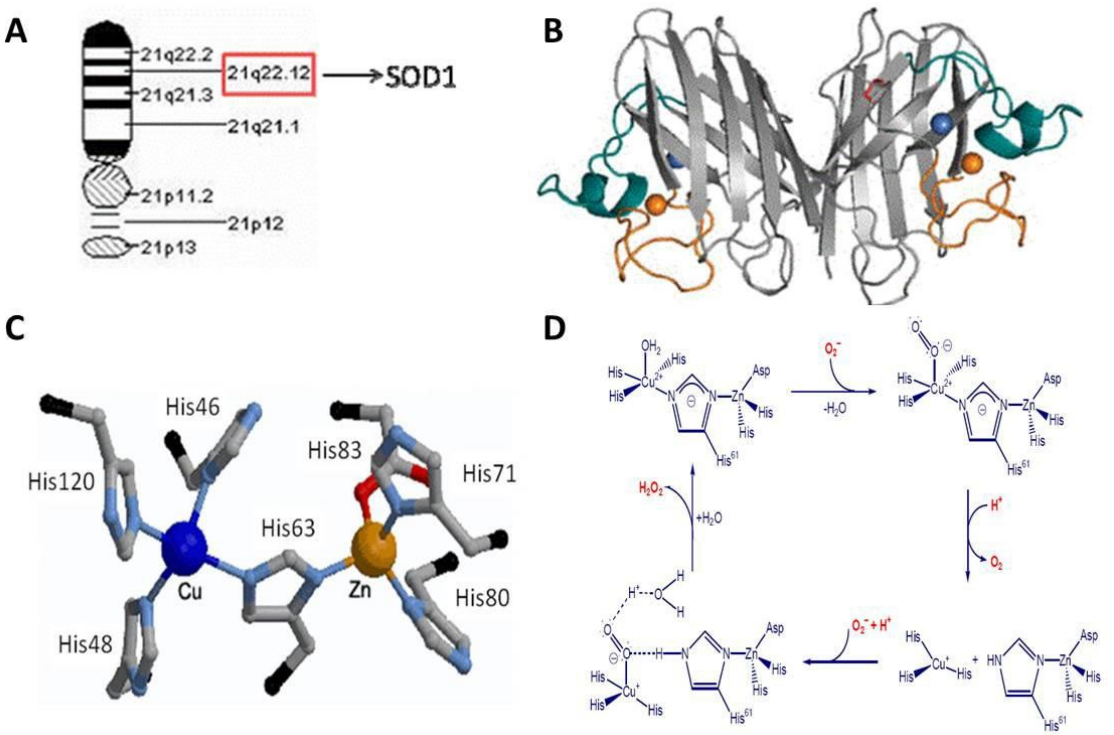


Figure 3.Structure and function of SOD1.A) SOD1 gene position on chromosome 21. B) Tridimensional structure of human SOD1. C) Active site of Cu/Zn SOD1. D) Mechanism cyclic oxidation-reduction catalyzed by SOD1.

Since the first description in 1993, more than 200 point mutations have been described, even if only a small percentage of them have been clearly linked to the pathology. In fALS linked to SOD1 mutations, the majority has a dominant inheritance, like G93A or H80R (Rosen, Siddique et al. 1993, Alexander, Traynor et al. 2002).

The pathological mechanism linked to SOD1 is complex. Since some mutations concern the metal binding residues at the active site, while others may concern correct folding or stability of the homodimer, the biochemical and biophysical properties of ALS-associated mutant SOD1 proteins are rather heterogeneous. In fact, studies on recombinant mutant SOD1 proteins have been proved that ALS-associated SOD1 mutations can be attributed at two mutant classes, the "wild-type like" (WTL) SOD1 mutants which retain the ability to bind copper and zinc and exhibit normal specific activity, indicate a native-like structure with only subtle changes to the backbone fold, in contrast the "metal-binding region" (MBR) SOD1 mutants that are deficient in copper and zinc and exhibit severe thermal destabilization and structural disorder of conserved loops near the metal-binding sites. G93A and H80R-SOD1 mutants belong respectively to the two classes of mutants described above (Tiwari and Hayward 2005).

To date, different hypothesis have been made to explain SOD1 toxicity. The aggregation hypothesis is particularly attractive because protein aggregates are frequently associated with neurodegenerative diseases such as ALS. Numerous studies revealed that SOD1 mutant is incline to misfolding and to form cytoplasmic aggregates (Rotunno and Bosco 2013). In turn, these aggregates could lead to cell death by sequestering other cytoplasmic proteins essential for neuronal survival, by clogging the ubiquitin/proteasome system, by chaperones depletion, or by disrupting mitochondria, cytoskeleton and/or axonal transport. Another hypothesis is that SOD1 misfolding induced by mutations would allow the access of abnormal substrates such as peroxynitrite to the catalytic site leading to the nitration of tyrosine residues (Beckman, Carson et al. 1993, Rotunno and Bosco 2013).

1.1.2.2 ALS6: FUS

Mutations in 16p11.2 locus encoding for FUS protein have been linked to a familiar form of ALS (Kwiatkowski, Bosco et al. 2009). FUS is a 75 kDa nuclear protein. It is ubiquitously expressed. FUS/TLS is a 526 amino acid protein encoded by 15 exons and

characterized by an N-terminal domain enriched in glutamine, glycine, serine and tyrosine residues (QGSY region), a glycine-rich region, an RRM domain, multiple arginine/glycine/glycine (RGG) repeats in an arginine and glycine-rich region and a C-terminal zinc finger motif. Most of the mutations are clustered in the glycine-rich region and in the extreme C-terminal part of the protein with evidence for mutations in each of the five arginine residues (Deng, Gao et al. 2014). Thirty mutations have now been reported in 4% of familial ALS and in rare sporadic patients with no apparent familial history. The inheritance pattern is dominant except for one recessive mutation (H517Q) found in a family of Cape Verdean origin. Most are missense mutations with a few exceptions (Kwiatkowski, Bosco et al. 2009).

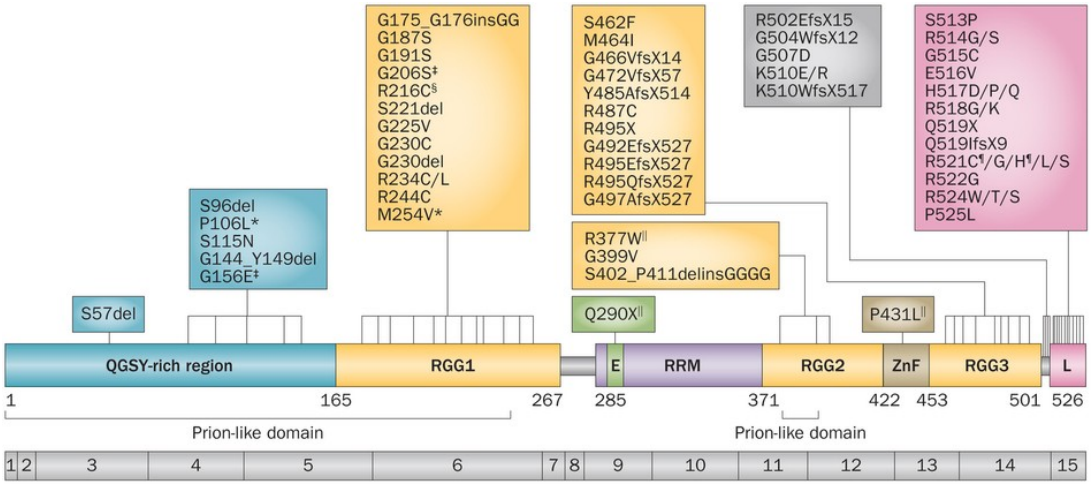


Figure 4.Genomic and structural organization of human FUS gene and protein.FUS gene is encoded by 15 exons that cover an 11.6 kb region on chromosome 16p11.2. QGSY rich: serine–tyrosine–glycine–glutamine rich domain; RRM: RNA recognition motif; ZnF: cysteine2/cysteine2 zinc finger motif; RGG rich: arginine–glycine–glycine rich domain.(Adapted from(Deng, Gao et al. 2014)

FUS functions are multiple and not completely understood. Many studies enlighten that FUS binds to single-strand and double-strand RNA; FUS seems to act at various levels in RNA metabolism, including transcription, splicing and translation (Prasad, Ouchida et al. 1994). In addition, FUS acts as a transcriptional regulator. FUS associates with products of RNA polymerase II transcription, forms complexes with hnRNPs, and represses RNA polymerase III transcription. Furthermore, FUS inhibits the acetyltransferase activities of CREB-binding protein (CBP) and p300 on cyclin D1 (CCND1) (Wang, Arai et al. 2008) and regulates the transcription factor nuclear factor kB (NF-kB) (Goransson, Andersson et al. 2009). FUS also engages in rapid nucleo-cytoplasmic shuttling, associates with actin-dependent motor protein myosin Va

(MyoVa) (Yoshimura, Fujii et al. 2006), and is a component of RNA granules that transport mRNAs (Belly, Moreau-Gachelin et al. 2005).

Many insights into FUS function come out studying FUS interactome with different experimental approach in a variety of models. FUS has been demonstrated to directly bind (i) to the U1-snRNP, reducing RNA processing, and SMN complexes activity; (Sun, Ling et al. 2015), (ii) to the C-terminal domain (CTD) of RNA polymerase II (RNA Pol II) in an RNA-dependent manner (Schwartz, Ebmeier et al. 2012), (iii) to the heterogeneous nuclear ribonucleoprotein (hnRNP) A1 and hnRNPA2B1 (Takanashi and Yamaguchi 2014), (iv) to histone deacetylase 1 (HDCA1) during DNA repair (Wang, Pan et al. 2013).

1.1.2.3 ALS10: TDP43

TDP-43 is a multifunctional protein involved in gene expression and regulation, including transcription, RNA splicing, transport, and translation. It is ubiquitously expressed, nuclear, 43 kDa. It is a multidomain protein: it has a N-terminal domain, with a NLS signal, two RRM domains (RRM1 and RRM2) and a glycine-rich domain, in the C-terminus of the protein. The majority of the mutations are localized in the C-terminal part of TDP43 (Sreedharan, Blair et al. 2008).

TDP-43 is also involved in the processing of small regulatory RNAs (micro RNAs) and in RNA maturation and splicing. TDP43 is a major component of the ubiquitin-positive neuronal inclusions that are the pathological hallmark of both ALS and frontotemporal dementia (FTD) (Neumann, Sampathu et al. 2006).

Mutations in TDP43 are responsible of 4–6% of familial and 0.7–2% of sporadic ALS. Although TDP43 mutations are found in ALS families across the globe, some regional variability does exist. For example, the A382T mutation of the protein is particularly frequent in Sardinia, reflecting the conserved nature of that island population combined with a historical founder effect (Chio, Borghero et al. 2011).

Mutations in TDP43 are mainly located with-in the C-terminal glycine-rich domain of the protein. Although the pathophysiological mechanisms by which TDP43 gene mutations result in neurodegeneration remain to be defined fully, emerging evidence suggests multiple mechanisms including gain of toxicity, loss of nuclear function, and the formation of large stress granules. Support for a toxic gain of function has been provided by studies in transgenic mouse models wherein increased expression of the

mutated TDP43 proteins leads to neurodegeneration through dysfunction of cellular organelles and proteins (Lee, Lee et al. 2011). The severity of cortical and spinal motor neuron degeneration appears to be proportional to TDP43 protein levels, suggesting a potential role for TDP-43 in regulating disease severity (Wils, Kleinberger et al. 2010). Alternatively, loss of nuclear TDP43 accompanied by accumulation of TDP43 aggregates in the cytoplasm has been well established in ALS patients, implying a potential role for a TDP43 loss of nuclear function mechanism in ALS pathogenesis (Lee, Lee et al. 2011). Of further relevance, TDP43 associates with cytoplasmic stress granules (McDonald, Aulas et al. 2011). Specifically, stress granules function to suppress mRNA translation temporarily and store pre-RNA complexes during periods of cellular stress, thereby safeguarding the coded RNA information from deleterious chemicals. Pathological TDP43 mutant protein appears to exhibit a greater propensity to associate with cytoplasmic stress granules and form larger stress granules with altered dynamics.

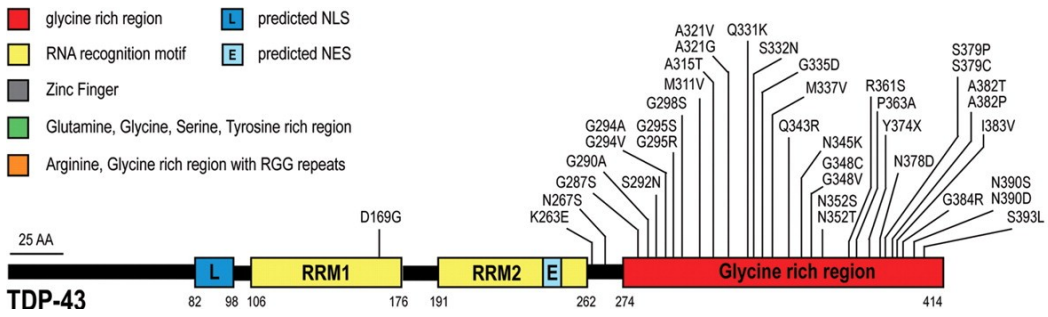


Figure 5.TDP43 mutations in ALS. Forty-four mutations have been identified in TDP43 in sporadic and familial ALS patients, with most lying in the C-terminal glycine-rich region. All are missense mutations, except for the truncating mutation TDP43^{Y374X}. Adapted from (Lagier-Tourenne, Polymenidou et al. 2010).

TDP43 interacts with a large amount of RNAs and proteins. For example, the glycine-rich C-terminus of TDP-43 has been shown to mediate interaction with several hnRNP proteins, specifically hnRNPs A1, A2/B1, C1/C2, and A3 (Buratti, Brindisi et al. 2005). TDP-43 interacts with nuclear and cytoplasmic proteins, and these interactions are often RNA-dependent. Freibaum and Chitta elaborated an interactome of TDP43, recognizing two classes of TDP43 binding protein: the first is a network of nuclear proteins that regulate RNA splicing and other aspects of nuclear RNA metabolism, and the second is a network of cytoplasmic proteins that regulate mRNA translation (Freibaum, Chitta et al. 2010). Moreover, they demonstrated that TDP43 pathological mutations M337V and

A315T do not alter the interaction. TDP43 interacts also with FUS (Ling, Albuquerque et al. 2010). TDP43 and FUS directly interact in a RNA/protein complex with HDAC6, regulating its expression levels (Kim, Shanware et al. 2010). TDP43 and HDAC6 interaction was demonstrated by Hebron and colleagues in 2013 (Hebron, Lonskaya et al. 2013).

Another interesting point to discuss is TDP43 changing functions when acetylated. As for others transcription factor, acetylation is one of the most important control mechanism. A well studied example is p53, whom acetylation has many important effects. It increases p53 protein stability, binding to low affinity promoters, association with other proteins, antiviral activities, and is required for its checkpoint responses to DNA damage and activated oncogenes (Reed and Quelle 2014). Since now, few is known about TDP43 acetylation. Cohen and colleagues demonstrated that TDP-43 acetylation impairs RNA binding and promotes accumulation of insoluble, hyper-phosphorylated TDP-43 species that largely resemble pathological inclusions in ALS and FTLD-TDP (Cohen, Hwang et al. 2015).

1.1.3 Environmental factors

In ALS onset and progression the interaction between genetic background and external factors is thought to play a major role (Bozzoni, Pansarasa et al. 2016). Exposure to heavy metals, such as lead, selenium, mercury, cadmium and iron, as a risk factor for ALS has long been studied, and the results produced are contradictory. Exposure to selenium has been largely studied and results indicate that there are in vitro evidences but not any statically significant association. Similar results emerged in studies about mercury and lead (Trojsi, Monsurro et al. 2013). Another factor that has recently been linked to sALS is the exposure to electromagnetic fields (EFMs), especially to low frequency EFMs. Despite interesting initial evidences, no any significant association has emerged in the latest studies (Vergara, Mezei et al. 2015). Exposure to a various spectrum of substances has been largely investigated. Cyanobacteria produce several cyanotoxins, divided into neurotropic (e.g. BMAA) and hepatotropic, such as cycasin, whose carcinogenic potential is well documented. The hypothesis that BMAA may have a role in neurodegenerative diseases was initially based on reports of elevated rates of ALS, Parkinson's disease and dementia in the island of Guam, where cycad seeds are

used to produce flour containing a remarkable amount of BMAA. Increased brain levels of BMAA were found in Guam ALS patients and similar BMAA levels were also found in the brains of ALS patient in Florida (Pablo, Banack et al. 2009). The latest *in vivo* studies confirm that BMAA can be incorporated into nerve cell proteins, causing misfolding, aggregates and cell death (Dunlop, Cox et al. 2013). BMAA role in causing worldwide sALS needs to be more detailed studied. Pesticides and precisely insecticides are the earlier studied fALS cause. Some of them, particularly organophosphate pesticides, can cause neurological damage, due to inhibition of acetylcholinesterase, the enzyme responsible for terminating the biological activity of acetylcholine. Moreover, most of these chemical compounds are known for their ability to induce oxidative stress, mitochondrial dysfunction, α-synuclein storage and neuronal loss.

Environmental toxins	Effect
Cigarette smoke	Increase the probability of developing ALS through inflammation, oxidative stress, and neurotoxicity by heavy metals contained in cigarettes.
Heavy metals	**Lead**: greater tibia and blood Pb levels were found to be associated with increased survival of ALS patients **Mercury**: Diet organic mercury, through fish and derivates, and occupational exposure have been shown to be linked to ALS onset. **Selenium**: Se exposure may considerably influence SOD1 accumulation into mitochondria, a somatic feature occurring in neurons during ALS pathogenesis.
Physical activity	Several genes (i.e., ciliary neurotrophic factor, leukemia inhibitory factor, and vascular endothelial growth factor 2) related to exercise have been recognized as possible ALS risks factors.
Agricultural chemicals	ALS risk is associated with use of organochlorine insecticides (e.g., dichloro-diphenyl-trichloroethane), pyrethroids, herbicides, and fumigants but not with other pesticide classes
Radiation/electromagnetic fields	Electromagnetic fields cause DNA strands to break in brain cells, leading to cell death and such reaction could be the reason for the association between electromagnetic fields and ALS risk.
Diet	Consuming high level of glutamate and fat can have adverse effects on ALS patients while Omega 3 fatty acids, Vitamin E, and fiber can have defensive impact.

Table 2. Environmental factors linked to ALS risk and pathophysiology. Adapted from (Zarei, Carr et al. 2015).

This data emerges by the more elevated risk to develop ALS in farmers, employees of chemical industries, inhabitants in rural areas (McGuire, Garrison et al. 1997). A big amount of case-control studies confirm that pesticides exposure is a risk factor for sALS (Sutedja, Fischer et al. 2009). ALS owes its colloquial name to baseball player Lou Gehrig, who contracted ALS at the age of 27 in 1939. A cohort study conducted on 24000 soccer player Italian players evidences that there is a high prevalence of ALS, approximately 5 or 6 folds more than global population. Causes are unclear yet, but many were postulated. Football players may be exposed to various risk factors that could contribute to neurodegenerative processes, namely excessive physical activity, repeated head injuries, exposure to pesticides and dietary supplements or illegal substances.

1.1.4 ALS experimental models

In order to better understand the pathophysiological processes of the ALS onset, several model systems have been created over the years such as cell lines and transgenic animals, expressing different ALS-causative genes (SOD1, FUS and TDP43) in the wild type form or with the several pathological mutations. Among the mammalian animal and cellular models Table 3 summarize the most relevant to this thesis.

	TARGETED GENE	DESCRIPTION	PHENOTYPE (Y/N)	NEURONAL DEGENERATION (Y/N)	PROTEINOPATHY / AGGREGATES (Y/N)
Animal model	SOD1	At least three mutations: SOD1 G93A, G87R, G85R G93A fALS mice: 20copies, hSOD1 promoter	Y; paralysis and premature death	Y; motor neuron degeneration, muscle denervation; implication of non-cell autonomous mechanisms; inflammation characterized by astroglial and microglial activation; muscle atrophy	Y: aggregated SOD1 can be detected with specific antibodies
	TDP-43	Overexpression of WT and mutated forms	Y; Motor deficits but no paralysis	Y; +/- MN loss, depending on the line. Overall mild effects	Not in all cases Sometimes Ub+, TDP43 nuclear and cytosol aggregates
	TDP-43	Conditional KO	Weight loss and age-dependent motor impairment	Y; MN degeneration	N
	FUS	PrP-hFUS WT overexpression	Y; motor impairment, paralysis and death	Y: MN loss	Y
	Transgenic mutated FUS rats		Motor axon degeneration, muscle denervation	Y; Motor axon degeneration, muscle denervation	Y
	Pharmacological model	Treatment with BMAA	Some motor phenotypes reported	?	?
Cellular Models	iPS cells (SOD1, TDP43, FUS C9ORF72)	Co-cultures MN/astrocytes/microglial cells	Y; cells degeneration	Y; used to evaluate non-cell autonomous processes by co-cultivating cells carrying or not-carrying the pathogenic mutation	Y
	Primary MN	Primary cultures with or w/o astrocytes	Y; cells degeneration	Y	Y
	SH-SY5Y	Human neuroblastoma cells	Apoptosis and necrosis	Y	Y
	NSC-34	Motoneuronal cells	Apoptosis and necrosis	Y	Y

Table 3. Characteristic of the most used mammalian animal and cellular models for ALS

1.1.5 Pathogenic mechanisms

Understanding ALS pathogenesis appears to be central for future development of diagnostic and therapeutic strategies in ALS. Pathogenesis of ALS is complex and many processes seem to contribute to the development of the disease. *In vitro* studies show that synaptosomes in neural tissue display a marked decrease in the maximal velocity of transport for high-affinity glutamate uptake in spinal cord, motor cortex, and somatosensory cortex compared to controls. The decrease in glutamate uptake was not observed in tissue from visual cortex, striatum, or hippocampus (Rothstein, Martin et al. 1992).Oxidative stress is another pathway lined to neurodegeneration in ALS. SODl mutations have been shown to enhance in vitro generation of hydroxyl radical from hydrogen peroxide. In addition to the dismutation of superoxide to hydrogen peroxide, wild-type CuZn-SOD1 can use hydrogen peroxide as a substrate to initiate a Fenton-like reaction with production in vitro of hydroxyl radical (Liu, Althaus et al. 1998). Several evidences report a link between protein misfolding and ALS. In particular, *SOD1* mutations induce conformational instability and misfolding of the SOD1 peptide, resulting in formation of intracellular aggregates that inhibit normal proteosomic function, disrupting axonal transport systems and vital cellular functions (Zetterstrom, Stewart et al. 2007). Recently, a functional study demonstrated that mutant ineffective Hsp104 impairs SOD1 or TDP43 aggregation and lead to the disgregation of misfolded aggregates (Jackrel and Shorter 2014). Of further relevance, TDP-43 was recognized as a major component of ubiquitinated cytoplasmic protein aggregates in almost all patients with sporadic ALS, but not in the nucleus, as in normal neurons. Given that TDP43 binds both DNA and RNA, mutations in *TDP43* could result in deregulation of RNA processing (Arnold, Ling et al. 2013). As with TDP43, the finding of cytoplasmic FUS-positive inclusions in ALS patients also implies loss of nuclear function as a potential pathogenic mechanism, and is supported by FUS expression studies in transgenic mouse models. Conversely, a toxic-gain of function has also been inferred from FUS expression studies. Of further relevance, TDP-43 and FUS associate with cytoplasmic stress granules. Specifically, stress granules function to suppress mRNA translation temporarily and store pre-RNA complexes during periods of cellular stress, thereby safeguarding the coded RNA information from deleterious chemicals (Van Deerlin, Leverenz et al. 2008). The role of RAN-proteins, for example the possible

translation of *C9Orf72* RNA, have recently been studied in ALS context. (Cleary and Ranum 2014). RAN translation of the sense GGGGCC expansion is predicted to result in the expression of three dipeptide proteins: GlyPro (GP), GlyArg (GR) and GlyAla (GA). Support for the accumulation of RAN-proteins in *C9ORF72* ALS/FTD autopsy brains was reported using antibodies against the predicted dipeptide repeat motifs (GP, GR and GA).Structural and functional abnormalities of mitochondria, impairment of axonal transport systems and endosomal trafficking, together with neuroinflammation and induction of the endoplasmic reticulum stress response, have all been implicated in ALS pathogenesis. Although these mechanisms contribute to neurodegeneration, they appear to be secondary events in ALS (Ferraiuolo, Kirby et al. 2011).

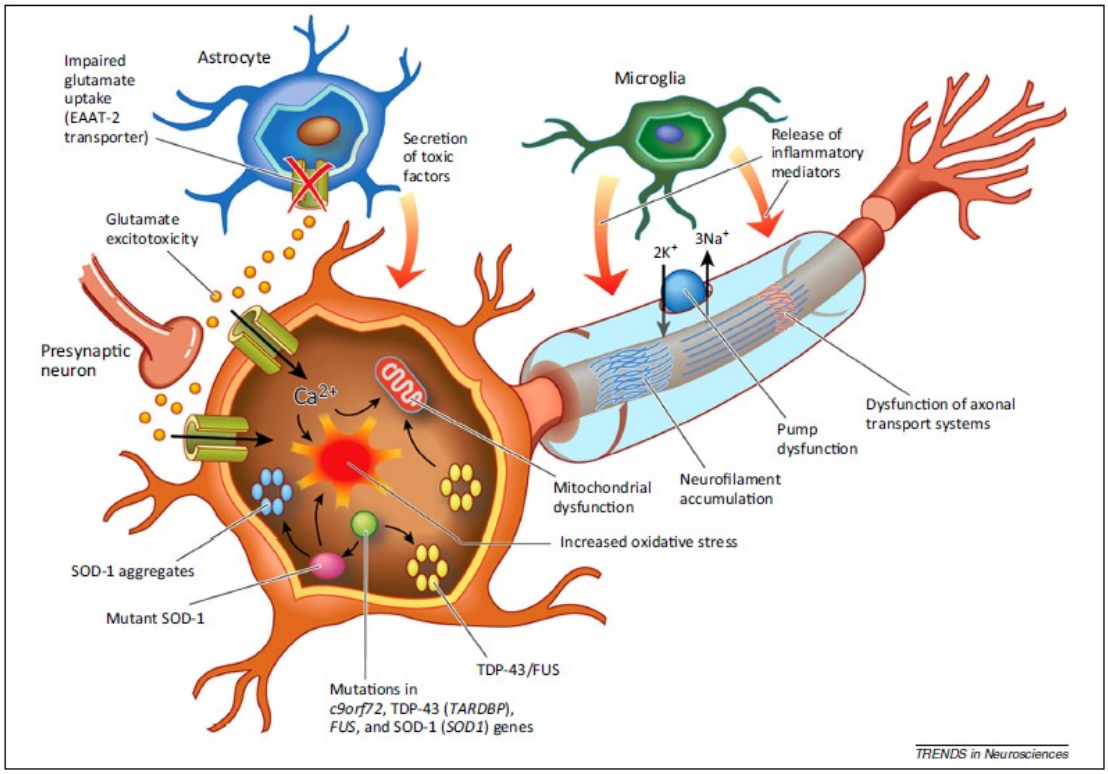


Figure 6.ALS in a multifactorial disease. Pathway involved are formation of SOD1 and TDP43 misfolded aggregates, increased oxidative stress, neuroinflammation, impaired glutamate uptake, glutamate excitotoxicity (Vucic, Rothstein et al. 2014).

In addition, alterations in epigenetic regulation are implicated in ALS pathogenesis (Al-Chalabi, Kwak et al. 2013). In fact in the last decade, the field of epigenomics has emerged, revealing that DNA modifications, including DNA-bound histones, DNA methylation, and chromatin remodeling, which may depend from environmental clues, such as lifestyle, diet and toxin exposure, also provide levels of gene regulation and alter gene expression. Epigenetic factors are probably much more suited than genetic

factors to explain disease onset and progression in ALS, since aberrant epigenetic, patterns may be acquired throughout life. In the next paragraphs I will review epigenetic modifications, focusing on their role in ALS.

1.2 EPIGENETICS

1.2.1 The epigenetic machinery

Historically, the word “epigenetics” was used to describe events that could not be explained by genetic principles. Conrad Waddington (1905–1975), who is given credit for coining the term, defined epigenetics as “the branch of biology which studies the causal interactions between genes and their products, which bring the phenotype into being” (Waddington 1959).The term *epigenetics* in its contemporary usage emerged in the 1990s, but for some years has been used in somewhat variable meanings (Berger, Kouzarides et al. 2009). A consensus definition of the concept of *epigenetic trait* as "stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence" was formulated at a Cold Spring Harbor meeting in 2008 (Berger, Kouzarides et al. 2009).

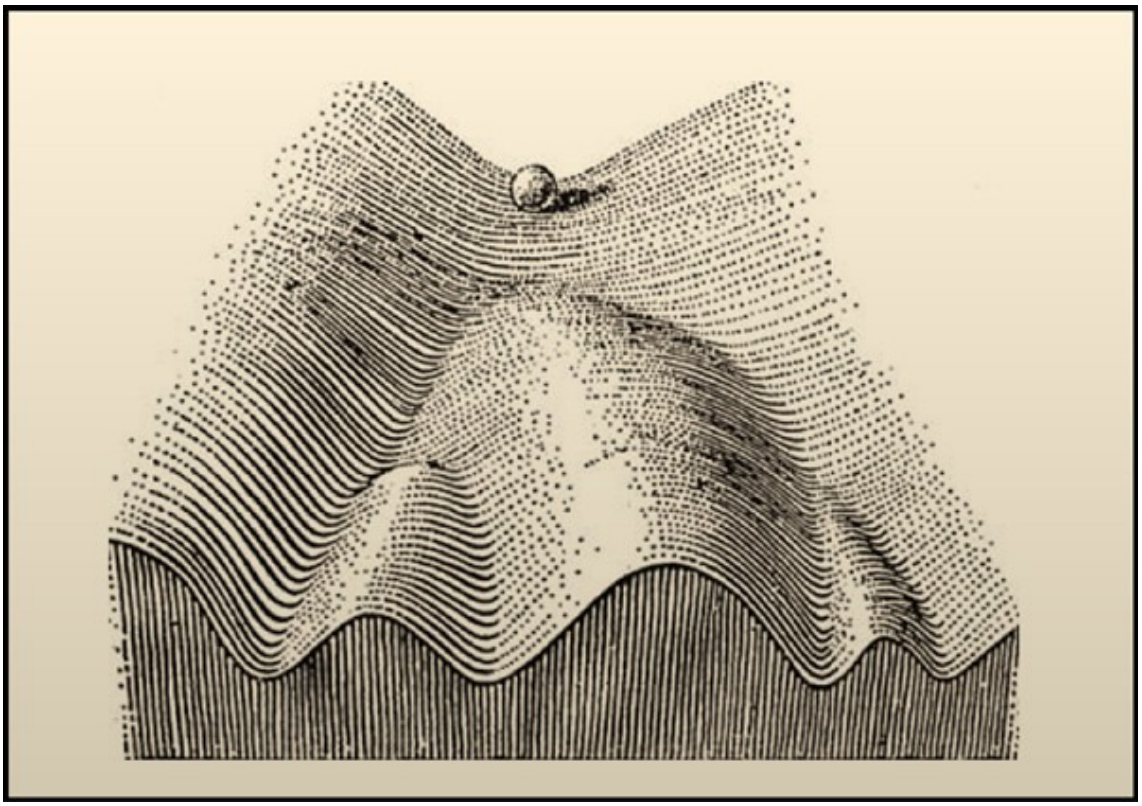


Figure 7.Waddington’s idea of epigenetics in 1942.

Epigenetics refers to patterns of gene expression that are heritable through cellular division (i.e., mitosis and meiosis) and are not directly attributed to any changes in the primary DNA sequence. Such effects on cellular and physiological phenotypic traits may result from external or environmental factors that switch genes on and off and affect how cells express genes. A wide variety of illnesses, behaviors, and other health indicators already have some level of evidence linking them with epigenetic mechanisms, including cancers of almost all types, cognitive dysfunction, and respiratory, cardiovascular, reproductive, autoimmune, and neurobehavioral illnesses (Goldberg, Allis et al. 2007). Known or suspected drivers behind epigenetic processes include many agents, including heavy metals, pesticides, diesel exhaust, tobacco smoke, polycyclic aromatic hydrocarbons, hormones, radioactivity, viruses, bacteria, and basic nutrients.

Many types of epigenetic processes have been identified. The most well-known epigenetic mechanisms in humans are DNA methylation and post-translational modifications of histone proteins. Other epigenetic mechanisms and considerations are likely to surface as work proceeds. Epigenetic processes are natural and essential to many organism functions, but if they occur improperly, there can be major adverse health and behavioral effects. Perhaps the best known epigenetic process, in part because it has been easiest to study with existing technology, is DNA methylation, namely the addition or removal of a methyl group (CH3). DNA methylation was first confirmed to occur in human cancer in 1983, and has since then been observed in many other illnesses and health conditions.

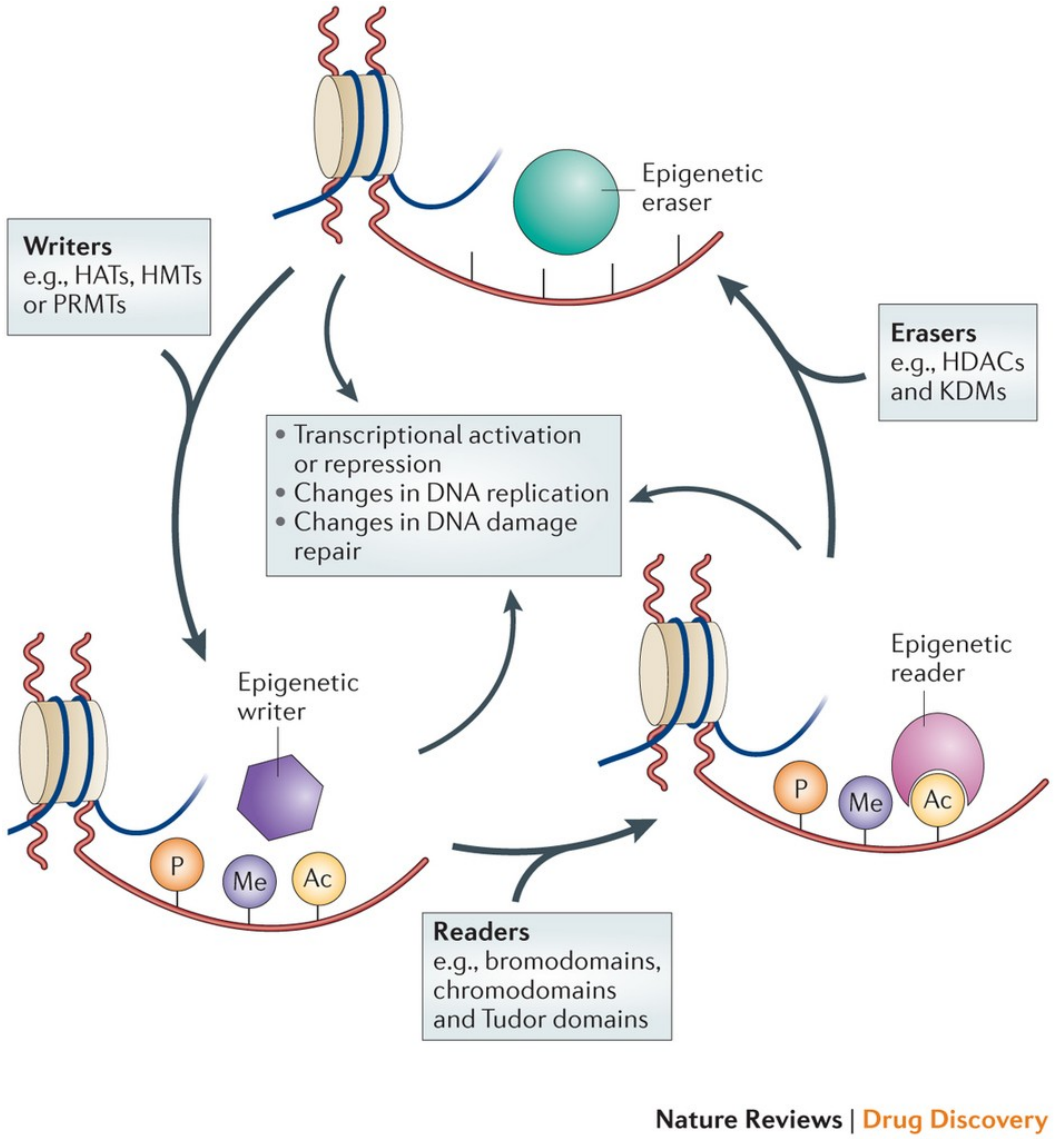


Figure 8. Epigenetic regulation is a dynamic process. A class of epigenetic regulators are "the writers", which modify chromatin laying down epigenetic marks on amino acid residues on histone tails. They are histone acetyltransferases (HATs), histone methyltransferases (HMTs), protein arginine methyltransferases (PRMTs) and kinases. The "epigenetic readers" such as proteins containing bromodomains, chromodomains and Tudor domains bind to these epigenetic marks and translate the messages. Finally, "epigenetic erasers" such as histone deacetylases (HDACs), lysine demethylases (KDMs) and phosphatases catalyse the removal of epigenetic marks. Addition and removal of these post-translational modifications of histone tails leads to the addition and/or removal of other marks in a highly complicated histone code. Together, histone modifications regulate various DNA-dependent processes, including transcription, DNA replication and DNA repair. Adapted from (Falkenberg and Johnstone 2014).

Histone modifications involve multiple post-transcriptional alterations of the N-terminal tails of histone proteins such as acetylation, methylation and phosphorylation, which can contribute to the 'open' or 'closed' transcriptional state of the chromatin. DNA methylation and histone-modification events are tightly interconnected. Histone-

modifying enzymes can recruit DNA-methylransferases to the genomic loci bearing a specific 'histone code' and, in turn, methylated DNA binding proteins can recruit histone-modifying enzymes to the hypermethylated loci, thus establishing a combined DNA/histone epigenetic mark self-perpetuated in cellular divisions.

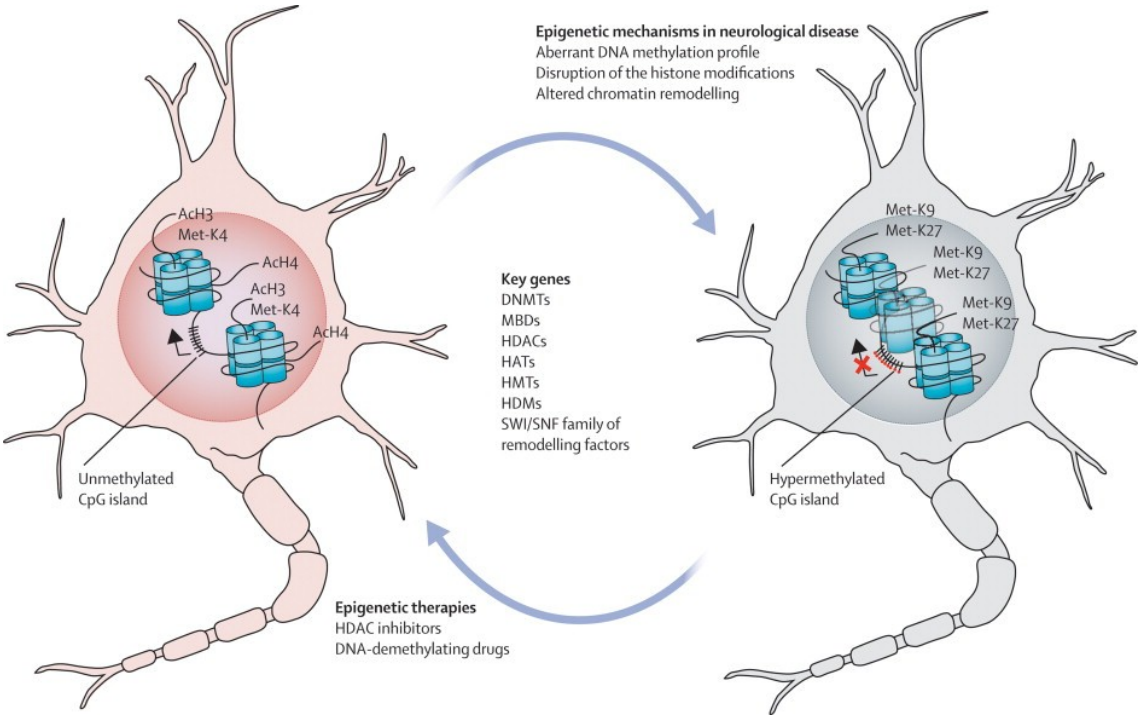


Figure 9. Epigenetic machinery in neurons. In healthy neurons or glia (left), transcriptionlly active gene promoter have unmethylated CpG island and a set of histone modifications associated with open "chromatin" conformation (eg, hyperacetylation and methylation of lysine 4 of histone H3). One the left, a neurodegenerated neuron carries "closed" chromatin conformation with dense hypermethylation of the CpG island promoter, repressive histone chemical modifications such as methylation of lysines 9 and 27 of histone H3. Epigenetic drugs such as DNA-demethylating drugs and HDAC inhibitors can partially rescue the distorted epigenetic processes. Ac=acetylation. DNMT=DNA methyltransferase. HAT=histone acetyltransferase. HDAC=histone deacetylase. HDM=histone demethylase. HMT=histone methyltransferase. MBD=methyl-CpG binding domain protein. Met-K4=methylation of lysine 4. Met-K9=methylation of lysine 9. Met-K27=methylation of lysine 27. SWI/SNF=switching/sucrose non fermenting chromatin-remodelling complex. Adapted from (Urdinguio, Sanchez-Mut et al. 2009).

1.2.2 Covalent histone-tails post-translational modifications

At the heart of chromatin structure are highly conserved histone proteins (H3, H4, H2A, H2B and H1) that function as building blocks to package eukaryotic DNA into repeating nucleosomal units that are folded into higher-order chromatin fibers. Covalent modifications of histone proteins play central roles in many types of epigenetic inheritance. The most common histone tails modifications are lysine acetylation, lysine

and arginine methylation, serine and threonine phosphorylation, but also lysine ubiquitination and sumoylation. The majority of this covalent modifications occurs in histone H3 and H4 tail (Strahl and Allis 2000). This modifications acts as an alphabet, a code that is read by protein complexes, which slide on DNA molecules and translate the code into a biological and physical response, which may be the switch to an active into a repressed state of the chromatin or vice versa. In addition, histone code differentially marks chromatin that is constitutively condensed, or facultative eterochromatin, which can be converted into a non-condensed form in certain moments of cell cycle.

Every single modification has a determined significance if considered in the context in which it is localized. That is, a modification that acts as a repressive message, changing context, can change its meaning. In general, considering the electrostatic requirements for folding the chromatin polymer, histone acetylation, through the neutralization of positive charge, and histone phosphorylation, through the addition of negative charge, would probably cause decondensation of the chromatin. On the contrary, lysine and arginine methylation usually causes chromatin condensation.

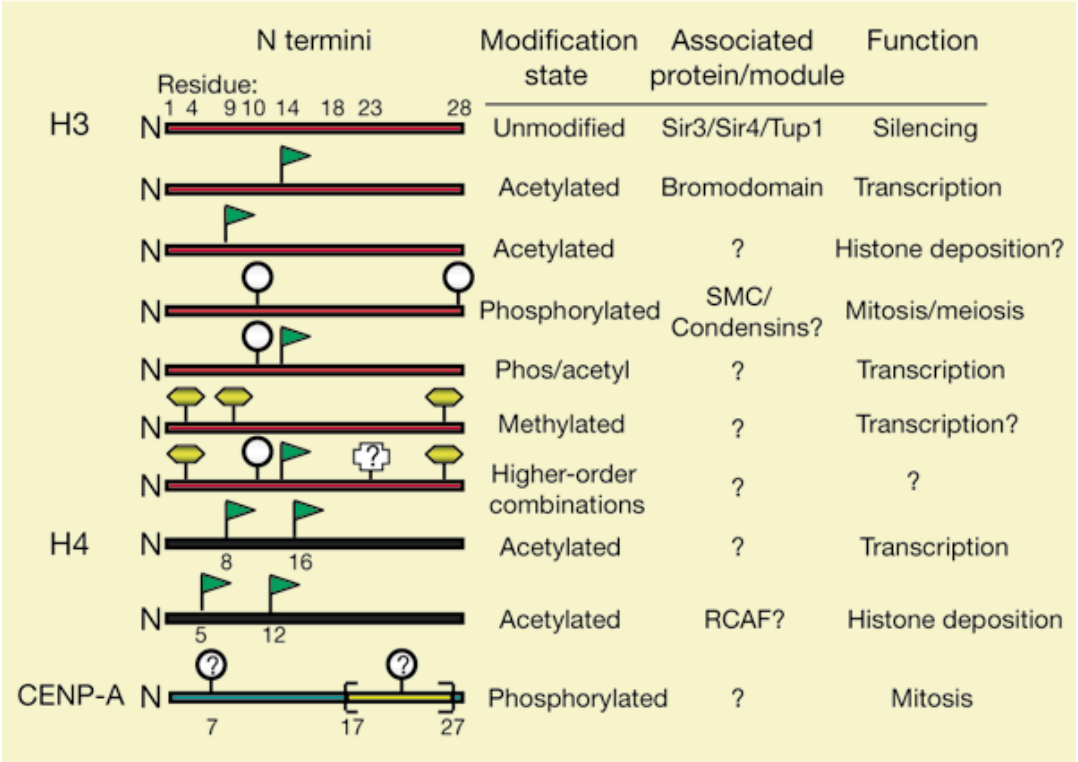


Figure 10. The most frequent covalent modifications on histone H3 and H4 tails and their relative meaning. S10 phosphorylation associated with K14 acetylation lead to transcriptional activation; K4 dimethylation is associated with transcriptional activation; K9 and K27 methylation are associated with transcriptional repression. From (Strahl and Allis 2000).

As histone code is written by a variety of enzymes (see below), it is read by complex of proteins. Recent evidence shows that the bromodomain of human PCAF (P300/CBP-associated factor), a domain of little known function which is shared between many, but not all HATs, binds acetylated lysine in the context of H3 and H4 tail sequences (Dhalluin, Carlson et al. 1999). For example, ATP-dependent chromatin remodeling complexes are specialized protein machinery able to restructure the nucleosome to make its DNA accessible during transcription, replication and DNA repair. ATP-dependent chromatin remodeling complexes specifically recognize these histones marks, and through ATP hydrolysis unwrap, mobilize, exchange or eject the nucleosome, and subsequently recruiting a transcriptional apparatus to nucleosomal DNA (Tang, Nogales et al. 2010). Among them, there is the SWI/SNF complex. The SWI/SNF family of chromatin remodeling complexes was initially discovered in yeast by two independent screenings aimed at identifying mutations in genes that affect the mating-type switching (SWI) and sucrose fermentation (Sucrose Non Fermenting - SNF) pathways. Chromatin remodeling complexes use the energy of ATP hydrolysis to slide the DNA around the nucleosome. The first step consists in the binding between the remodeler and the nucleosome. This binding occurs with nano molar affinity and reduces the digestion of nucleosomal DNA by nucleases. Upon ATP hydrolysis, the torsion subdomain carries out a directional DNA translocation. This event destroys histone-DNA contacts and creates a transient DNA loop that propagates around the nucleosome and resolves when it reaches the exit site on the other side of the nucleosome resulting in nucleosome repositioning. The tracking domain ensures that the waves of DNA loops can move only in one direction blocking any backward movement. The remodeler then resets its original position ready for a new remodeling cycle.

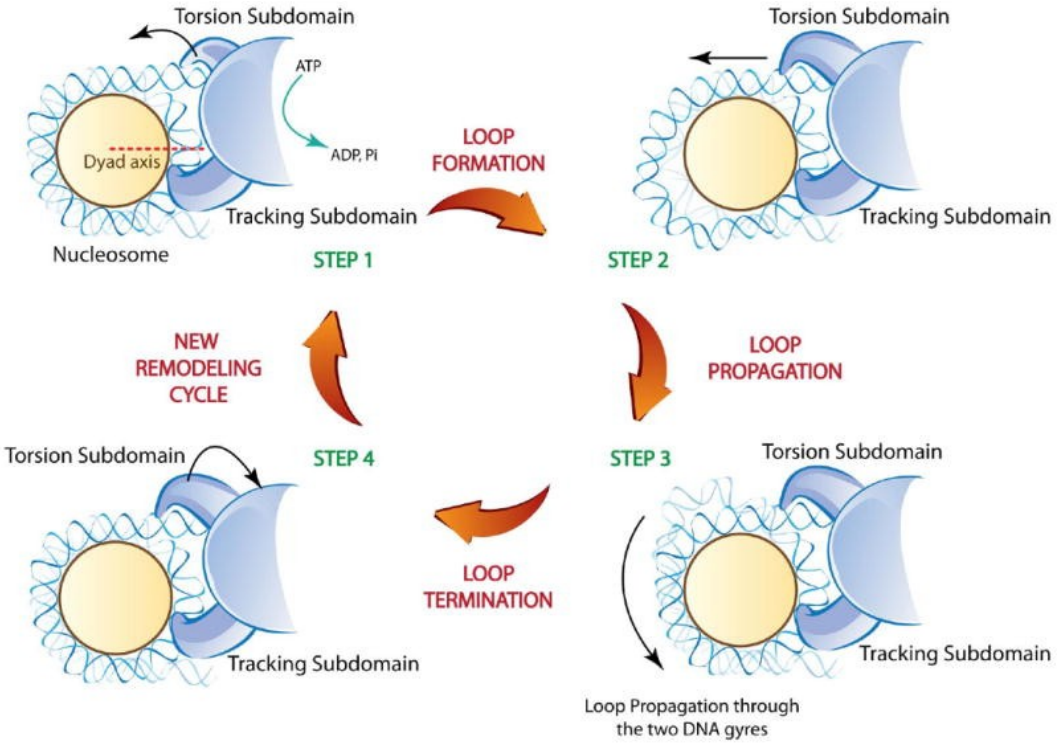


Figure 11. Schematic representation of the SWI/SNF dependent nucleosome remodeling process. During Step1, the translocase domain binds the nucleosome two turns away from the dyad. Upon ATP-dependent hydrolysis, the torsion sub-domain generates a DNA loop that translocates through the tracking subdomain and the dyad, continuing in the second gyre (Step 2-3). The loop resolves when it reaches the exit site on the other side of the nucleosome (Step 4). The combination of these steps results in nucleosome repositioning. The complex is then ready for a new remodeling cycle (Step1). Adapted from (Tang, Nogales et al. 2010).

1.2.2.1 Histone acetylation

Histone acetylation occurs on lysine residues on H3 and H4 histone tails. It is the well-studied histone modification. The mechanism for acetylation and deacetylation takes place on the NH3+ groups of Lysine amino acid residues. These residues are located on the tails of histones that make up the nucleosome of packaged dsDNA. The process is aided by factors known as Histone Acetyltransferases (HATs). HAT molecules facilitate the transfer of an acetyl group from a molecule of Acetyl Coenzyme-A (Acetyl-CoA) to the NH3+ group on Lysine. When a Lysine has to be deacetylated, factors known as Histone Deacetylases (HDACs) catalyze the removal of the acetyl group with a molecule of H2O. Acetylation has the effect of changing the overall charge of the histone tail from positive to neutral. Nucleosome formation is dependent on the positive charges of the H4 histones and the negative charge on the surface of H2A histone fold domains. Acetylation of the histone tails disrupts this association, leading to weaker

binding of the nucleosomal components. By doing this, the DNA is more accessible and leads to more transcription factors being able to reach the DNA. Thus, acetylation of histones is known to increase the expression of genes through transcription activation. Deacetylation performed by HDAC molecules has the opposite effect. By deacetylating the histone tails, the DNA becomes more tightly wrapped around the histone cores, making it harder for transcription factors to bind to the DNA. This leads to decreased levels of gene expression and is known as gene silencing (Verdone, Agricola et al. 2006). Histone acetyl-transferases (HATs) are a protein family that mediate the acetylation of lysine residues on histone tails. The most important are CBP/p300, MYST family and GNAT family. Histone deacetylase (HDACs) mediate the opposite reaction, that is the removal of acetyl group from lysine residues on histone tails. HDACs are divided into 4 families. Class I includes HDAC1, 2, 3 and HDAC8. Class IIa includes HDAC 4, 5, 7 and 9, whereas Class IIb includes HDAC 6 and 10. HDAC Class III includes the Sirtuins and Class IV contains HDAC 11 (Gallinari, Di Marco et al. 2007).

	Histone Acetyl-transferases (HATs)	Histone deacetylases (HDACs)
H3K9, K14, K18, K36	GNAT family (Gnc5, PCAF)	SIRT1, Clr-3
H3K14, K18, H2AK5, H2BK12, K15	p300, CBP	HDAC3, class I HDACs,
H4K5, K8, K12, K16, H3K14	MYST family (Tip60, MOZ, HBO1)	SIRT1, SIRT3
H3K9, K14, K18	TFIIC, TAF1,	HDAC1, SIRT1

Table 4. Principal histone acetyltransferase (HATs) and deacetylases (HDACs) and their relative target. (Seto and Yoshida 2014).

The most studied histone acetylation are acetylation of lysine 9, 14, 18 and 23 on H3 tail and lysine 5, 8, 12 and 16 on H4 tail (Strahl and Allis 2000). Every of this signals correlate with transcriptional activation.

1.2.2.2 Histone methylation

Histone methylation consists in the addition of a methyl group to lysine or arginine residues. It preferentially occurs on histone H3 and H4 tails. Lysine and arginine residues both contain amino groups, which confer basic and hydrophobic characteristics. Lysine is able to be mono-, di-, or trimethylated with a methyl group replacing each hydrogen of its NH3+ group. With a free NH2 and NH2+ group, arginine can be mono-, di- or tri-methylated. Methylation is not studied as well as acetylation, because it's technically more difficult to detect (doesn't change the total charge of the histone). The majority of methylation occurs on lysine 4, 9 and 27 on H3 tail. Histone methylation can be associated with either transcriptional repression or activation. For example, dimethylation and trimethylation of histone H3 at lysine 4 (H3K4me3) is an active mark for transcription and is upregulated in hippocampus one hour after contextual fear conditioning in rats. However, dimethylation or trimethylation of histone H3 at lysine 9 (H3K9me2), a signal for transcriptional silencing, is increased after exposure to either the fear conditioning or a novel environment alone (Gupta, Kim et al. 2010).

Histone methylation is mediated by enzymes that are specific for a single lysine or arginine residue. They catalyze the transfer of one, two, or three methyl groups to lysine and arginine residues of histoneproteins. Lysine methyltransferase can be divided into two groups: the ones which possess a SET domain (Su(var)3-9, Enhancer of Zeste, Trithorax), and the ones who don't have a SET domain. Both use S-adenosyl-methionine as cofactor. There are two different types of protein arginine methyltransferases (PRMTs) and three types of methylation that can occur at arginine residues on histone tails. The first type of PRMTs (PRMT1,PRMT3,CARM1/PRMT4, and Rmt1/Hmt1) produces monomethyl arginine and asymmetric dimethyl arginine. The second type (JBP1/PRMT5) produces monomethyl or symmetric dimethyl arginine. The differences in the two types of PRMTs arise from restrictions in the arginine binding pocket (Branscombe, Frankel et al. 2001).

	Histone Methyltransferase (HMTs)	Histone demethylase (HDMs)
H3K4me1/2/4	SET1, MLL	PHF8
H3K9me3	Suv3-9H1/H2, SETDB1	KDM2B
H3K27	EZH1, EZH2	KDM6

Table 5. Principal histone methyltransferases (HMTs) and demetylase (HDMs) and their relative target (Khare, Habib et al. 2012)

1.2.2.3 Histone phosphorylation

Phosphorylation, particularly that of histones H1 and H3, has long been implicated in chromosome condensation during mitosis (Koshland and Strunnikov 1996). However, converging evidence suggests that H3 phosphorylation (specifically serine 10) is also directly correlated with the induction of immediate-early genes such as *c-jun, c-fos* and *c-myc* (Mahadevan, Willis et al. 1991). Histone phosphorylation can occur on serine, threonine and tyrosine residues and constitutes an essential part of the histone code. Phosphorylation of H2A(X) is an important histone modification that plays a major role in DNA damage response (Rossetto, Truman et al. 2010). A substantial number of phosphorylated histone residues are associated with gene expression. Interestingly, these are often related to regulation of proliferative genes. Phosphorylation of serines 10 and 28 of H3 and serine 32 of H2B has been associated with regulation of epidermal growth factor (EGF)-responsive gene transcription. H3S10ph and H2BS32ph have been linked to the expression of proto-oncogenes such as *c-fos*, *c-jun* and *c-myc* (Lau, Lee et al. 2011). Indeed, in EGF-stimulated cells, phosphorylation of H3S10 is tightly coupled to H3 K9ac and K14ac, both marks of transcriptional activation. It has been shown that phosphorylation of H3S10 promotes acetylation of H3K14 by the Gcn5 acetyltransferase in vitro and allows Gcn5-regulated gene transcription in vivo (Lo, Trievel et al. 2000). Phosphorylation is a chimeric modification, and its meaning depends on the chromatin conformation. It is interesting to observe that the same phosphorylation events can be implicated in multiple cellular processes involving chromatin modulation. The same phosphorylated residue can have significantly distinct effects on chromatin structure depending on the context in which it occurs. Phosphorylation of H3S10 and H3S28 is a good example of this duality: both phosphorylated residues are involved in chromatin condensation associated with mitosis and meiosis, as well as in chromatin relaxation linked to transcription activation.

1.2.3 DNA methylation

DNA methylation is a process by which methyl groups are added to DNA. Methylation modifies the function of the DNA, altering its expression. When located in a gene promoter, DNA methylation typically acts to repress gene transcription. DNA methylation is essential for normal development and is associated with a number of key processes including genomic imprinting, X-chromosome inactivation, repression of repetitive elements, aging and carcinogenesis (Jin, Li et al. 2011).

DNA methylation occurs at the cytosine bases of eukaryotic DNA, which are converted to 5-methylcytosine by DNA methyltransferase (DNMT) enzymes. The altered cytosine residues are usually immediately adjacent to a guanine nucleotide, resulting in two methylated cytosine residues sitting diagonally to each other on opposing DNA strands. The addition of methyl groups is controlled at several different levels in cells and is carried out by a family of enzymes called DNA methyltransferases (DNMTs). Three DNMTs (DNMT1, DNMT3a and DNMT3b) are required for establishment and maintenance of DNA methylation patterns. Two additional enzymes (DNMT2 and DNMT3L) may also have more specialized but related functions. DNMT1 appears to be responsible for the maintenance of established patterns of DNA methylation, while DNMT3a and 3b seem to mediate establishment of new or *de novo* DNA methylation patterns (Bestor 2000). Methylation can be directly observed by staining cells with an immunofluorescently labeled antibody for 5-methylcytosine. In mammals, methylation is found sparsely but globally, distributed in definite CpG sequences throughout the entire genome, with the exception of CpG islands, or certain stretches (approximately 1 kilobase in length) where high CpG contents are found. In human DNA, 5-methylcytosine is found in approximately 1.5% of genomic DNA, on the contrary, In embryonic stem (ES) cells, a substantial amount of 5-mC is also observed in non-CpG contexts (Lister, Pelizzola et al. 2009).

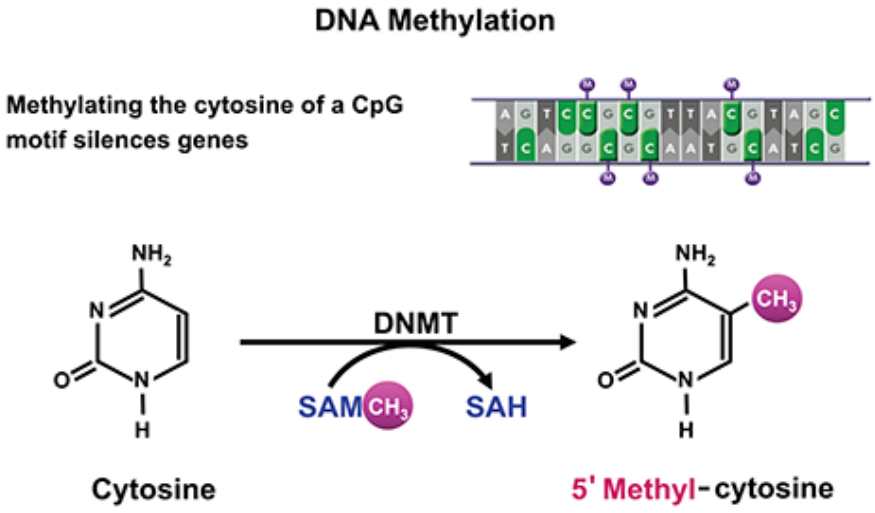


Figure 12. Methylation of cytosine residues in 5'-methyl cytosine happens thanks to DNA methyl transferases (DNMTs) which uses S-adenosylmethyonine as a cofactor.

Although patterns of DNA methylation appear to be relatively stable in somatic cells, patterns of histone methylation can change rapidly during the course of the cell cycle. Despite this difference, several studies have indicated that DNA methylation and histone methylation at certain positions are connected. Indeed, evidence has been presented that in some organisms, such as *Neurosporacrassa* (Tamaru and Selker 2001) and *Arabidopsis thaliana* (Soppe, Jasencakova et al. 2002), H3-K9 methylation is required in order for DNA methylation to take place. Given the critical role of DNA methylation in gene expression and cell differentiation, it seems obvious that errors in methylation could give rise to a number of devastating consequences, including various diseases. Indeed, medical scientists are currently studying the connections between methylation abnormalities and diseases such as cancer, lupus, muscular dystrophy, and a range of birth defects that appear to be caused by defective imprinting mechanisms (Robertson 2005).

DNA methylation can alter gene expression in two ways. First, the methylation of DNA itself may physically impede the binding of transcriptional proteins to the gene, and second, and likely more important, methylated DNA may be bound by proteins known as methyl-CpG-binding domain proteins (MBDs). MBD proteins then recruit additional proteins to the locus, such as histone deacetylases and other chromatin remodeling proteins that can modify histones, thereby forming compact, inactive chromatin, termed

heterochromatin. This link between DNA methylation and chromatin structure is very important. In particular, loss of methyl-CpG-binding protein 2(MeCP2) has been implicated in Rett syndrome; and methyl-CpG-binding domain protein 2 (MBD2) mediates the transcriptional silencing of hypermethylated genes in cancer (Soppe, Jasencakova et al. 2002).

1.3 EPIGENETICS AND ALS

Since epigenetic mechanisms were discovered and subsequently well described, a variety of physiological and pathological conditions were linked to alterations in the epigenetic regulation. Beyond the well-known epigenetic imprinting, that lead for example to Angelman and Prader-Willis disease (Knoll, Nicholls et al. 1989), several studies had clarified the connection between many human diseases and epigenetics. Among them, cancer and several neurodegenerative disorders had been linked to epigenetics alterations (Sharma, Kelly et al. 2010, Landgrave-Gomez, Mercado-Gomez et al. 2015). Addiction from abuse of drugs had been correlated to changes in normal epigenetic regulation (Renthal and Nestler 2008).

ALS is one of the neurodegenerative diseases that has been linked to epigenetics. In the next paragraphs I will discuss what is known until now about this link.

1.3.1 Acetylation and deacetylation of histonic and non histonic proteins

1.3.1.1 Targets

Beyond the most studied role of histone acetylation, lysine acetylation of non histonic proteins has emerged as a major covalent modification controlling diverse cellular processes and has been implicated in Alzheimer's disease (AD) and other neurodegenerative disorders. In particular, acetylation of misfolded tau proteins marks mature neurofibrillary tangles (NFTs) in AD and related tauopathies and represents a disease-specific marker of AD pathology (Cohen, Guo et al. 2011).

A recent global proteomics study identified ~1750 proteins that are subject to lysine acetylation, including a distinct subset of RNA-binding proteins and associated factors (Choudhary, Kumar et al. 2009). Among them, the case of p53 is the well studied. Acetylation of p53 is important in regulating DNA damage response. The acetylation of p53 regulates its stability through crosstalk with the ubiquitination machinery, modulates interactions with TAF1, and regulates its transcriptional activity (Yang and Seto 2008).

Moreover, recent evidences demonstrated that TDP43 functions are regulated by acetylation. Cohen and colleagues demonstrated that TDP-43 acetylation impairs RNA binding and promotes accumulation of insoluble, hyper-phosphorylated TDP-43 species that largely resemble pathological inclusions in ALS and FTLD-TDP43 (Cohen, Hwang et al. 2015).

1.3.1.2 Histone acetyltransferase

Rouaux et al. observed that in a mouse model of ALS, it is present a severe decrease in the levels of histone acetyltransferase CBP (CREB-cAMP response element-binding protein-binding protein) (Rouaux, Jokic et al. 2003) in lumbar spinal cord motor neurons. Even if a decrease in H3 acetylation level was not revealed in the ALS mouse model, it was detected in cellular model of apoptosis. This imbalance induce a relative increase in global histone deacetylation, leading to the silencing of several anti-apoptotic genes and the contemporary overexpression of pro-apoptotic ones (Saha and Pahan 2006).

1.3.1.3 Histone deacetylases

Several HDACs alterations have been linked to ALS. Among them, HDAC4 mRNA and protein greater levels in ALS patients have been linked to a severe phenotype a negatively correlates with re innervation processes (Bruneteau, Simonet et al. 2013). HDAC6 directly interacts with TDP43, and is deacetylated by it (Cohen, Hwang et al. 2015), and, in association with FUS; regulates its mRNA (Kim, Shanware et al. 2010). Valle and colleagues reported the global deregulation of HDACs in two mouse models of ALS (Valle, Salvatori et al. 2014). Interestingly, they demonstrated that HDACs expression pattern changes from spinal cord to muscle.

HDAC1 is a nuclear ubiquitous histone deacetylases class I of 55 kDa. It deacetylatesHDAC1 knockout embryos are lethal (Lagger, O'Carroll et al. 2002). Silencing of HDAC1 via RNAi lead to inhibition of proliferation associated with the upregulation of cyclin-dependent kinase (cdk) inhibitors. In addition, HDAC1 along with HDAC2 are recruited at the double stranded break sites and play an important role in DNA damage repair to promote non homologous end joining (NHEJ) (Miller, Tjeertes et al. 2010) . HDAC1 directly interacts with FUS in DNA damage sites (Wang, Pan et al. 2013). In my work, I demonstrated that HDAC1 interacts also with TDP43.

1.3.1.4 HDAC inhibitors and other ALS therapy approaches

There are several evidences that restoring the correct acetylation/deacetylation balance using histone deacetylases inhibitors (HDACi) like Sodium Valproate (VPA), Sodium Butyrate (NaB) and Trichostatin A (TSA), contrasts MNs death and the neurodegenerative process (Sugai, Yamamoto et al. 2004). Anyway, their efficacy hasn't been proved in clinical yet. In my work, I tested the efficacy two HDACi, sodium 4-phenylbutyrate and trichostatin A, in improving cell viability in a cellular model of ALS.

Classification	Examples	Specificity to HDAC
1. Aliphatic fatty acids	Butyrate	Classes I and IIa
	Valproic acid	Classes I and IIa
2. Hydroxamate	SAHA (vorinostat)	Pan inhibitor
	PXD101 (belinostat)	Pan inhibitor
	LBH589 (panobinostat)	Classes I and II
	ITF2357 (givinostat)	Pan inhibitor
	4SC-201 (resminostat)	Pan inhibitor
	PCI 24781 (abexinostat)	Classes I and II
	Tubacin	Class IIb
3. Benzamides	MS-275 (entinostat)	Class I
	MGCD0103 (mocetinostat)	Class I
	CI-994 (tacedinaline)	Class I
	MGCD-0103	Classes I and IV
4. Cyclic peptides	Depsipeptide/FK228 (romidepsin)	Class I
	Apicidin	Class I
5. Mercaptoketone	KD5170	Classes I and II

Table 6.Principal HDAC inhibitors and their relative target.

Sodium 4-phenylbutyrate (PBA) is an orally bioavailable, blood brain barrier (BBB) permeable, short-chain fatty acid that has been approved by the Food and Drug Administration (FDA) for treatment of urea cycle disorders. PBA has potential benefit for a wide variety of diseases like cancer, cystic fibrosis, thalassemia, spinal muscular atrophy as well as protein folding diseases such as type 2 diabetes mellitus, ALS and other neurodegenerative disesases. PBA and sodium valproate promoted motorneuron survival in mouse models of ALS, and compound efficacy was at least partially

attributed to the amelioration of abnormal histone hypoacetylation and transcriptional dysregulation, which is implicated in ALS (Ryu, Smith et al. 2005) (Rouaux, Panteleeva et al. 2007). Similarly, combined lithium and valproate treatment delayed disease onset, reduced neurological deficits and prolonged survival (Feng, Leng et al. 2008), and treatment with trichostatin A (Yoo and Ko 2011), or valproate (Sugai, Yamamoto et al. 2004), delayed disease progression and/or increased survival in the SOD1-G93A mice. A phase II study in ALS individuals revealed that sodium phenylbutyrate was safe and tolerable, and histone acetylation was significantly increased after sodium phenylbutyrate administration (Cudkowicz, Andres et al. 2009). Conversely, a trial using valproic acid did not show a beneficial effect on survival or disease progression in patients with ALS (Piepers, Veldink et al. 2009). An open-label study, involving 40 patients with ALS, has been performed to establish the safety and pharmacodynamics of escalating dosages of phenylbutyrate in 26 participants who completed a 20-week treatment phase. From this study emerges that phenylbutyrate is safe and the majority of subjects tolerated higher dosages (21 g/day) of this drug, but the lowest dose (9 g/day) was therapeutically efficient in improving histone acetylation levels. No deaths or clinically relevant laboratory changes occurred with phenylbutyrate treatment. Histone acetylation decreased by approximately 50% in blood buffy-coat specimens at screening and significantly increased after phenylbutyrate administration. In addition, blood levels of phenylbutyrate and the primary metabolite (PAA) increased with dosage (Piepers, Veldink et al. 2009).

At present there is a clinical trials from Duke University (USA) recruiting patients to test the effect of Lunasin, a 43-amino acid polypeptide originally discovered in soy (ClinicalTrials.gov Identifier: NCT02709330). Lunasin can modify the epigenome and using an HAT assays, with acid-extracted histones as templates, it has been demonstrated that Lunasin specifically inhibited H4K8 acetylation while enhanced H4K16 acetylation catalyzed by HAT enzymes p300, PCAF, and HAT1A (Galvez, Huang et al. 2011).

HDAC inhibition is not the only therapeutic strategy that is in course of study. Rodriguez-Paredes and colleagues reviewed all the molecules that have an effect in

targeting HATs, HMT, HDM, and other epigenetic modifiers (Rodriguez-Paredes and Esteller 2011).

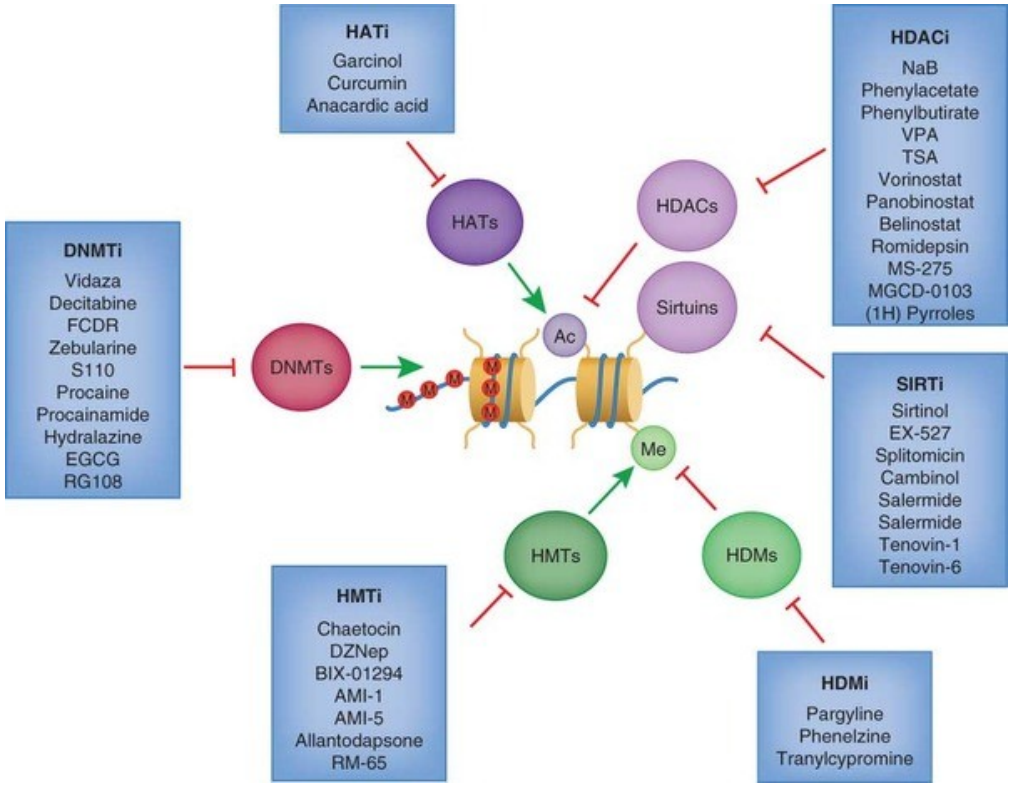


Figure 13. A summary of all molecules targeting DNMTs, HMT, HDMs, SIRTs, HDACs and HATs. Adapted from (Rodriguez-Paredes and Esteller 2011)

1.3.2 Methylation and demethylation

Histone methylation of some particular residues is involved in the development of ALS. In particular, the trimethylation of lysine residues within histones H3 and H4 is a mechanism involved in reducing *C9orf72* mRNA expression in expanded repeat carriers of c9FTD/ALS patients blood. The residues involved are lysine 9 and 27 on histone H3 (H3K9, H3K27) (Belzil, Bauer et al. 2013).

Recent evidences reported that FUS in methylated on a specific arginine residue. Arginine methylation modulates nuclear import of FUS via a TRN-binding epitope and chemical or genetic inhibition of arginine methylation restores TRN-mediated nuclear import of ALS-associated FUS mutants (Dormann, Madl et al. 2012).

1.3.3 Histone phosphorylation

Very few data are available on histone phosphorylation and neurodegenerative mechanisms. Phosphorylation of histone H2AX on serine 139 is correlated to DNA damage response (Rogakou, Nieves-Neira et al. 2000) in a variety of conditions, such as in Alzheimer disease (Myung, Zhu et al. 2008). No any alterations in histone phosphorylation pattern has been demonstrated since now in ALS patients. Nevertheless, several evidences induce to speculate that histone phosphorylation may be involved in ALS pathogenesis, considering that DNA damage is one of the marker of ALS (Qiu, Lee et al. 2014).

1.3.4 DNA methylation

Another epigenetic hypothesis of ALS pathogenesis involves DNA methylation. Recent genome-wide analyses have found differential gene methylation in human ALS. Neuropathologic assessments have revealed that motor neurons in human ALS show significant abnormalities in Dnmt1, Dnmt3a, and 5-methylcytosine. Similar changes are seen in mice with motor neuron degeneration, and Dnmt3a was found abundantly at synapses and in mitochondria (Martin and Wong 2013). Furthermore, Chestnut and colleagues demonstrated that during apoptosis of cultured motor neuron-like cells, Dnmt1 and Dnmt3a protein levels increase, and 5-methylcytosine accumulates. In addition, enforced expression of Dnmt3a, but not Dnmt1, induces degeneration of cultured neurons (Chestnut, Chang et al. 2011). These data suggest that DNA methylation may be a therapeutic target in ALS. As a matter of fact, inhibition of Dnmt catalytic activity with small molecules RG108 and procainamide protects motor neurons from excessive DNA methylation and apoptosis in cell culture and in a mouse model of ALS (Chestnut, Chang et al. 2011).

2. AIMS OF THE WORK

Up to date, several experimental evidences underline the link between epigenetic and neurodegenerative diseases, such ALS. In particular, it is widely demonstrated that both in sporadic and in familiar ALS there is a severe decreasing in acetylation of N-terminal tail of histone H3 and H4. In parallel, many evidences suggest how there is a contemporary decreasing in the expression of histone-acetyl transferases such as CBP. Finally, in ALS patients several studies show an increase in DNA methylation. This data lead me to speculate on the role of ALS causative genes SOD1, FUS and TDP43 on epigenetic deregulations. For this reason, I focused my PhD project on 3 main objectives:

1) Understand the role of SOD1, FUS and TDP43 in regulating histone post-translational modifications and the possible pathological implications. In this sense, I performed western blot analysis on cells lysates expressing SOD1, FUS or TDP43, with primary antibodies targeting histonic modifications linked to transcriptional activation or to transcriptional repression; in addition, we measured DNA methylation levels.
2) Investigate the possible interaction between TDP43 and HDAC1. We performed co-immunoprecipitation experiments to evidence the interaction, the role of point pathological mutations and which is TDP43 interacting domain.
3) Analyze the possible therapeutic effect of HDAC inhibitors such as Sodyum butyrate and Trichostatin A, performing cells vitality assays.

3. MATERIALS AND METHODS

Bacterial strain.

Competent cells used are E. coli DH5-α. Bacterial cells are defective for the restriction and have mutations in relA1 and recA1 genes, to improve the stability and quality of recombinant plasmids.

Cell lines.

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

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

SH-SY5Y-HDAC1 cell line: SHSY-5Y cell line stably expressing HDAC1-FLAG created in our lab. Selection is maintained with 200 ug/ml of G1418.

Adenoviral vectors.

All adenoviral vectors (*pAdenoX-hFUS*$^{WT/R495X/H517Q/R521G/P525L}$; *pAdenoX-hTDP43*$^{WT/Q331K/M337V/A382T}$and *pAdenoX-hSOD1*$^{WT/H80R/G93A}$) were generated using the Adeno-X Expression System 1 (Clontech). Their production is completed in two stages. First, generation of mammalian expression cassette by cloning gene of interest into pShuttle2.Second, excision of expression cassette from pShuttle2 and insertion into I-Ceu I and PI-Sce I sites of BD Adeno-X Viral DNA. All constructions were verified by automated sequencing.

Plasmids and oligonucleotides.

Name	Expression cassette	Resistance	Tag
pMTK-hTDP43^{WT,M337V,A382T}	hTDP43^{WT,M337V,A382T}	Ampicillin	5x-myc
pMTK-hFUS^{WT,R521G,P525L,R495X}	hFUS^{WT,R521G,P525L,R495X}	Ampicillin	5x-myc
pcDNA3-hHDAC1	hHDAC1	Ampicillin	1x-FLAG
pMTK-hTDP43-ΔN-term	hTDP43-ΔN-term	Ampicillin	5x-myc
pMTK-hTDP43-ΔRRM1	hTDP43-ΔRRM1	Ampicillin	5x-myc
pMTK-hTDP43-ΔRRM2	hTDP43-ΔRRM2	Ampicillin	5x-myc
pMTK-hTDP43-ΔG-rich	hTDP43-ΔG-rich	Ampicillin	5x-myc
pMTK-hTDP43-ΔRRM1/RRM2	hTDP43-ΔRRM1/RRM2	Ampicillin	5x-myc

Table 7. Plasmids used in transfection experiments.

ΔN-term forw	5'-GAATTCAAGGCCTCTCGAGCCATAGTGTTGGGTCTCCCATGG-3'
ΔN-term rev	5'-CCATGGGAGACCCAACACTATGGCTCGAGAGGCCTTGAATTC-3'
ΔRRM1 forw	5'-CAGAAAACATCCGATTTAATAGTGCCTAATTCTAAGCAAAGC-3'
ΔRRM1 rev	5'-GCTTTGCTTAGAATTAGGCACTATTAAATCGGATGTTTTCTG-3'
ΔRRM2 forw	5'-GAGCCTTTGAGAAGCAGAAAACACAATAGCAATAGACAG-3'
ΔRRM2 rev	5'-CTGTCTATTGCTATTGTGTTTTCTGCTTCTCAAAGGCTC-3'
ΔG-rich forw	5'-CAGTTAGAAAGAAGTGGAAGAGGAATGTAGCTCGAG-3'
ΔG-rich rev	5'-CTCGAGCTACATTCCTCTTCCACTTCTTTCTAACTG-3'
ΔG-rich EcoRI forw	5'-ATTGAATTCTTTGGTGGTAATCCAGGTGGC-3'
TDP G-rich EcoRI F	5'-ATTGAATTCTTTGGTGGTAATCCAGGTGGC-3'
RRM1-EcoRI-forw	5'-ATTGAATTCGTGTTGGGTCTCCCA-3'
RRM1-XhoI-STOP-rev	5'-AATTCTCGAGCTAAAGTTTGCAGTCACACCA-3'
RRM2-EcoRI forw	5'-ATTGAATTCGTGTTTGTGGGGCGC-3'
RRM2-XhoI-STOP-rev	5'-AATTCTCGAGCTACTTAGGTTCGGCATTGGA-3'

Table 8.Oligos used for site-directed mutagenesis and to clone TDP43 domains.

Plasmids carrying hTDP43^{WT,M337V,A382T}and hFUS^{WT,R521G,P525L,R495X} were already present in the lab. Plasmid carrying hHDAC1 was bought from Addgene (#13820). TDP43 deletion mutants were made by site-directed mutagenesis protocol (Agilent Technologies Inc, Stratagene, QuikChange® Site-Directed Mutagenesis #200518).

Antibodies.

In the present study we used the following antibodies: mouse anti-myc (clone 9E10 Sigma-Aldrich, 1:5000 for western blot and 1:10000 for immunofluorescence), rabbit anti-FLAG (Sigma Aldrich, F7452), rabbit anti-Cu/Zn SOD1 (Enzo Life Science, 1:2000 final dilution), rabbit anti-TARDBP (Proteintech, 1:1500 final dilution), mouse anti-FUS/TLS (Santa Cruz Biotechnology, 1:1000 final dilution), mouse anti-β-actin (Sigma-Aldrich, 1:5000 final dilution), anti-phospho (Ser10)-acetyl (Lys14)-Histone H3 (MilliporeMerck, 07-081,), anti-Histone H3 (diMethyl) (Lys4) (MilliporeMerck, 07-030), anti-trimethyl Histone H3(Lys9) (MilliporeMerck, 05-1250), anti-Histone H3 (MilliporeMerck,05-499), Alexa Fluor®488 goat anti-mouse IgG (Thermo Fisher Scientific), Alexa Fluor®647 goat anti-rabbit IgG (Thermo Fisher Scientific), Goat anti-Mouse IgG Peroxidase conjugated (Millipore), Goat anti-Rabbit IgG Peroxidase conjugated (Millipore).

HDAC inhibitors

To inhibit HDAC1 activity, we used Sodium Butyrate (B5887, Sigma Aldrich) and Trichostatin A (T8552, 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 tock concentration of 1 mg/ml, and then diluted at the final concentration of 10 and 25 nM.

Standard technique of molecular biology.

All standard technique of molecular biology were performed according to *Molecular Cloning–A Laboratory Manual* (Sambrook, Russell et al. 2006).

DNA purification from agarose gel.

Whenever it was necessary to purify DNA from agarose gel was used the commercial *Kit Wizard ® SV Gel and PCR Clean-Up System* (Promega) according to the manual instructions.

Mutagenesis and PCR reactions

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

95 °C for 5’

95° C for 3’
54-56 °C for 30’’ } for 18 cycles
72°C (1’ for Kb)

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.

Plasmid DNA purification.

When it has been requested a greater quality of plasmidic DNA (i.e. sequence analysis or cell's transfection), was used the commercial kit *Wizard®Plus SV Minipreps DNA Purification System* (Promega) and *PureLink® HiPure Plasmid Midiprep Kit* (Thermo Fisher Scientific) according to the manual instructions.

Cell cultures.

SH-SY5Y were cultured in Dulbecco MEM/F12 ground (Thermo Fisher Scientific), HEK293 in Dulbecco MEM (Thermo Fisher Scientific), always in the presence of 10% fetal calf serum, free of tetracycline contamination (Tet-free FCS, Clontech) and inactivated at 56 °C for 30'. Medium contained100 units/ml penicillin G and 100 µg/ml streptomycin (Thermo Fisher Scientific). The cells were grown in an incubator at 37 °C in a humidified atmosphere containing 5% CO_2. Trypsin (0.5 g/ml, 68 mM EDTA) was added to split cells, then diluted in fresh medium.

Transfection of eukaryotic cells.

Transient expression of each plasmid (1.5 µg DNA/5–7×10^5 cells) was obtained transfecting cells with lipofectamine LTX and PLUS reagent (Thermo Fisher Scientific). We diluted the optimized amount of plasmid DNA in OPTIMEM (Thermo Fisher Scientific) and then we added the optimized volume of PLUS™ Reagent directly to the diluted DNA. After mixing gently, we incubated for 5' at room temperature. Then, the optimized volume of Lipofectamine™ LTX was added directly to the diluted DNA, incubated for 30' at room temperature. DNA-lipid complexes are stable for 6 hours at room temperature. DNA-lipid complex were added dropwise to the well containing cells. Medium was changed 4-6 hours later.

Production of recombinant adenovirus.

We plated HEK 293 cells at a density of 1–2 x 10^6 cells per 60-mm culture plate 12–24 hours before transfection and incubated at 37°C in a humidified atmosphere maintained at 5% CO_2. Each 60-mm culture plate was transfected with 5 µg of Pac I-digested BD Adeno-X DNA using standard transfection method. One day later, and periodically

thereafter, we check for cytopathic effect (CPE). One week later, cells were transferred to a sterile 15-ml conical centrifuge tube (without using trypsin: infected cells that still adhere to the bottom or sides of the culture plate can be dislodged into the medium by gentle agitation). We centrifuged the suspension at 1,500 x g for 5 min at room temperature. Pellets were resuspended in 500 µl sterile PBS. Cells were lysed with three consecutive freeze-thaw cycles: freeze cells in a dry ice/ethanol bath; cells were thawed by placing the tube in a 37°C water bath (do not allow the suspension to reach 37°C). and vortexed after each thaw. The lysate was transferred to a clean, sterile centrifuge tube and used immediately to infect a fresh 60-mm culture by adding 250 µl (50%) of the cell lysate directly to the medium, then incubated as normal. CPE was usually evident within one week. We repeated these steps two or three time until we reached a high titer viral preparation.

Determination of adenoviral titer: End-Point dilution assay.

Approximately 24 hours before beginning the titration protocol, we plate HEK 293 cells in two 96-well plates. We carefully seeded all wells at the same density (~10^4 cells per well) in 100 µl of growth medium. Then, we prepare serial dilutions of your virus as follows:

- 1:100 dilution by adding 10 µl virus stock to 990 µl sterile growth medium.

- Starting with the 1:100 dilution, we prepared serial 1:10 dilutions by transferring 100 µl diluted virus to 900 µl sterile growth medium.

In general, an appropriate range of dilutions for testing is 10^{-3}, 10^{-10}. We removed the 96-well culture plate from the incubator and inspected the wells to ensure that the cells have attached to form an even monolayer. We added 100 µl diluted virus to each well in columns 1–10 and add 100 µl of virus-free growth medium to wells in columns 11–12, these wells serve as controls for the viability of non-infected cells. We incubated cells in a humidified CO_2 (5%) incubator for 10 days at 37°C. Using a microscope, we checked each wells for cytopathic effect (CPE). For each row, we counted the number of wells having CPE. A well is scored as CPE positive even if only a few cells show cytopathic effects. We calculated the fraction of CPE-positive wells in each row. Finally, we calculated Viral Titer:

- Titer (pfu/ml) = 10(x + 0.8)

x = the sum of the fractions of CPE-positive wells.

The assay is a reliable indicator of viral titer only if the following three conditions are met: i) the negative control wells show no visible signs of CPE or growth inhibition; ii) wells infected with the least dilute virus (10^{-3}) are all CPE-positive; iii) wells infected with the most dilute virus (10^{-10}) are all CPE-negative.

Adenoviral infection of eukaryotic target cells.

We plated target cells (SH-SY5Y or SHSY-5Y-HDAC1) 12–24 hours before infection in the culture plates desired. 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 CO2 (5%) incubator at 37°C for 1 hour to allow the virus to infect the cells. Then, we added fresh complete growth medium and incubated in a humidified 5% CO2 at 37°C. We analyzed gene expression at different time points required and then we proceeded to further experimental manipulations.

Assessment of cell viability.

The viability of control SH-SY5Y cells or SH-SY5Y cells infected with adenoviruses encoding for fALS-causative genes was calculated after 72 hours through an MTS assay. Cell viability was assessed by a colorimetric assay using the 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 the manufacturer's instructions. Absorbance at 490 nm was measured in a multilabel counter (Victor X5, PerkinElmer).

Immunofluorescence

1×105 SH-SY5Y cells grown on a cover-glass were washed twice with PBS 1X and then fixed with 1 ml of 4% paraformaldehyde/PBS 1X for 10'. Cells were permeabilized with 0.1% Triton X-100 in PBS and non-specific binding was blocked with 5% bovine serum albumin, 0.1% Triton X-100 diluted in PBS for 1 h at room temperature. Cells were incubated with primary antibodies diluted in blocking solution, overnight at 4°C and then with secondary antibodies and nuclear marker (TO-PRO3, Thermo Fisher Scientific or Hoechst 33342, Sigma-Aldrich), diluted in blocking

solution for 1 h at room temperature. Glasses were analyzed with a Leica TCS SP5 confocal microscopy with LAS lite 170 image software.

SDS-PAGE and western blot analysis

Cells were lysed in Laemmli Buffer (10% SDS, 50% glycerol, 25% 2-mercaptoethanol, 0.02% bromophenol blue and 0.3125 M Tris HCl, pH approx. 6.8)- 10 ug of total extract were run on a SDS-PAGE 15% at 120 V in running buffer (25 mM Tris, 190 mM glycine, 0,1% SDS), then protein were transferred on a nitrocellulose membrane, at 186 mA, in transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol). Membranes were incubated with primary antibodies dissolved in non-fat milk 3% in PBS-Tween 0,05%, ON. Secondary antibodies conjugated to peroxidase were used at room temperature, for 1 hour. Peroxidase reactions were revealed by ECL solutions (Thermo Fisher).

Analysis of modified-histone H3/total H3 ratio

We acquired H3-modified signal, then we stripped the signal incubating with Glycine 1M-ph 2,5. We re-incubated the membrane with total H3 antibody. We analyzed intensity of the signal using Gel Doc XR software. Intensity data of modified histone H3 and total histone H3 were used to calculate the ratio of modified histone and for the further statistic analysis.

Co-immunoprecipitation analysis

$1x10^6$ HEK-293 cells were plated on a 60mm dish. 24 hours later, cells were co-trasnfected with pMTK $TDP43^{WT/M337V/A382T}$ and pCDNA3-HDAC1. 48 hours after transfection, cells were collected in a 1,5 centrifuge tube and lysed in lysis buffer (NaCl 150mM,Tris-HCl 20 mM pH 7,5, NP40 1%) with protease inhibitors 1:1000. Lisates were washed and centrifuged to discard pellet debris. Protein lisates were incubated with protein-A-sepharose for 30' in agitation at 4°C to pre-clear the lysates and reduce non-specific binding. Then, protein-A-sepharose was discarded andante-myc primary antibody 1:1000 was added. Lysates with anti-myc were incubated ON at 4°C in agitation. Then, new fresh protein-A-sepharose was added, to precipitate protein-antibody complexes. The complexes were incubated for 2 hours at 4°C in agitation. Finally, the complexes were centrifuged, washed four times in lysis buffer, and then

protein-A-sepharose was lysed in Laemmli Buffer 1X to be processed in western blot analysis with anti-FLAG antibody.

Cells viability assay with HDAC1 inhibitors

$2x10^4$ SHSY-5Y cells were plated on a 96 well dish 24 hours before infection. Cells were transduced as described above with adenoviruses carrying $TDP43^{WT/M337V/A382T}$. 1 hour after the transduction, cells were treated with NaB 0,2 or 0,04 mM or TSA 10 or 25 nM. Treatment was repeated 24 hour after transduction. 48 hours after transduction, MTS assay was performed, as described above.

Genomic DNA extraction

Genomic DNA was purified from cells expressing $SOD1^{WT/G93A/H80R}$, $FUS^{WT/R521G/P525L/R495X}$, $TDP43^{WT/M337V/A382T}$ with a commercial kit (G1N70, Sigma Aldrich).

P/ACE capillary electrophoresis

To measure 5'-methylcytosine amount in genomic DNA, we dried the samples (eluted in mqH_2O) to lyophilize them. Then they were mixed with formic acid (100-µL final volume, 90% final concentration) and incubated at 130 °C for 80 min. After hydrolysis, the samples were exsiccated at 60 °C under a vacuum and the dry residue containing free bases was dissolved in 100 µL of acetonitrile/ water (50:50). Finally, they were stored at −20 °C or analyzed immediately. Capillary electrophoresis and next methylation analysis was performed by a PACE MDQ system according to Zinellu et al (Zinellu, Sotgia et al. 2011).

Nuclear-cytoplasmic separation.

$1/2\times10^6$ SH-SY5Y cells were transduced with TDP43 adenoviral vectors. 48 h after transduction cells were wash twice with cold PBS 1X, harvested in 500 µl of PBS 1X and centrifuged at 1000 x g for 3 min at 4 °C. The pellet were resuspended gently in 500 µl of S1 buffer (10mM Hepes pH 7.9, 10mM KCl, 1.5mM MgCl2, 0.1mM EGTA pH 7.0, 1M DTT (1 µl/2ml), and protease inhibitors mixture, Sigma-Aldrich). We syringed ten times at 4 °C and centrifuge at 1000 x g for 3' at 4 °C, supernatant (cytoplasmic

fraction) was collected and the pellet was resuspended gently in 500 µl of S1 buffer. We centrifuged at 3000 rpm for 3' at 4 °C, the pellet was resuspended with 30µl of S2 (10mM Hepes pH 7.9, o.4M NaCl, 1.5mM MgCl2, 0.1mM EGTA pH 7.0, 5% glycerol 1M DTT (1 µl/2ml), and protease inhibitors mixture, Sigma-Aldrich). We shaked for 30 min at 4 °C, centrifuge for 10 min at 4 °C at 12,000xg and collected the supernatant (nuclear fraction).

HDAC1 enzymatic assay

HDAC1 activity was measured with a commercial kit (Epiquik HDAC1 assay kit (colorimetric), Epigentek, P-4005-96). We first plated $2x10^6$ SHSY-5Y cells, transduced them, and 48 hours later we purified the nuclear fraction. On 5 ug of protein from the nuclear extract, according to manufacturer's instructions, we performed the assay. Colorimetric results were read on a multiplate reader.

4. RESULTS

4.1 ANIMAL AND CELLULAR MODELS FOR ALS

In order to study epigenetic modifications induced by the expression of 3 differentALScausative-gene,SOD1 (OMIM # 105400), TDP43 (OMIM # 612069) and FUS (OMIM # 608030), we decided to use the following cellular and animal models able to mimic the genetic alterations that cause ALS:

(i) *Transgenic mice SOD1-G93A*. Different transgenic mice bearing mutations on the sod1 gene have been developed as ALS models, among which there is the G93A mouse strain. These mice die within about 140 days, presenting typical ALS symptoms: motoneuronal death, muscular atrophy, and paralysis.

(ii) *Adenoviral delivery of ALS causative-gene* Although cell lines over-expressing mutant SOD1 have been widely used as model of ALS, it is well known that stable cell lines tend to show adaptive effects towards toxic genes. In order to prevent it we plan to obtain high level of expression using an adenoviral delivery strategy, one of the most reliable methods for introducing genes in mammalian transformed cell lines as well as into primary culture.In particular, both WT and mutant variants of the mentioned genes were expressed.

fALS	OMIM	Gene	Pathological mutations
ALS1	# 105400	SOD1	G93A, H80R
ALS6	# 608030	FUS	R495X, R521G, P525L
ALS10	# 612069	TDP43	M337V, A382T

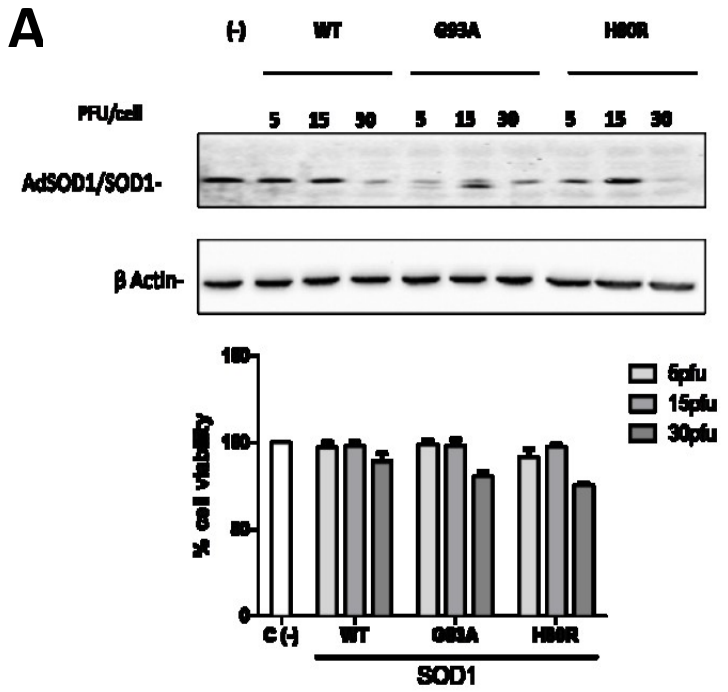
Table 9.Familiar ALS causative genes and relative pathological mutations.

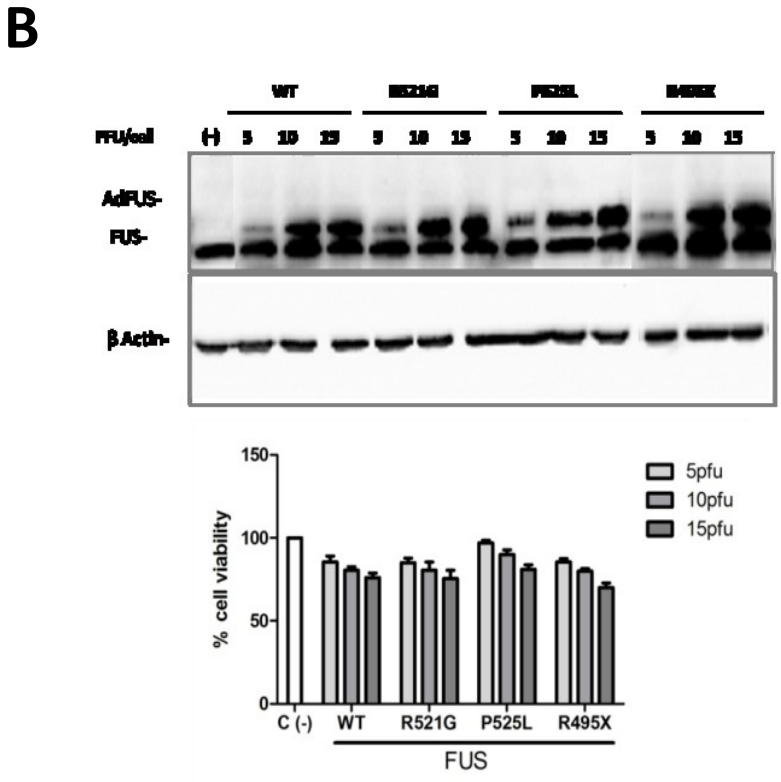
To drive the expression of ALS-causative genes, the recombinant adenoviruses encoding for hSOD1, hTDP43 (WT or mutant) and hFUS (WT or mutant) genes were produced. Recombinant adenoviruses provide a versatile system for gene expression studies and therapeutic applications. Since infection by adenovirus is not cell-cycle dependent, it is possible to deliver the gene of interest to primary as well as to transformed cell lines. Following infection, target gene was transiently expressed at high levels since many cells received multiple copies of the recombinant genome. Expression is transient because adenoviral DNA normally does not integrate into the cellular genome. The assembly of recombinant adenoviruses is completed in few stages: first the cDNA coding for WT or pathological mutant SOD1, TDP43 and FUS was

cloned into pShuttle2 vector (in fusion with five repeats of myc epitope 5xMyc for TDP43 and FUS). Then, the expression cassette was excised from recombinant pShuttle2 plasmid DNA by digesting with the homing endonucleses I-Ceu I and PI-Sce I and ligated into Adeno-X Viral DNA. Recombinants were selected with kanamycin and screened by restriction endonuclease digestion. Third, the recombinant adenoviral construct was cleaved with PacI to expose its inverted terminal repeats and transfected into a packaging cell line Adeno-X 293. After 7–10 days, viruses were harvested and amplified by infecting packaging cells for three times to obtain high titer virus stock. By performing an end-point dilution assay, the final yields were evaluated generally around 10^8 plaque-forming particles per ml. Recombinant adenoviruses were finally used to infect neuronal SH-SY5Y with a multiplicity of infection (M.O.I.) of 5-30 pfu/cell. At the indicated time after infection cells were harvested and protein extracts analyzed by western blot.

4.1.1 Characterization of ALS cellular models

As an initial step to characterize the effects of adenoviral delivery of fALS genes into neuronal cells we evaluated metabolism of SH-SY5Y cells after infection with scalar concentration of infectious adenoviruses encoding for WT or mutant fALS genes through western blot analysis on total lysates. The cellular viability was analyzed performing a viability assay using a commercial kit (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega). Neuronal SH-SY5Y cells were infected with increasing concentrations of adenoviruses encoding for WT or mutant fALS genes and 72 hours post-infection the MTS assay was performed, according to the manual instruction. As expected, SOD1, both WT and mutant forms, doesn't induce any decrease in cells vitality, except for the infection with 30 pfu/cell, where the toxic effect could be due to the infection itself and not to the over expression of the SOD1 gene. Concerning FUS, we saw a mild decrease in cells vitality, for WT, R521G and R495X forms. TDP43 is extremely toxic, reducing cells vitality of the 20%, both in WT and mutant forms (see figure 14).


A
(-)
WT
G93A
H80R
PFU/cell
5 15 30 5 15 30 5 15 30
AdSOD1/SOD1-
β Actin-
% cell viability
5pfu
15pfu
30pfu
C (-)
WT
G93A
H80R
SOD1


B
WT
PFU/cell
(-) 5 10 15 5 10 15 5 10 15 5 10 15
AdFUS-
FUS-
β Actin-
% cell viability
5pfu
10pfu
15pfu
C (-)
WT
R521G
P525L
R495X
FUS

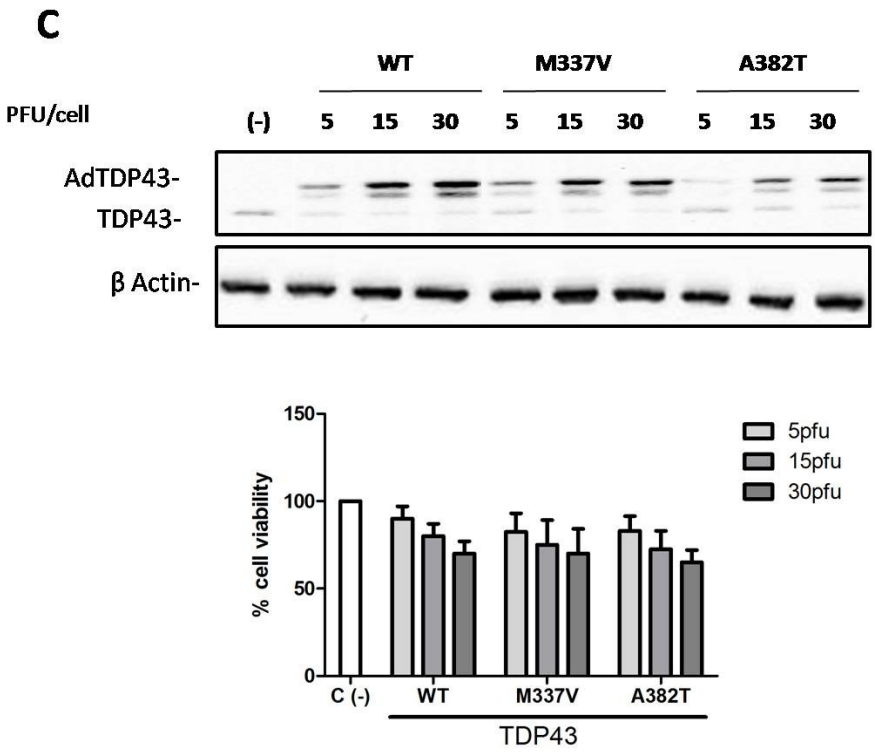


Figure 14.ALS causative genes SOD1, FUS and TDP43 overexpression alter cells viability. A) MTS assay on SHSY-5Y cells transduced by SOD1 adenoviruses, at a final concentration of 5, 15 and 30 PFU/cell. B) MTS assay on SHSY-5Y cells transduced by FUS adenoviruses, at a final concentration of 5, 10 and 30 PFU/cell. C) MTS assay on SHSY-5Y cells transduced by TDP43 adenoviruses, at a final concentration of 5, 15 and 30 PFU/cell.

4.2 ANALYSIS OF HISTONE POST-TRANSLATIONAL MODIFICATIONS H3-K14ac-S10ph, H3K14me2 AND H3K9me3

As stated in general introduction (paragraph 3) in both ALS experimental models and patients, a general misregulation of the epigenome had been observed (Rouaux, Jokic et al. 2003, Paez-Colasante, Figueroa-Romero et al. 2015). In order to evaluate if ALS causing genes SOD1, FUS and TDP43 toxicity could be linked to alterations in the epigenetic machinery, we analyzed some histone post-translational modification linked to:

1. transcriptional activation:

- dimethylation of lysine 4 on H3 tail (H3-K14me2), that is associated to active transcription and splicing (H3K4 trimethylation could facilitate pre-mRNA maturation via bridging of the spliceosomal components to actively transcribed gene).

- phospho-acetylation of serine 10 and lysine 14 on H3 tail (H3-K14ac-S10ph is associated to transcriptional activation of immediate early-genes in neuronal populations in response to a variety of stimuli);

2. transcriptional repression: trimethylation of lysine 9 on H3 tail leads to transcriptional repression (H3-K9me3).

We tested, in a preliminary experiment, some histone modifications (H3-K14ac-S10ph and H3K4me2) in cells under oxidative stress conditions, which is considered one of the most important causes of death of motor neurons in ALS. SHSY-5Y cells were treated with scalar concentrations of H_2O_2 and analyzed 30' and 3 h later. Equal amount of total protein lysates were separated by SDS-PAGE and epigenetic markers aforementioned were analyzed by western blot analysis. As shown in figure 15, H3-K14ac-S10ph is

strongly reduced, according to what mentioned by Rouax et al, 2007. H3K4me2 levels do not show any significant variation.

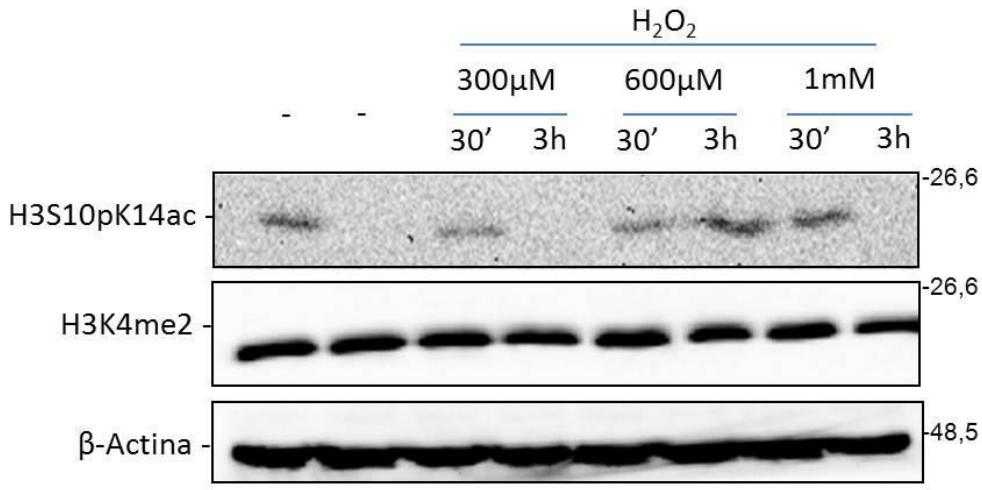


Figure15: Western blot analysis on 10 ug of proteic extracts of SHSY-5Y cells treated at different times with scalar concentrations of H_2O_2. From the left: control cells, 30' treatment with H_2O_2 300 uM, 3h with H_2O_2 300 uM, 30 ' with H_2O_2 600 uM, 3h with H_2O_2 600 uM, 30' with H_2O_2 1 mM, 3h with H_2O_2 1 mM.

We then decided to transduce SHSY-5Y with adenovirus coding for WT or mutant SOD1, FUS and TDP43 and 48 hours later total cell lysates were analyzed by western blot.

4.2.1 SOD1 induce an in vitro decrease in transcriptional activation markers and an increase in repression markers

As shown in figure 17, expression of SOD1 pathological mutants G93A and H80R induce a significant decrease in transcriptional activation markers H3-S10Ph-K14Ac and H3-K14me2 ($p<0,05$). Nevertheless, SOD1 WT overexpression induces an increase in transcriptional repression marker H3-K9me3. A similar effect is not observed in pathological mutant forms. Data on histone modification levels were normalized by total/ modified H3 ratio.

4.2.2 SOD1 reduction of H3-14Ac-S10Ph marker is in proportion with the time of infection

We then, performed a time-course experiment, analyzing H3K14acS10ph marker at 24, 48 and 72 hours form transduction with SOD1$^{WT/G93A/H80R}$. We observed that the reduction in the marker is in proportion with the increasing in transduced SOD1 expression, which accumulates over time (see figure 16). Results are similar to what observed above, i.e. that point pathological mutations of SOD1 gene lead to a reduction in transcriptional activation marker H3-K14ac-S10ph levels.

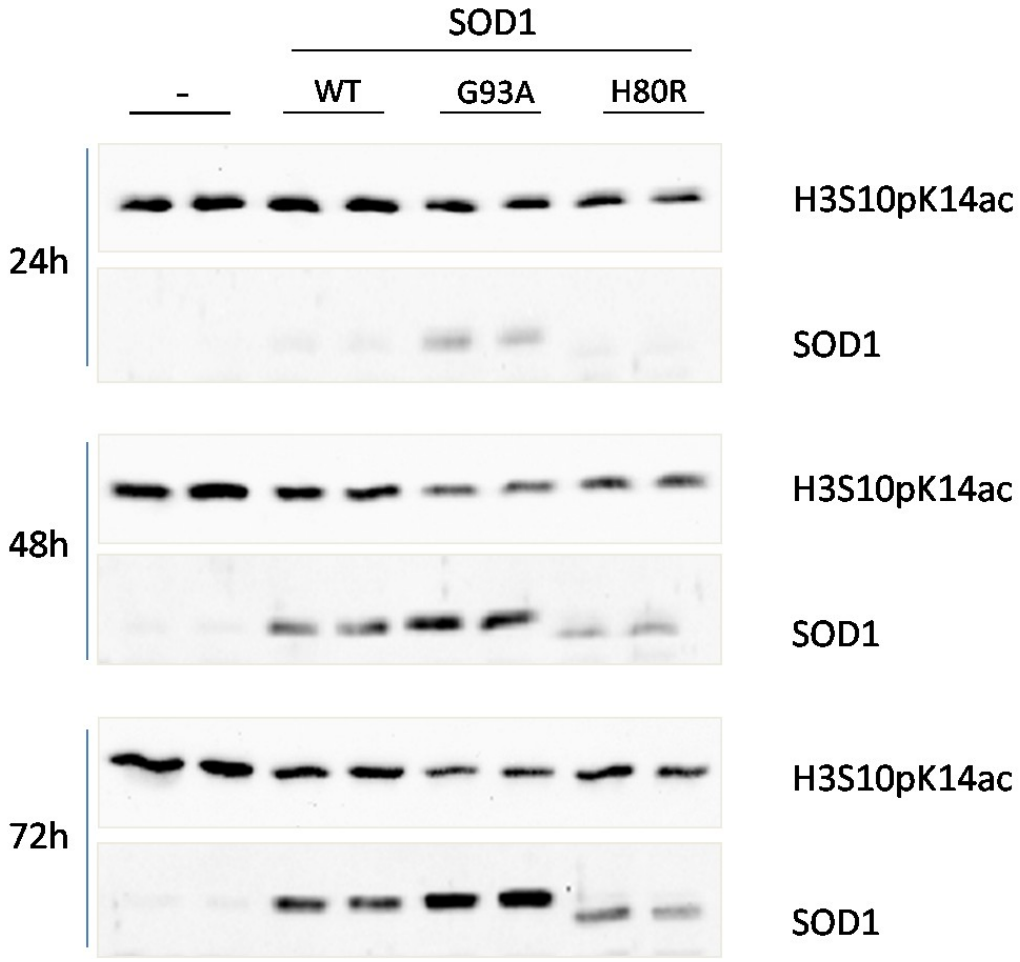


Figure16. Increasing expression in time with SOD1 correlates with the decrease of H3-K14acS10ph marker level. SHSY-5Y cells were transduced with SOD1$^{WT/G93A/H80R}$ and expression level of H3-K14acS10ph marker were analyzed by western blot with 10ug of total extract form cells at 24 h, 48 h or 72 h after transduction.

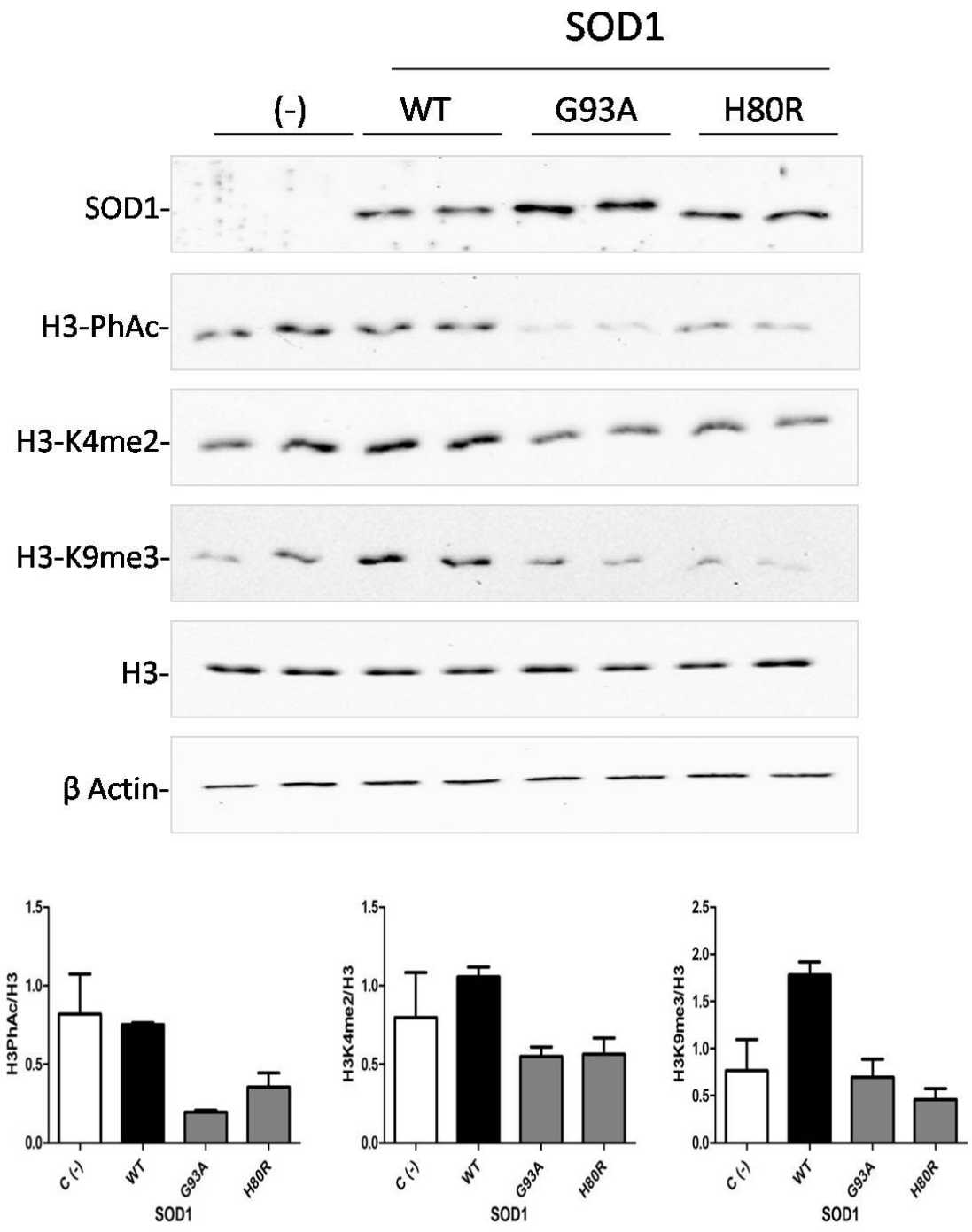


Figure17. Pathological point mutation in SOD1 induce a decrease in transcriptional activation markers whereas SOD1^{WT} induce an increase in transcriptional activation marker. SHSY-5Y cells were transduced with 10 PFU/cell of SOD1$^{WT/G93A/H80R}$ adenoviral vectors. Total extract were collected 48 h later. SDS-PAGE and western blot analysis were performed according to Materials and Methods. From the top: expression of SOD1$^{G93A/H80R}$ induce a decrease in transcriptional activation markers H3-K14acS10ph and H-K14me2. Overexpression of SOD1^{WT} induce an increase in transcriptional activation marker H3-K9me3. Data were analyzed by ONE-WAY ANOVA with a level of significance of 0.05.

4.2.3 SOD1 induce a decrease in transcriptional activation markers in SOD1 G93A spinal cord mice

We demonstrated that H3-K14Ac-S10-Ph and H3-K14me2 transcriptional activation signal are reduced in motor neurons spinal cord of $SOD1^{G93A}$ mice when compared to non transgenic mice (see figure 18). Spinal cords were collected from non-symptomatic(70 days from birth),pre-symptomatic (100 days from birth) and symptomatic mice (140 days from birth) $SOD1^{G93A}$mice. Non transgenic littermates were used as controls. As shown in 18, we performed an hematoxylin and eosin stain to mark motor neurons death areas. In non-transgenic mice, motor neurons are evident, on the contrary, transgenic $SOD1^{G93A}$ spinal cord shows a significant reduction in motor neurons, both in the pre-symptomatic (100 days after birth) as in the symptomatic mice (140 days after death), where motor neuronal death is almost complete.

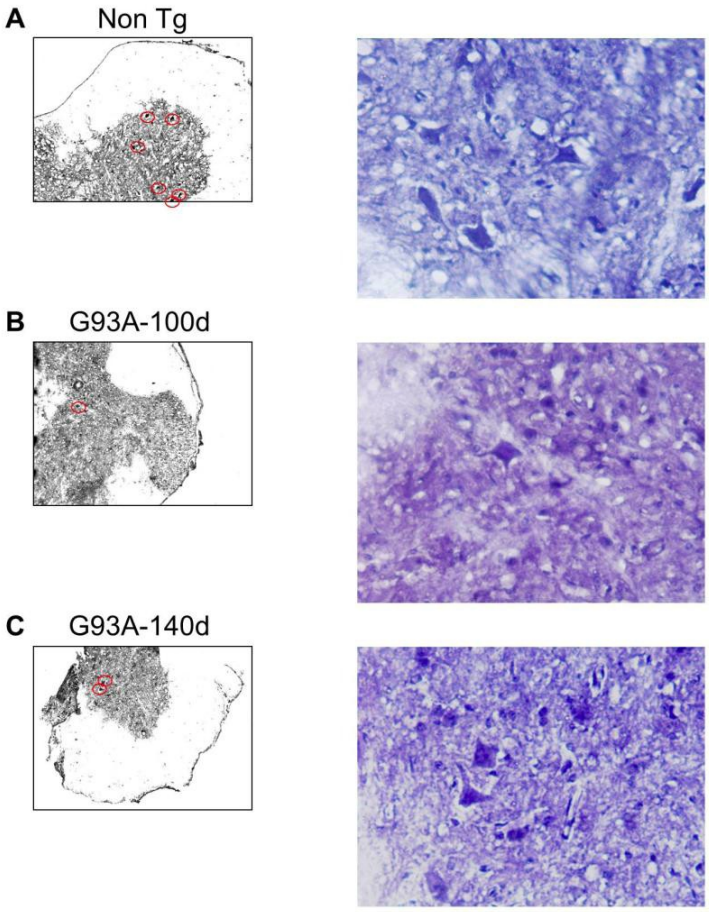


Figure 18 Ematoxylin-eosin stain of 10 um slides of nTg 100 days mice (A), or Tg SOD1^{G93A} in pre symptomatic form (B) or at 140 days after birth, simptomatic form (C).

Then, we performed immunofluorescence analysis on spinal cord slides collected from the same mice. We used monoclonal antibody directed against H3-K14Ac-S10ph and against SMI-32, a specific marker of neurofilaments to stain neurons. Confocal microscopy allowed us to evidence a reduction in H3K14Ac-S10ph levels in motor neurons of SOD1^{G93A}presymptomatic and moreover of symptomatic mice, compared to control non-transgenic mice. H3K4me2 levels too decrease in transgenic mice compared to control non-transgenic mice, even if the reduction is less intense (figure 19). These *in vivo* evidences confirm what observed in *in vitro* experiments, i.e. that SOD1^{G93A} induce a decrease in histonic markers associated with transcriptional activation.

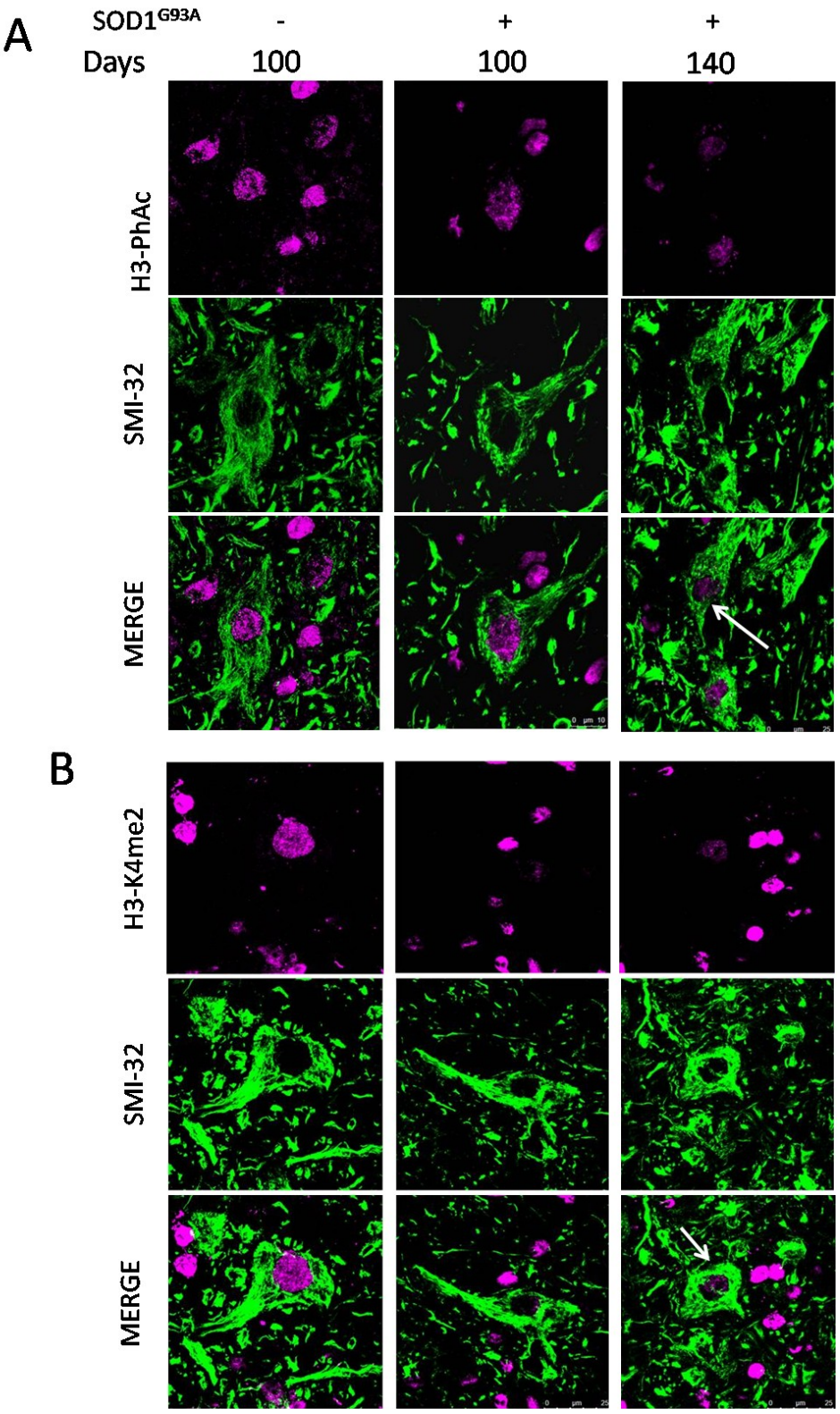


Figure 19. Immunofluorescence analysis on spinal cord sections SOD1^{G93A} demonstrated that SOD1^{G93a} induce a significant decrease in transcriptional activation markers. A) of SOD1^{G93A} mice, pre symptomatic at 100 days, and symptomatic, at 140 days. White arrows indicates that H3-PhAC signal is drastically reduced in both cases compared to control wild type mice. B) H3K4me2 signal is reduced in pre symptomatic and symptomatic mice compared to wild type control mice (white arrows).

4.2.4 FUS and TDP43 overexpression induce an increase in transcriptional repression marker

As for SOD1, different lines on evidenced linked FUS and TDP43 to alteration in gene expression, that can be linked to directly or indirectly to alteration in epigenetic markers. We thus decided to test if also FUS or TDP43 pathological mutants can globally influence histone modifications. As shown in figure 20, both WT or pathological mutants FUS or TDP43 overexpression doesn't induce a similar effect compared to SOD1,since we do not observe a decrease in transcriptional activation markers. Moreover, we could observe any statistically significant increase in transcriptional repression marker H3-K9me3 in cells expressing $SOD1^{WT}$but not mutant forms. This trend is similar to what observed for FUS^{WT} and $TDP43^{WT}$, even if in these cases the data are not statistically significant. (see figures 20, 21).

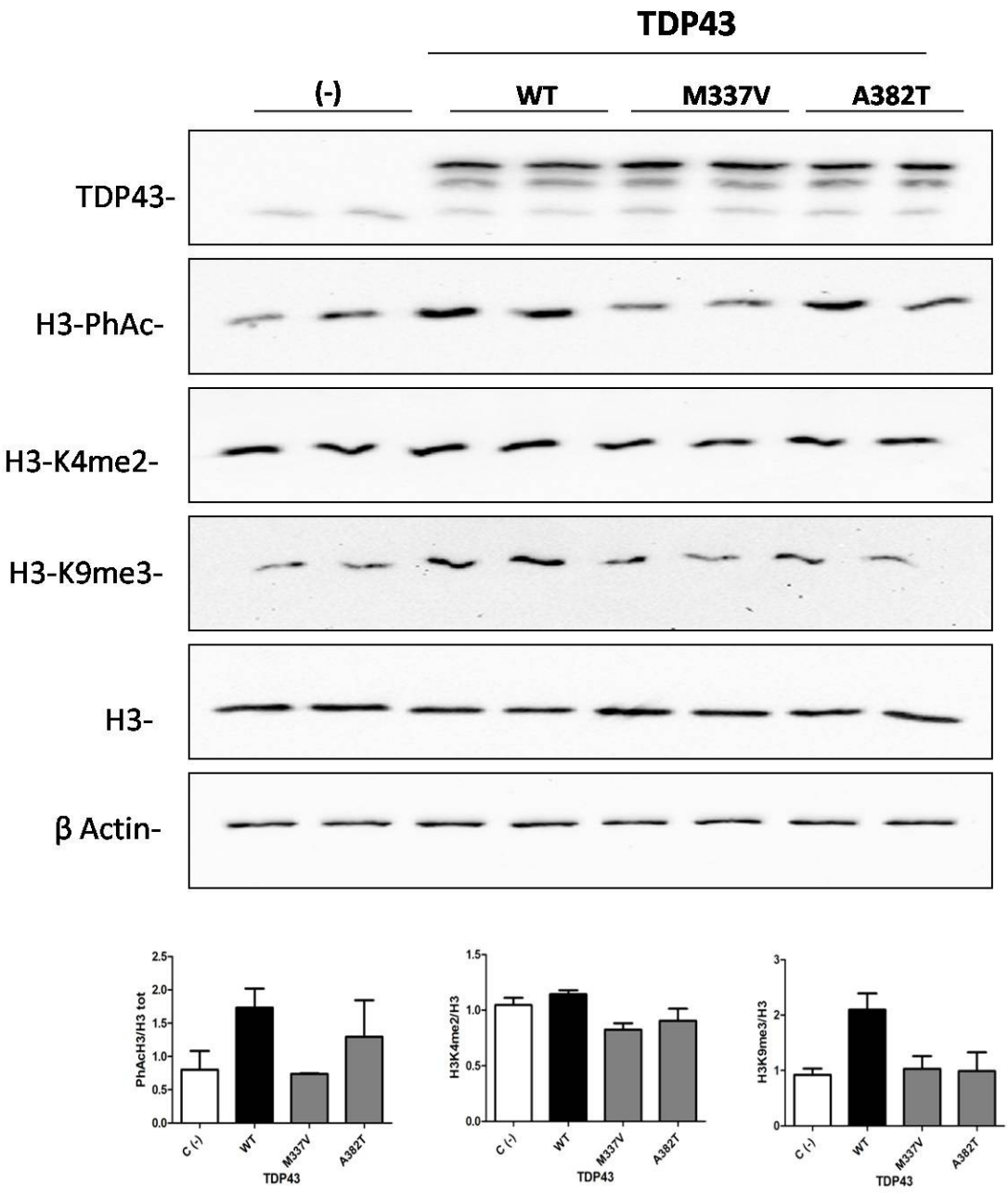


Figure 20. Pathological point mutation in TDP43 doesn't alter transcriptional activation markers whereas TDP43^{WT} induce an increase in transcriptional activation marker. SHSY-5Y cells were transduced with 10 PFU/cell of TDP43$^{WT/M337V/A382T}$ adenoviral vectors. Total extract were collected 48 h later. SDS-PAGE and western blot analysis were performed according to Materials and Methods. From the top: expression of TDP43$^{M337V/A382T}$doesn't induce any decrease in transcriptional activation markers H3-K14acS10ph and H-K14me2. Overexpression of TDP43^{WT} induce an increase in transcriptional activation marker H3-K9me3. Data were analyzed by ONE-WAY ANOVA with a level of significance of 0.05.

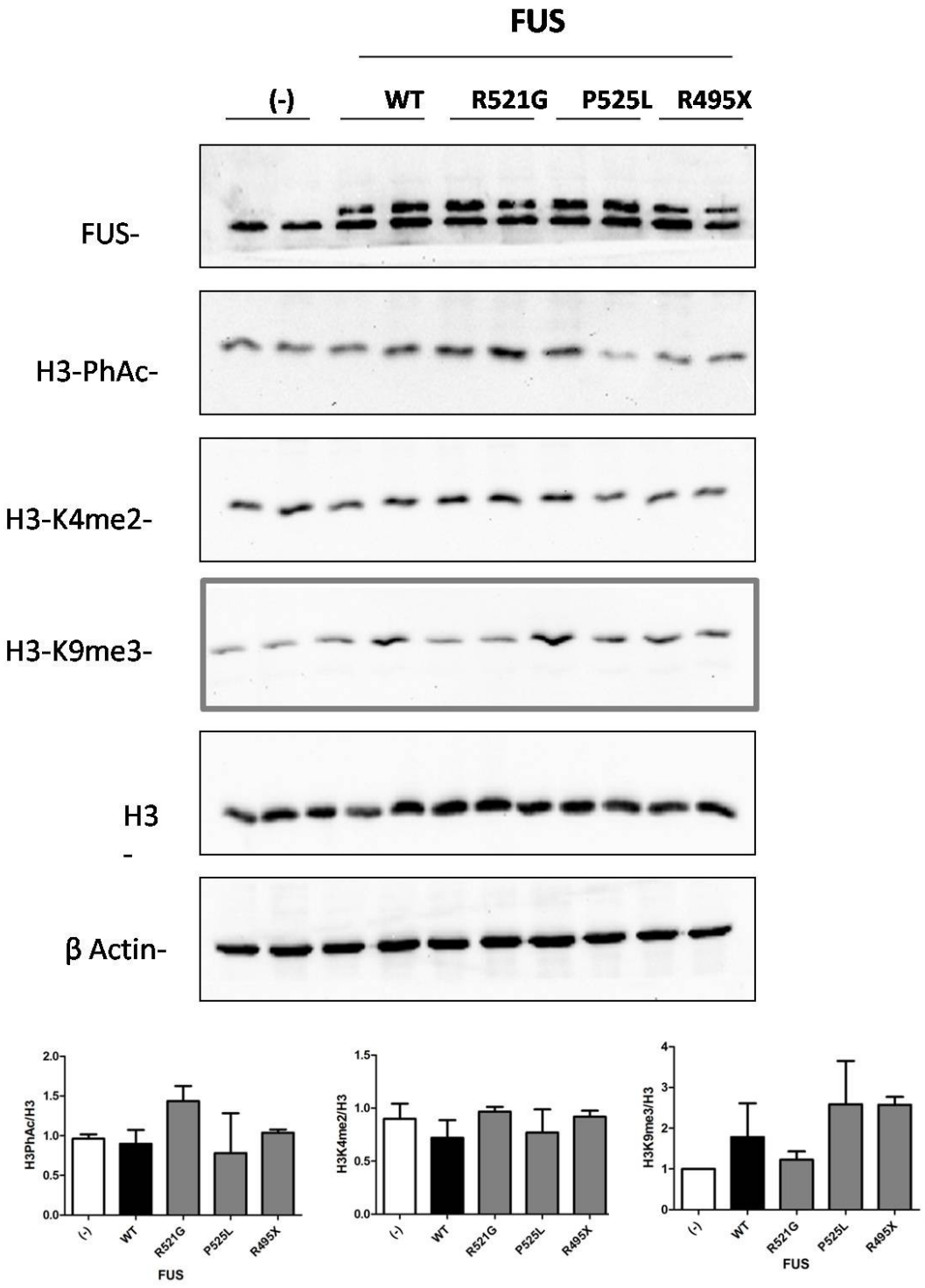


Figure 21. Pathological point mutation in FUS doesn't alter transcriptional activation markers whereas FUS^{WT} induce an increase in transcriptional activation marker. SHSY-5Y cells were transduced with 10 PFU/cell of $FUS^{W/R521G/P525L/R495X}$ adenoviral vectors. Total extract were collected 48 h later. SDS-PAGE and western blot analysis were performed according to Materials and Methods. From the top: expression of $FUS^{R521G/P525L/R495X}$ doesn't induce any decrease in transcriptional activation markers H3-K14acS10ph and H-K14me2. Overexpression of FUS^{WT} induce an increase in transcriptional activation marker H3-K9me3. Data were analyzed by ONE-WAY ANOVA with a level of significance of 0.05.

4.3 SOD1G93A OVER EXPRESSION INDUCES A GLOBAL INCREASE IN DNA METHYLATION

Several evidences shows that 5-methylcytosine increase in ALS patients (Martin and Wong 2013). To further understand the possible role of WT or pathological mutant SOD1, FUS or TDP43, we analyzed global DNA methylation levels by a HPLC-P/ACE approach. We purified genomic DNA from cells transduced by adenoviral particles, and we analyzed 2,5 ug, as described in Materials and methods. Data were analyzed by Graph Pad software, in a ONE-WAY ANOVA. Again, SOD1 pathological mutant G93A induces a significant increase in 5-met-cyt (figure 22). No significant alterations were observed in DNA of cells transduced by FUS or TDP43 (data not shown).

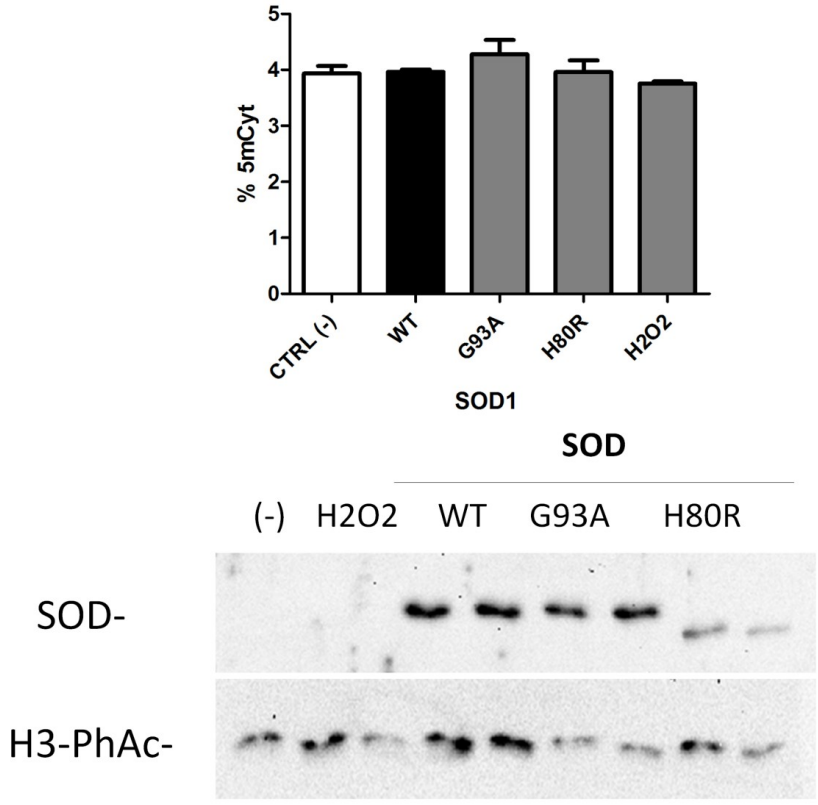


Figure 22. SOD1^{G93A} induce a significant increase in global DNA methylation. SHSY-5Y cells were transduced with 10 PFU/cell of SOD1 adenoviral vectors. Genomic DNA were extracted with a commercial kit (See Materials and Methods). Levels of 5mCyt were quantified with a F/ACE approach. ONE-WAY ANOVA followed by Bonferroni test evidenced a statistically significant increase in 5mCyt levels in genomic DNA of cells expressing SOD1^{G93A}.

4.4 TDP43 INTERACTS WITH HDAC1

4.4.1 TDP43^{WT} interacts with HDAC1 in vitro

Histone deacetylases is a complex class of proteins involved in the regulation of the epigenome, linked to ALS onset and progression, at many different levels: (i) class III histone deacetylases SIRT1 decreases in the spinal cord, but increases in muscle during the progression of the disease, while SIRT2 mRNA expression increases in the spinal cord in both G93A-SOD1 and G86R-SOD1 mice but protein expression is substantially unchanged in all the models examined (Valle, Salvatori et al. 2014);(ii) treatment with HDAC inhibitors phenylbutyrate and sodium valproate promoted motor neuron survival in mouse models of ALS (Kazantsev and Thompson 2008); (iii) FUS interacts with HDAC1 by its G-rich and C-terminal domains (Wang, Pan et al. 2013); (iv) TDP43 is deacetylated by HDCA6 (Cohen, Hwang et al. 2015). We thus decided to perform co-immunoprecipitation experiments between TDP43 and three different HDCAs, namely HDAC1, HDAC6 and SIRT1, expressed in frame with a FLAG epitope (figure 23). Despite numerous assays we were not able to express at high levels neither HDAC6 or SIRT1 (figure 23), and in the co-ip experiment we were not able to detect a positive signal (figure 24).

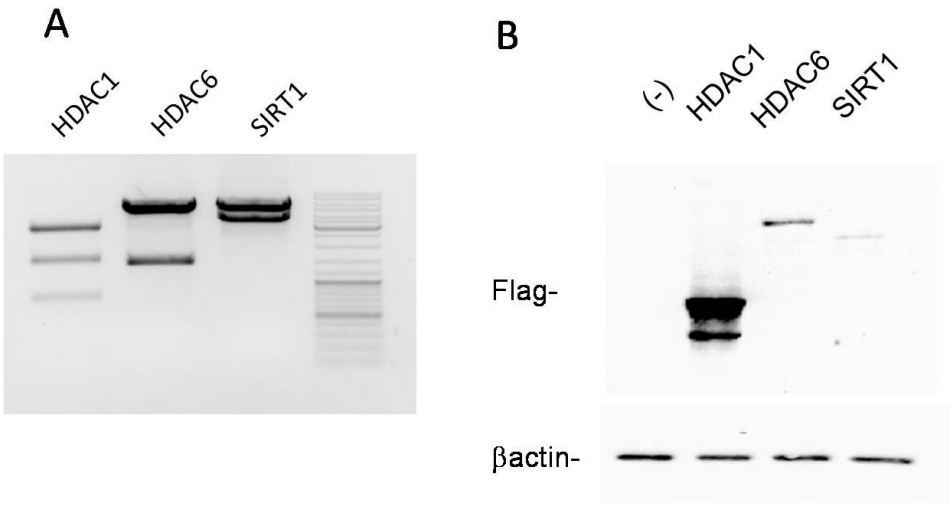


Figure 23. Histone deacetylase 1, 6 and Sirtuin 1 are the major representative of HDAC families. A) Agarose gel analysis of pCDNA3-HDAC1-FLAG, pCDNA3-HDAC6-FLAG and pECE-SIRT1-FLAG plasmids. B) Anti-FLAG western blot analysis on protein total extract from SHSY-5Y cells transfected with 500 ug of plasmids above.

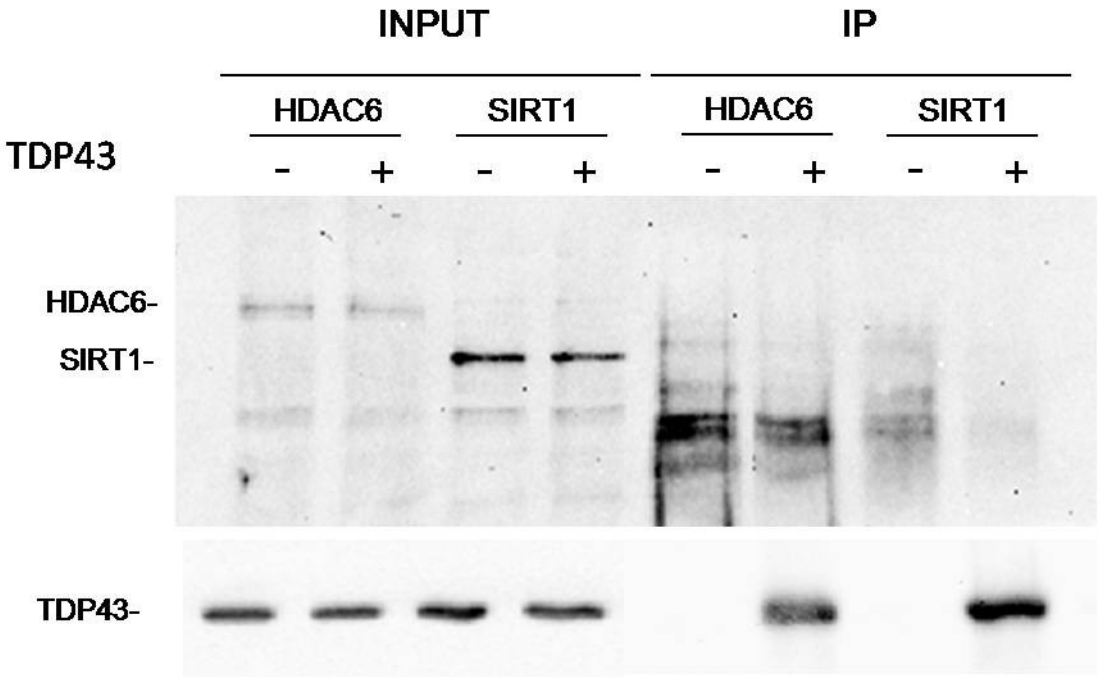


Figure 24. TDP43 doesn't interact with HDAC6 or SIRT1. HEK 293T cells were transiently co-transfected with FLAG-tagged HDAC6 (FLAG-HDAC6) or FLAG-tagged SIRT1 (FLAG-SIRT1). 48 hours after transfection, total cell lysates were subjected to immunoprecipitation with anti-c-myc monoclonal antibody 9E10. Binding of HDAC1 to TDP43 was analyzed with western blotting with the indicated antibody.

We thus focalized our attention on HDAC1. We transfected FUS or TDP43 fused with myc-tag in association with HDAC1 fused with FLAG-tag. We immunoprecipitated the protein lysates with an anti-myc monoclonal antibody and we revealed the interaction by western blot analysis with a anti-FLAG monoclonal antibody. We confirmed the interaction between FUS and HDAC1, and we observed that also $TDP43^{WT}$ interacts with HDAC1 (fig. 25).

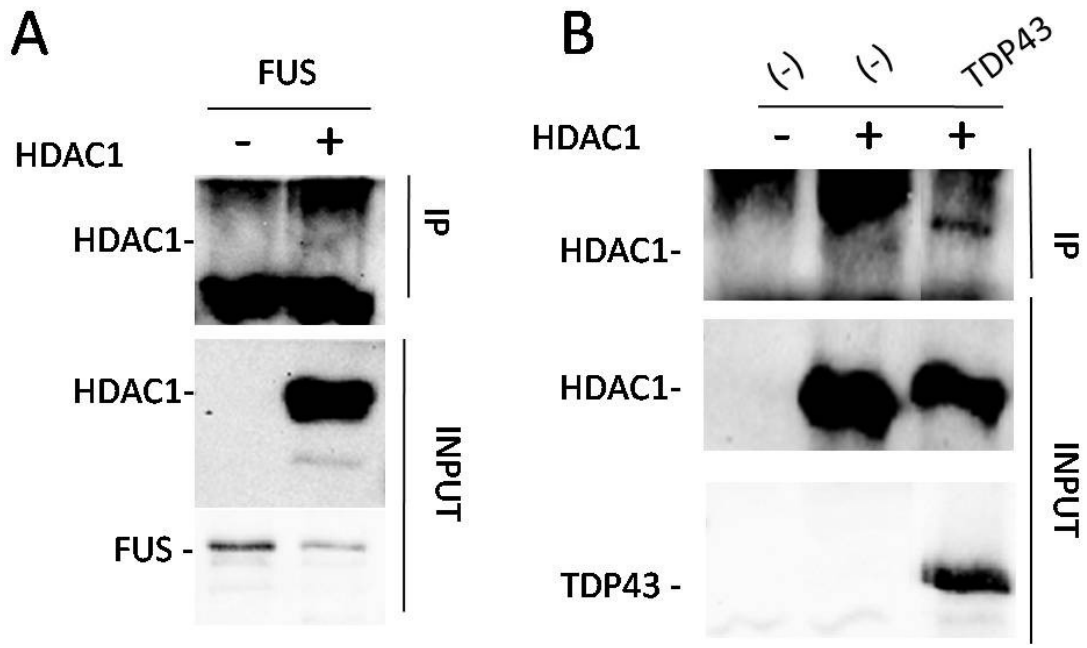


Figure 25. TDP43 interacts with HDAC1. HEK 293T cells were transiently co-transfected with FLAG-tagged HDAC1 (FLAG-HDAC1), (A) c-myc-tagged FUS (myc-FUS) or c-myc-tagged TDP43 (myc-TDP43) (B) 48 hours after transfection, total cell lysates were subjected to immunoprecipitation with anti-c-myc monoclonal antibody 9E10. Binding of HDAC1 to TDP43 was analyzed with western blotting with the indicated antibody.

4.4.2 Pathological mutant $TDP43^{M337V}$ and $TDP43^{A382T}$ interact with HDAC1 in vitro

Unlike wild-type (WT) TDP43, pathogenic TDP43 mutants are mislocalized in the cytoplasm. Therefore, to determine whether the binding of HDAC1 to pathogenic mutant HDAC1 is altered, we used WT TDP43 and pathogenic TDP43 variants (M337V and A382T) found in familial ALS patients. As shown in figure 26, also pathological mutants $TDP43^{M337V}$ and $TDP43^{A382T}$ interact with HDAC1 and we found no significant difference in HDAC1 binding affinity between mutants and WT TDP43,

while, at least in our experimental paradigms, TDP43/HDAC1 interaction seems to be stronger than the one with FUS (figure 26).

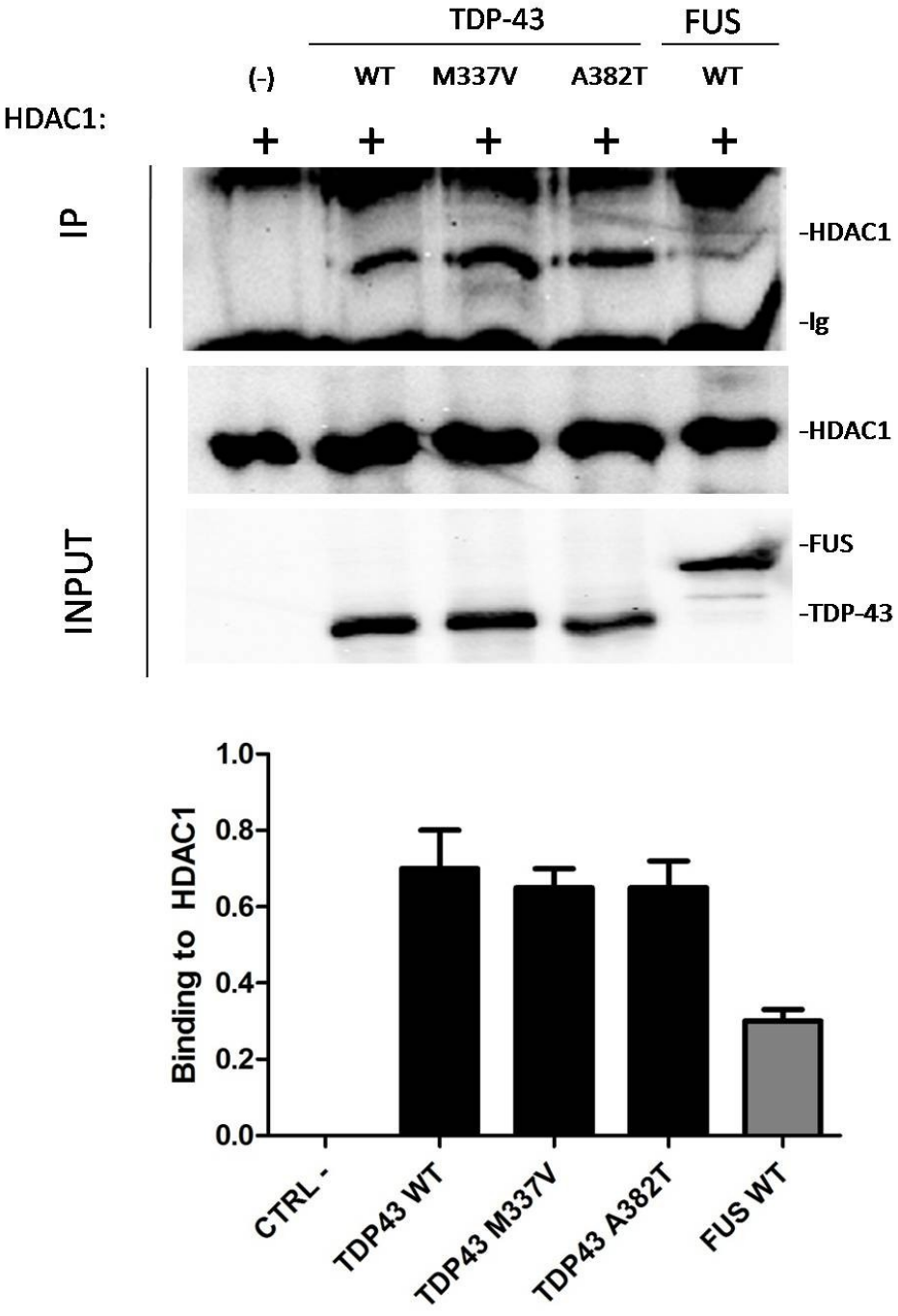


Figure 26.TDP43 interacts with HDAC1. HEK 293T cells were transiently transfected with myc-taggedTDP43 WT or mutant expression constructs (M337V or A382T) and FLAG-taggedHDCA1. Co-immunoprecipitation was performed as shown in figure 25.B. Bar graph shows the relative binding of HDAC1 to mutantTDP43, normalized to TDP43 WT. The data were obtained from four independent experiments; n.s .indicates $p > 0.05$ versus WT binding, analyzed with one-way ANOVA.

4.4.3 HDAC1 localizes in the nucleus and partly co-localizes with TDP43

Axonal damage described in brains of humans with multiple sclerosis and of mice with cuprizone-induced demyelization, has been associated with HDAC1 nuclear export, a critical event for impaired mitochondrial transport (Kim, Shanware et al. 2010). To investigate if mutant TDP43 expression can affect HDCA1 localization we decided to use different experimental approach. First of all we produce a stable SHSY-5Y cell line stably expressing HDAC1 fused in frame with FLAG tag. Single clones were isolated and tested for HDAC1 expression by western blot and confocal microscopy (Figure 27). FLAG-positive selected clones were clone 8, clone 9 and clone 10.

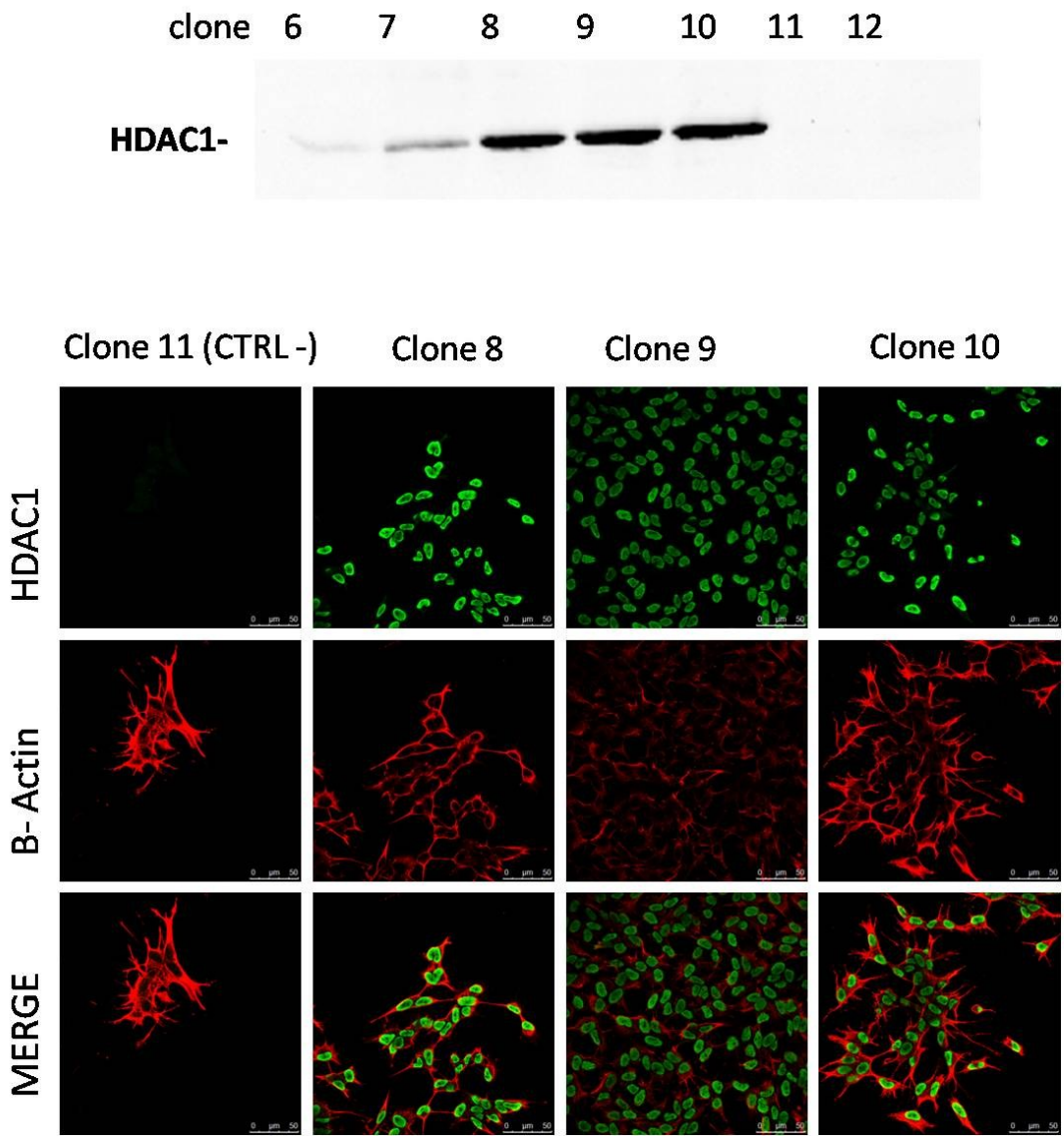


Figure 27. Western blot and immunofluorescence analysis on screened clones of SHSY-5Y cells stably expressing HDAC1-FLAG. Clone 11 were used as a negative control. HDAC1 was revealed with anti-FLAG monoclonal antibody; β-actin was used as a control for the cytoplasm.

We then look at HDCA1 localization in 3 different cellular models:

- SHSY-5Y-HDCA1 (clone 8 and 10) and SHSY5Y transduced with WT or pathological mutant TDP43

- SHSY-5Y co-transfected with HDAC1 and with WT or pathological mutant TDP43.

As show in figure 28 in all the experimental condition tested, HDAC1 retains its nuclear localization (Data shown refers only to HDAC1-clone 10 transfected with wild type or pathological mutants TDP43, figure 28).

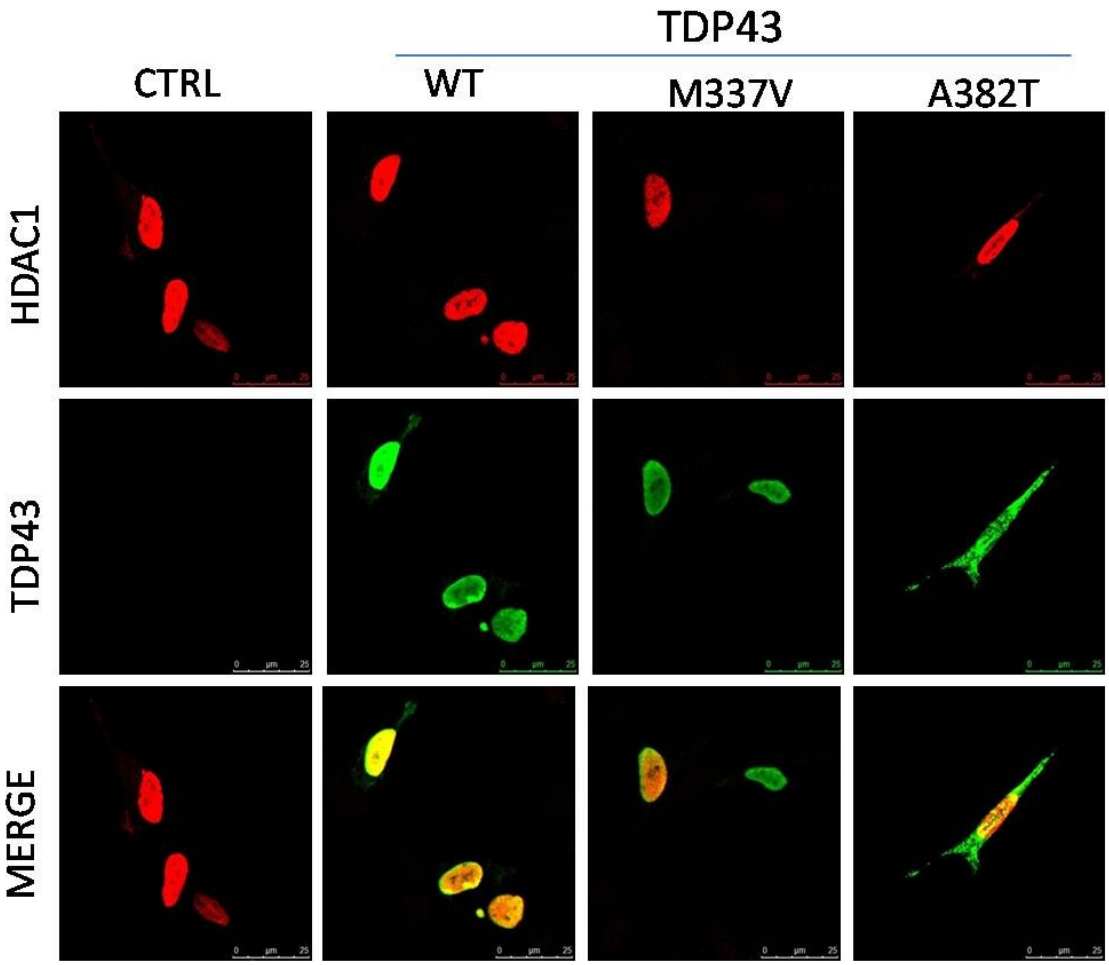


Figure 28. HDAC1 is localized in the nucleus also in presence of TDP43 pathological mutants. SHSY-5Y cells were co-transfected with pMTK-TDP43^{WT}, pMTK-TDP43^{M337V} or pMTK-TDP43^{A382T}, and pCDNA3-HDAC1. TDP43 signal was revealed by anti-myc primary antibody and secondary anti-mouse-ALEXA 488, HDAC1 was revealed by anti-FLAG antibody and secondary anti-rabbit ALEXA 647. Slides were analyzed by Leica confocal microscope.

4.4.4 Development of TDP43 deletion mutants

For determining which TDP43 functional domain interacts with HDAC1, we produced deletion mutants of TDP43 lacking of one or two protein domain (See fig. 29).We designed couples of deletion oligos (see Materials and Methods, table 7) and we performed site-directed mutagenesis according to Agilent Quick-change II manual. After PCR, DNA was DpnI-digested and transformed into DH5α. DNA was extracted from positive colonies, sequenced and amplified. We created deletion mutants for every functional domain, i.e. TDP43 lacking N-terminal domain, RRM1 domain, RRM2 domain or glycine-rich domain; finally, we developed the double-mutant lacking both RRM1 and RRM2 domain (see picture 29). DNA plasmids were then transfected into SHSY-5Y and SHSY-5Y-HDAC1 cells to be characterized. By western blot analysis, we confirmed protein dimension.

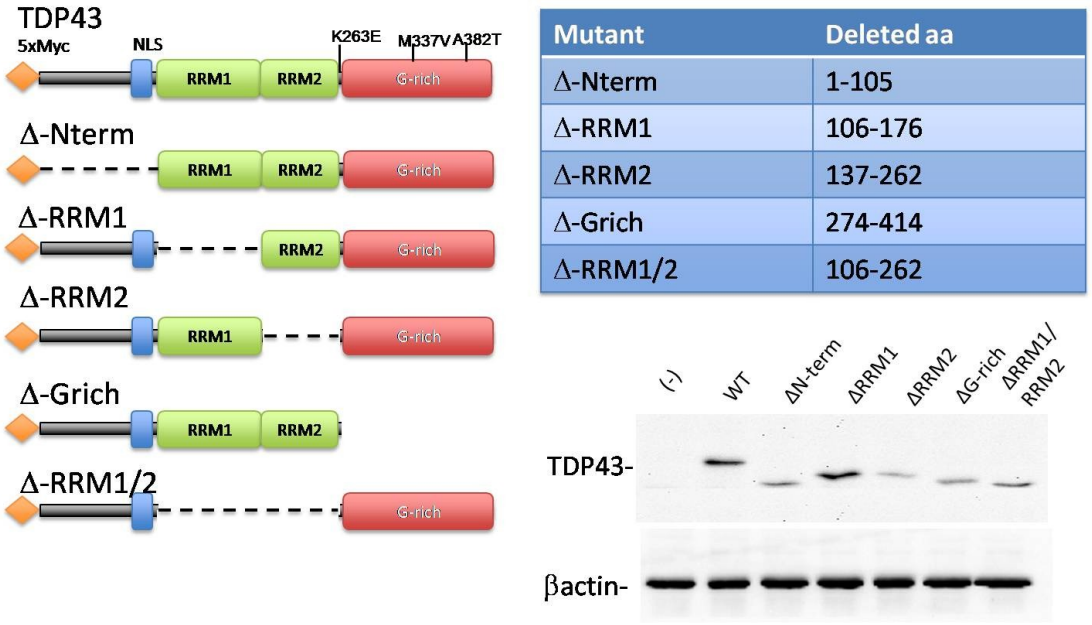


Figure 29. TDP43 deletion mutants lacking one or two protein domain. By in situ directed mutagenesis we produced 5 TDP43 deletion mutants, named TDP43-ΔN-term, TDP43-ΔRRM1, TDP43-ΔRRM2, TDP43-ΔGrich, TDP43-ΔRRM1/RRM2.Western blot analysis with anti-myc antibody confirmed the expected size of the mutant proteins.

Immunofluorescence analysis demonstrates that also deletion mutants localizes in the nucleus, except for TDP43-N-term, that lacks NLS signal and is particularly unstable (figure 30).

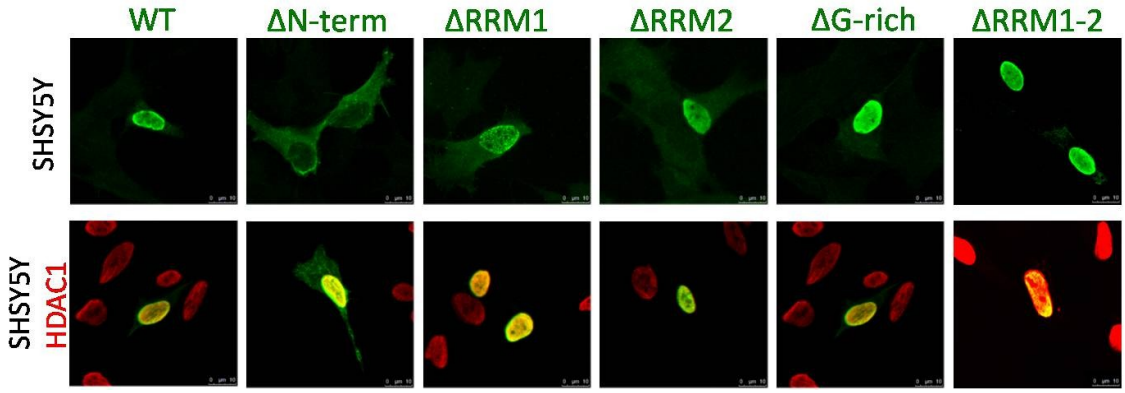


Figure 30. Immunofluorescence analysis of TDP43 deletion mutants, in SHSY-5Y cells and in a stable cell line SHSY-5Y-HDAC1. The top line shows SHSY-5Y cells expressing TDP43 deletion mutants and their localization. The bottom line shows TDP43 deletion mutants expressed in SHSY-5Y-HDAC1 clone 10.

4.4.5 RRM1 and RRM2 domains are both necessary to mediate TDP43-HDAC1 interaction

For determining which TDP43 domain interacts with HDAC1, the myc-tagged TDP43 fragments, previously described, were co-immunoprecipitated with FLAG-tagged HDAC1 in HEK 293T cells. We didn't test the N-terminal domain deletion mutant due to its completely citoplasmatic localization. We found that full-length HDAC1 strongly interacts with G-rich, while the interaction is reduced when one of the two RRM domains is missing and abolished only when both RRM1 and RRM2 are deleted. This result showed that HDAC1 physically interacts with TDP43 through the RRM1 and RRM2 region of TDP43 (figure 31).

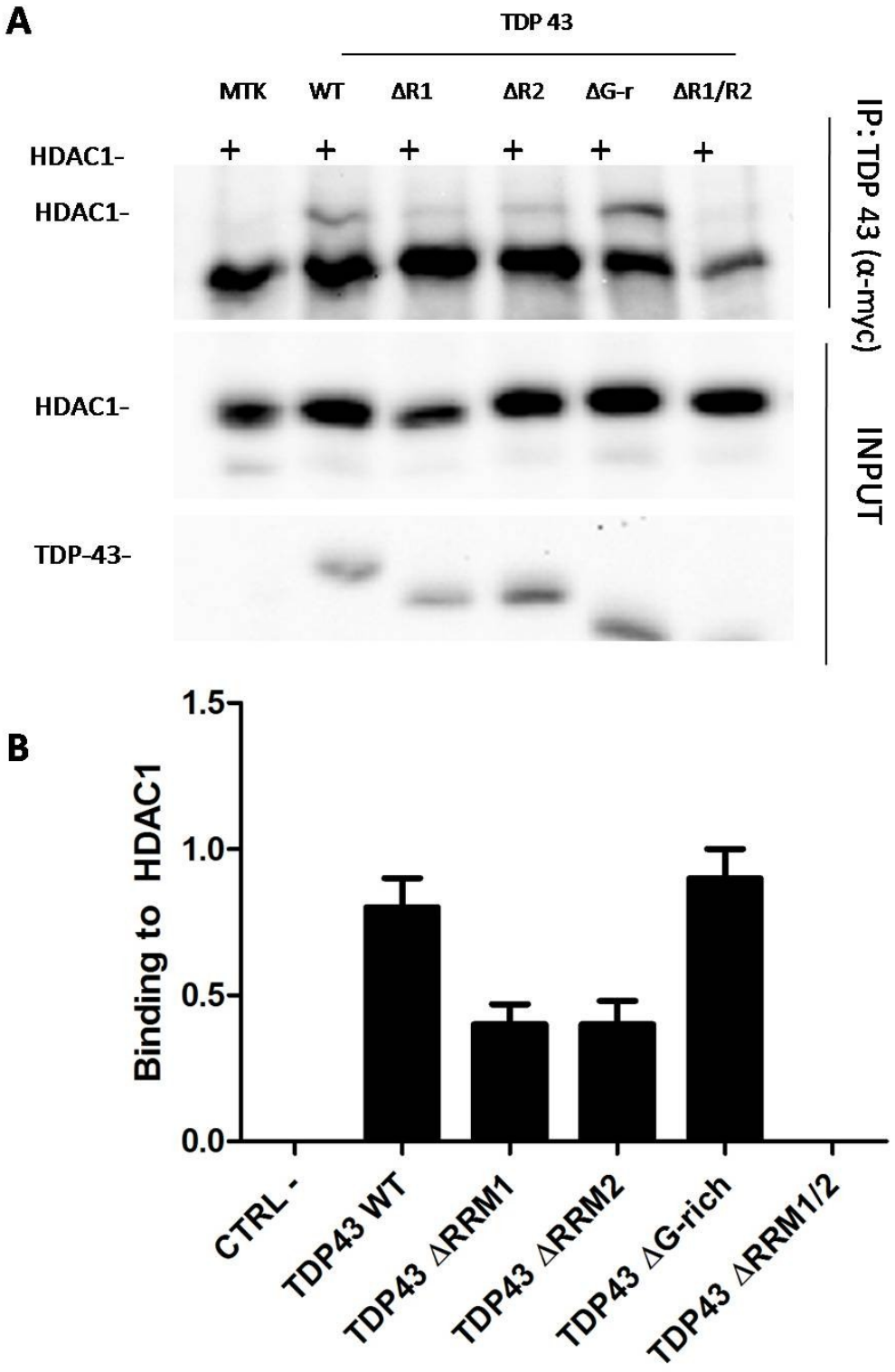


Figure 31.Coimmunoprecipitation between TDP43 deletion mutants and HDAC1. A) HEK 293T cells were transiently transfected with myc-tagged TDP43 WT or deletion mutants expression constructs (ΔRRM1, ΔRRM2, ΔG-rich, ΔRRM1/RRM2) and FLAG-taggedHDCA1. Co-immunoprecipitation was performed as shown in figure 25 .B) Bar graph shows the relative binding of HDAC1 to mutantTDP43, normalized to TDP43 WT. The data were obtained from four independent experiments; n.s.indicates $p > 0.05$ versus WT binding, analyzed with one-way ANOVA.

4.5 TDP43 DOESN'T ALTER HDAC1 ACTIVITY IN VITRO

In order to better understand the possible physiological link between TDP43 and HDAC1, we performed enzymatic assay based on a commercial kit (see materials and methods), to test HDAC1 activity. We transduced SHSY-5Y and SHSY-5Y-HDAC1 cells with adenoviral particle coding for WT or pathological mutant TDP43, and 48h later we performed, according to manufacturer's instructions, a colorimetric assay to detect HDAC1 activity. We observed that, in both cell lines, $TDP43^{WT}$ seems to reduce HDAC1 activity, comparing that to cells expressing mutant forms $TDP43^{M337V/A382T}$. Anyway, no any significant difference was observed (see figure 32).

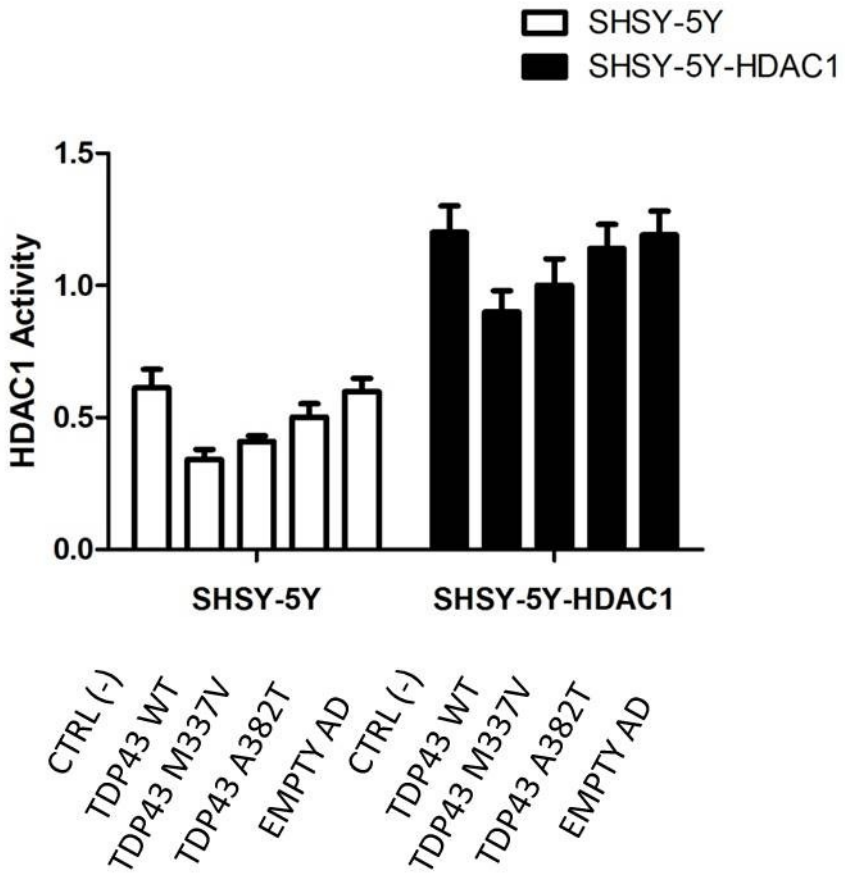
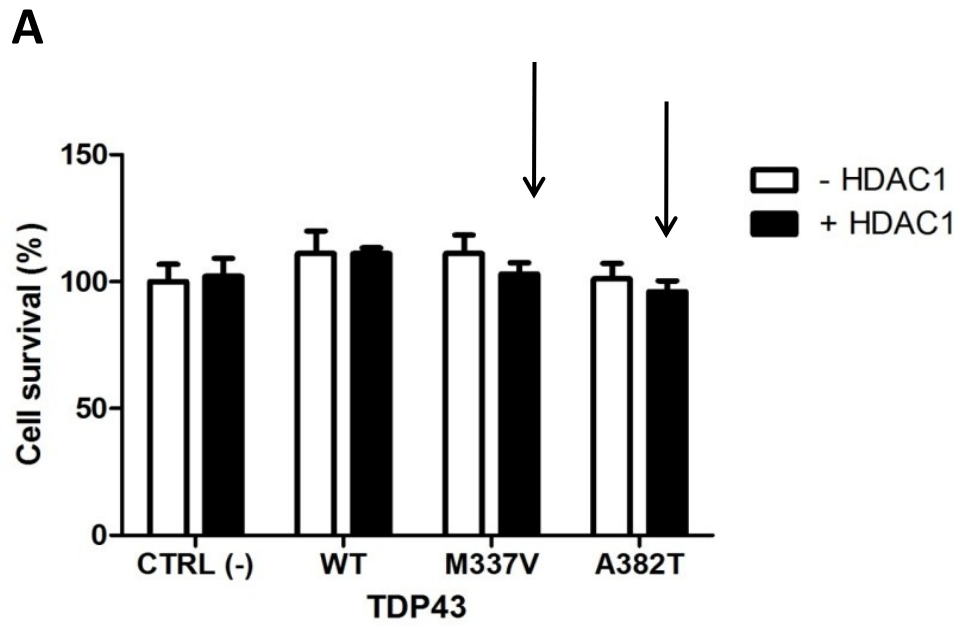


Figure 32.Wild-type or pathological mutant of TDP43 overexpression doesn't alter HDAC1 activity *in vitro*. SHSY-5Y or SHSY-5Y-HDAC1 cells were transduced with adenoviral vectors carrying TDP43 WT and mutant forms (M337V or A382T), or with an empty adenovirus, as a control. 5 ug of nuclear extracts was processed with a commercial kit as indicated in Materials and Methods. HDAC1 enzymatic activity was indirectly calculated from the colorimetric level measured by a multi-plate reader. ONE-WAY ANOVA doesn't indicate any significant difference ($p>0,05$).

4.6 TDP43 AND HDAC1 HAVE A SYNERGIC EFFECT IN DECREASING CELLS VITALITY

To enlighten the possible functional role of HDAC1 in TDP43 pathway, we performed MTS vitality assay in cells expressing or not HDAC1, in a transient or stable way. First of all, we trasnfected pMTK-TDP43^{WT}, pMTK TDP43^{M337V} or pMTK pMTK-TDP43^{A382T}, and we measured cellular vitality in presence or in absence of HDAC1, expressed transfecting pCDNA3-HDAC1 (figure 33, table A). We observed a mild, not statistically significant ulterior decrease in cells expressing both TDP43^{M337V} or TDP43^{A382T} and HDAC1. Even if we couldn't demonstrate a statistically significant difference, we observed this trend several times. In order to evaluate if HDAC1 way of expression could interfere with our results, we designed a similar experiment, but transducing TDP43 in SHSY-5Y cells or in the stable cell line SHSY-5Y-HDAC1 (figure 33, panel B). Once again, TDP43^{M337V} and TDP43^{A382T} toxicity is worsened by the presence of HDAC1.



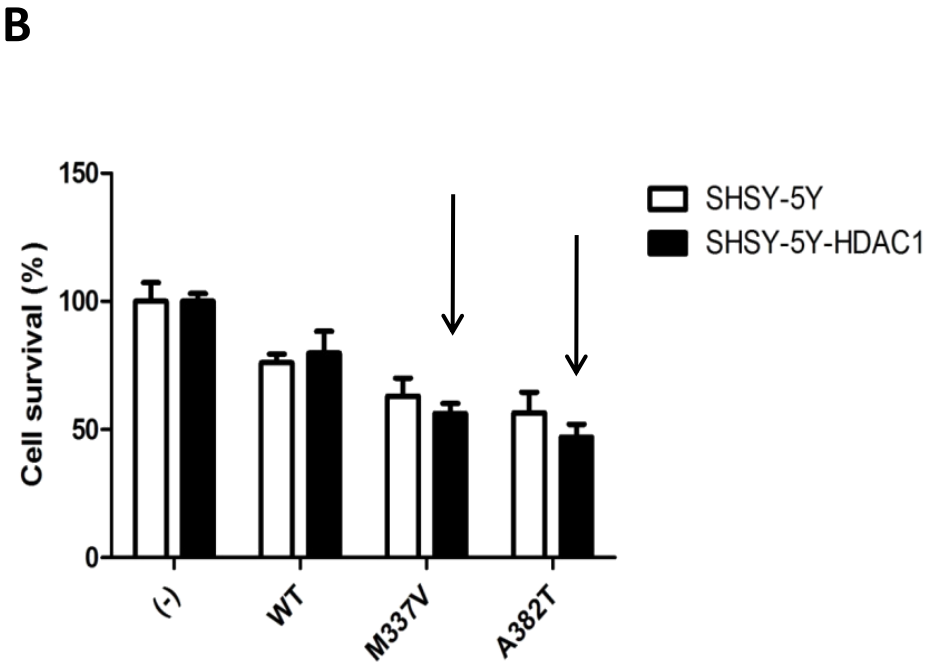


Figure 33. TDP43 and HDAC1 have a synergic effect in promoting cell toxicity. A) SHSY-5Y cells were transfected with plasmids carrying TDP43 WT or pathological mutants (M337V or A382T) and were transfected (black bars) or not trasnfected (white bars) with plasmids carrying HDAC1 gene. TDP43 confirms its toxicity, and so does HDAC1. Black arrows indicates cells in with the co-expression of the two proteins induce a worsening in cell viability. B) HSY-5Y (white bars) or SHSY-5y-HDAC1 cells (black bars) were transfected with plasmids carrying TDP43 WT or pathological mutants (M337V or A382T). Black arrows indicates cells in which the co-expression of TDP43 and HDAC1 induce a worsening in cell viability.

4.6.1 Possible therapeutic effect of HDAC inhibitors

HDAC inhibitors (HDACi) Sodium butyrate (NaB) and Trichostatin A (TSA) are known to have several therapeutic effect in epigenetic correlated pathologies, like cancer, neurological disease, immune disorders, and also in ALS (Petri, Kiaei et al. 2006, Falkenberg and Johnstone 2014). One of the reasons why we investigated on the possible therapeutic role of HDACi is that ALS is characterized by a severe histone deacetylation. In this part of our study, we tested the effect of two of these inhibitors, NaB and TSA in our ALS TDP43-related cellular model. As described in Materials and Methods, we transduced $TDP43^{WT}$, $TDP43^{M337V}$ or $TDP43^{A382T}$ in SHSY-5Y cells, we treated cells and we analyzed if HDACi had been able to reduce TDP43 induced mortality.

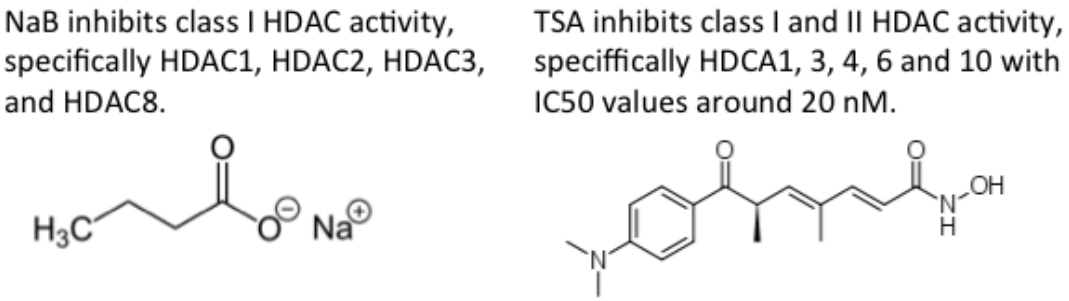


Figure 34.Molecular formula of HDACi Sodyum butyrate (on the left) and Trichostatin A (on the right).

4.6.1.1 HDACi Sodyum butyrate and Trichostatin A reduce TDP43 induced mortality

As described in Materials and methods, we treated TDP43 expressing cells with two different concentration of HDACi NaB, i.e. 0.2 mM and 0.04 mM, at the moment of the transduction and 24 hours later. We measured cells vitality 48 hours after transduction. We observed a statistically significant increase of vitality in cells treated with 0,2 mM of NaB, reaching, in some case, non infected cells vitality levels. 0,04 mM NaB induces a milder effect (Figure 35, panel B).Trichostatin A has a similar effect when used at the final concentration of 25 nM; on the contrary, 10 nM of TSA doesn't have any effect on survival(figure 35, panel C).

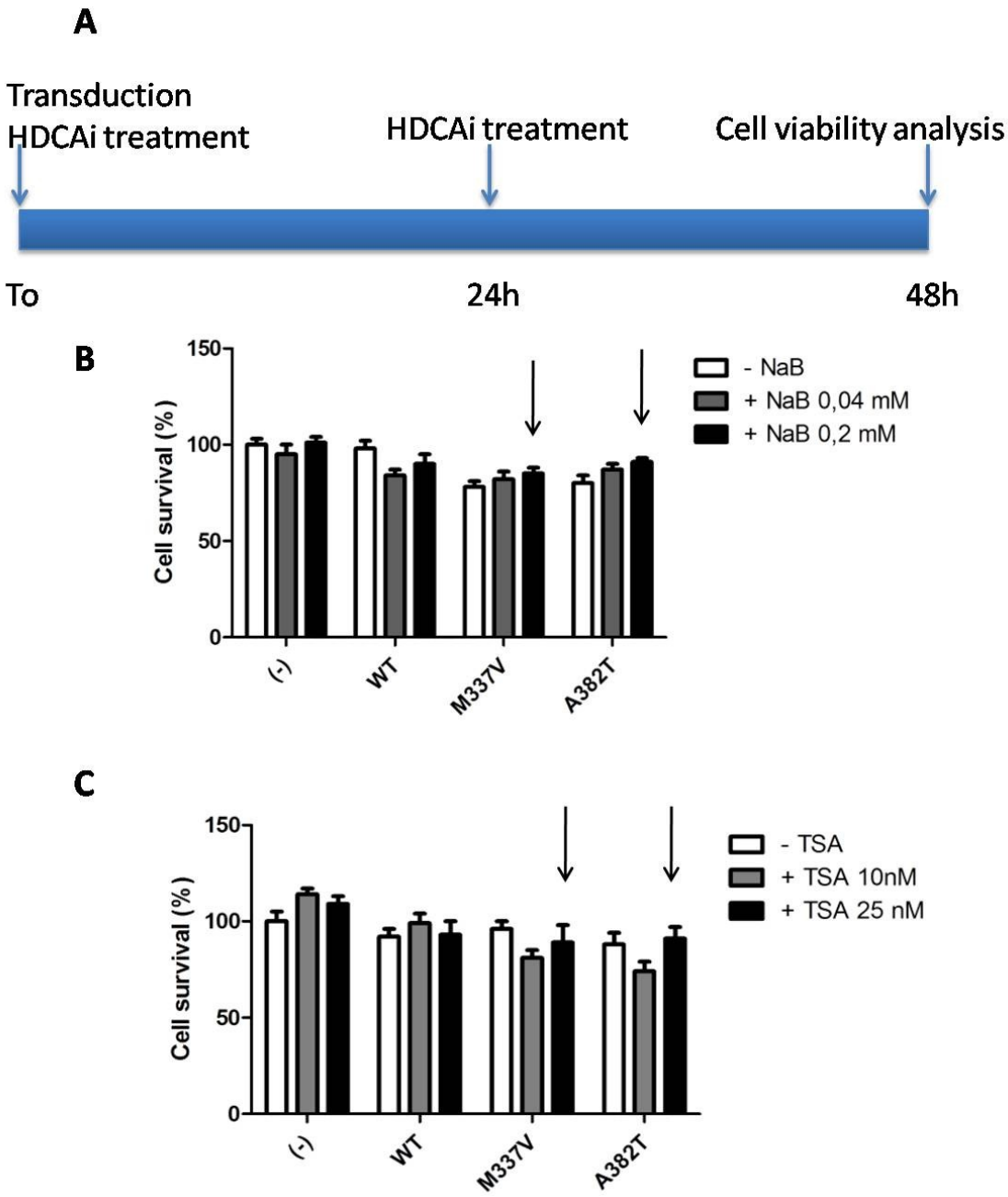


Figure 35. HDAC inhibitors Sodyum butyrate and Trichostatin A ameliorates cells viability in cells overexpressing TDP43 pathological mutant forms. A) Experimental approach. SHSY-5Y cells were plated on a 96-well multiplate dish and transduced 24 hours later with adenoviral vectors carrying TDP43 WT or mutant forms (M337V or A382T). Cells were treated immediately with HDACi after the transduction and then treated again 24 hours later. 48 hours after transduction, cell viability was analyzed as illustrated in materials and methods. B) SHSY-5Y cells treated with NaB 0,2 mM show an increase in cell viability, especially in those transduced with mutant TDP43 (black arrows). C) TSA 25 mM induce ameliorates cells viability in cells overexpressing mutant TDP43 (black arrows).

5. DISCUSSION

5.1 ALS AND EPIGENETIC ALTERATIONS

Several evidences indicates that in ALS in patients as well as in cellular and animal models there are remarkable alterations in the epigenetic equilibrium. The epigenetic hypothesis of ALS finds in the alteration of acetylation/deacetylation balance of histones tails a possible explanation for the pathogenesis. A decrease in global acetylation levels of histone H3 and H4 has been well described. A possible explanation of this phenomenon is the decrease in the expression levels of CBP/p300, one of the principal histone acetyltransferase. This decrease is not casual. It has been observed a severe deacetylation of the promoter of pro-survival genes, which leads to a decrease in transcriptional activation, and a contemporary increase in acetylation of lysine residues of histone tails in the promoter of pro-apoptotic genes.

DNA methylation is an another epigenetic pathway that has been linked to ALS pathogenesis. A significant body of literature evidences that in ALS there is a significant upregulation of some de novo methyltransferases (DNMTs), especially Dnmt1 and Dnmt3a, leading to an increase in DNA methylation. The increase in DNA methylation is a marker of transcriptional repression.

Up to now evidence of alterations in global acetylation and deacetylation levels were obtained mainly in ALS models linked to SOD1. For this reason, I have dealt with generating and characterizing ALS cellular models in which the transient expression of fALS causative genes is mediated by high titer infectious recombinant adenoviruses. I produced adenoviral vector carrying genes for $SOD1^{WT/G93A/H80R}$, $FUS^{WT/R521G,P525L,R495X}$ and $TDP43^{WT/M337V/A382T}$. I demonstrated that cell viability is reduced in neuronal cell lines that express SOD1, FUS and TDP43 genes, and so I confirmed that our model is a good model of fALS. Transgenic mice $SOD1^{G93A}$ was our animal model of fALS. Then, I selected histonic markers linked to transcriptional activation (phospho-acetylation on serine 10 and lysine 14 on H3 tail- H3-K14acS10ph, and the dimethylation of lysine 4 on H3 tail- H3K14me2), or to transcriptional repression (the trimethylation of lysine 9 on H3 tail). I performed western blot analysis of total protein extract and immunofluorescence analysis on spinal cord of transgenic mice. In addition, I measured global DNA methylation on genomic DNA from transduced cells.

Analyzing histonic markers, I could observe a severe significant reduction in transcriptional activation marker H3-K14acS10ph and H3K14me2 in cells expressing $SOD1^{G93A}$ and $SOD1^{H80R}$. Concerning SOD1, I observed a specular increase in transcriptional repression marker 5metCyt, in cells expressing $SOD1^{G93A}$. This lead us to speculate that there is a close link between SOD1 pathological mutations and epigenetic alterations.

On the contrary, I couldn't observe any significant reduction in transcriptional activation marker in cells expressing pathological mutant forms of FUS and TDP43. I observed, in both cases, a significant increase in transcriptional activation marker H3K9me3 in cells expressing FUS^{WT} and $TDP43^{WT.}$ This is probably why the pathological link between FUS, TDP43 and motor neuronal cell death is not directly linked the epigenetic alterations analyzed.

I were also able to demonstrate that the levels of transcriptional activation markers H3-K14acS10ph and H3-K4me2 are significantly reduced also *in vivo*, in a ALS mouse model, the transgenic mice $SOD1^{G93A}$.

Further analysis could clarify the link between SOD1 and the epigenetic machinery.

5.2 TDP43 AND HDAC1: POSSIBLE FUNCTIONAL ROLE OF THEIR INTERACTION

Starting from the evidence that FUS interacts with HDAC1 on DNA damage sites and considering that FUS and TDP43 share many functional domains, I decided to verify if also TDP43 could interact with HDAC1. It is important to consider also that TDP43 directly interact with HDAC6, as illustrated in Introduction, paragraph 1.2.3, and is deacetylated by this histone deacetylases. Starting from these evidences, by co-immunoprecipitation experiments between TDP43 and HDAC1 I was able to demonstrated that, as FUS, TDP43 WT interacts with HDAC1 in cellular models of ALS. I moved on this way and I could observe that also pathological mutant forms of TDP43 (M337V and A382T) *in vitro* interacts with HDAC1. By immunofluorescence experiments, I confirmed that TDP43 and HDAC1 co-localize into the nucleus is SHSY-5Y and SHSY-5Y-HDAC1 cells.

Since pathological mutants TDP43^{M337V} and TDP43^{A382T} interacts with HDAC1 as well as TDP43^{WT} , I looked at HDAC1 enzymatic activity in the presence of TDP43, in SHSY-5Y and SHSY-5Y-HDAC1 cells. Even if I observed a trend in both cell lines in which it seems that the enzymatic activity changes in the presence of mutated TDP43, I couldn't prove any significant difference in HDAC1 enzymatic activity in presence of TDP43.

To further understand how this interaction is mediated, I built up five TDP43 deletion mutants, lacking of 1 or 2 functional domain: Δ N-terminal, Δ-RRM1, Δ-RRM2, Δ-G-rich, Δ-RRM1/RRM2. I analyzed the interaction between these truncated forms of TDP43 and HDAC1 by co-immunoprecipitation techniques, and these data revealed that the only mutant that doesn't interact with HDAC1 is the double deletion mutant lacking both the RNA binding domains (Δ-RRM1/RRM2). Thanks to this data, I demonstrated that TDP43 interacts with HDAC1 via its RNA binding domains RRM1 and RRM2.

Starting from the evidence that TDP43 does not alter HDAC1 enzymatic activity, according to these evidences, I decided to analyze the effect of TDP43 and HDAC1 on cell viability. I observed that the co-expression of pathological mutant forms of TDP43 and HDAC1 in SHSY-5Y cells have a synergic effect in decreasing cellular viability, compared to cells expressing only TDP43. Furthermore, I confirmed this data also in cells stably expressing HDAC1 (SHSY-5Y-HDAC1 cell line).

Taken together, this data permitted us to imagine a model of the pathological role of TDP43 in ALS, in which TDP43 and HDAC1 can possibly interacts on promoters on DNA, and alter gene transcription mediating a change in acetylation/deacetylation equilibrium. This data is in accordance with what observed in the reduction of H3 acetylation in cells expressing SOD1$^{G93A/H80R}$. This model needs to be confirmed by several other experiments.

Even if I could demonstrate that TDP43 interacts in vitro with HDAC1 via its RRM1/2 domain, many points remain unclear. It will be necessary to verify if TDP43 and HDAC1 interacts also *in vivo.* Then, it will be interesting to analyze the functional aspect of this interaction and verify if our proposed model can be confirmed. One possible perspective can be to study if TDP43 and HDAC1 interacts on gene promoters by chromatin immunoprecipitation.

Future studies should analyze the role of TDP43 as a transcription factor, by luciferase assay on a predicted promoter.

In addition, it will be necessary to analyze the role of TDP43 in splicing of selected genes.

Finally, one possible point to clarify could be if HDAC1 directly deacetylate TDP43, analyzing TDP43 acetylation pattern by 2D gel electrophoresis, to discover if this covalent modification can be the signal that alter TDP43 functions. This last point may lead to clarify if TDP43 and HDAC1 interaction is direct and TDP43 is deacetylated by HDAC1, or if the interaction is indirect and they co-interact with other proteins in a chromatin remodeling complex.

5.3 HDAC INHIBITORS IMPROVE VITALITY IN ALS CELLULAR MODELS

HDAC inhibitors Sodium butyrate (NaB), Sodium Valproate (VPA) and Trichostatin A (TSA) have been widely studied as potential therapeutic resources in ALS treatment. Despite many promising preliminary data and clinical trials, they didn't proved any consistent therapeutic improving.

Starting from this unhopeful assumptions, I decided to analyze the effect of HDAC inhibitions in cellular models of ALS, in particular in TDP43 ALS model. I were particularly interested in the effect of HDAC inhibition, not in the possible therapeutic effect. Anyway, HDACi NaB and TSA improve cellular vitality in cells expressing $TDP43^{M337V}$ and $TDP43^{A382T}$. In particular, they are able to restore cellular vitality at the levels of non infected cells.

This enthusiastic *in vitro* preliminary data doesn't consider any of the possible problems in using HDACi in therapy, but they can be an interesting starting point.

To confirm the possible therapeutic effect of reducing HDAC activity, further experiments will be necessary. For example, it will be interesting analyzing the effect of the inhibition of HDACs mediated by RNA interference.

Anyway, more therapeutic inhibitors, specific for one HDAC, will be more adapt to study.

Finally, to confirm this data, it will be necessary to analyze if this therapeutic effect is visible also in mouse models or in iPS cells.

5.4 CONCLUSIONS

The vulnerability of motor neurons to neurodegeneration in ALS patients cannot be explained by considering separately genetic causes and environmental risk factors. On the contrary their contribution to post-transcriptional changes or epigenetic mechanisms can probably be crucial to understanding ALS pathogenesis, and establishing a path to treatment. The data presented in this PhD thesis indicates that is worthwhile to investigate the link existing between a determined epigenetic modification and a single ALS-causing gene, to identify an effective "epigenetic therapy".

6. BIBLIOGRAPHY

Al-Chalabi, A., O. Hardiman, M. C. Kiernan, A. Chio, B. Rix-Brooks and L. H. van den Berg (2016). "Amyotrophic lateral sclerosis: moving towards a new classification system." Lancet Neurol **15**(11): 1182-1194.

Al-Chalabi, A., S. Kwak, M. Mehler, G. Rouleau, T. Siddique, M. Strong and P. N. Leigh (2013). "Genetic and epigenetic studies of amyotrophic lateral sclerosis." Amyotroph Lateral Scler Frontotemporal Degener **14 Suppl 1**: 44-52.

Alexander, M. D., B. J. Traynor, N. Miller, B. Corr, E. Frost, S. McQuaid, F. M. Brett, A. Green and O. Hardiman (2002). ""True" sporadic ALS associated with a novel SOD-1 mutation." Ann Neurol **52**(5): 680-683.

Arnold, E. S., S. C. Ling, S. C. Huelga, C. Lagier-Tourenne, M. Polymenidou, D. Ditsworth, H. B. Kordasiewicz, M. McAlonis-Downes, O. Platoshyn, P. A. Parone, S. Da Cruz, K. M. Clutario, D. Swing, L. Tessarollo, M. Marsala, C. E. Shaw, G. W. Yeo and D. W. Cleveland (2013). "ALS-linked TDP-43 mutations produce aberrant RNA splicing and adult-onset motor neuron disease without aggregation or loss of nuclear TDP-43." Proc Natl Acad Sci U S A **110**(8): E736-745.

Beckman, J. S., M. Carson, C. D. Smith and W. H. Koppenol (1993). "ALS, SOD and peroxynitrite." Nature **364**(6438): 584.

Beleza-Meireles, A. and A. Al-Chalabi (2009). "Genetic studies of amyotrophic lateral sclerosis: controversies and perspectives." Amyotroph Lateral Scler **10**(1): 1-14.

Belly, A., F. Moreau-Gachelin, R. Sadoul and Y. Goldberg (2005). "Delocalization of the multifunctional RNA splicing factor TLS/FUS in hippocampal neurones: exclusion from the nucleus and accumulation in dendritic granules and spine heads." Neurosci Lett **379**(3): 152-157.

Belzil, V. V., P. O. Bauer, M. Prudencio, T. F. Gendron, C. T. Stetler, I. K. Yan, L. Pregent, L. Daughrity, M. C. Baker, R. Rademakers, K. Boylan, T. C. Patel, D. W. Dickson and L. Petrucelli (2013). "Reduced C9orf72 gene expression in c9FTD/ALS is caused by histone trimethylation, an epigenetic event detectable in blood." Acta Neuropathol **126**(6): 895-905.

Berger, S. L., T. Kouzarides, R. Shiekhattar and A. Shilatifard (2009). "An operational definition of epigenetics." Genes Dev **23**(7): 781-783.

Bestor, T. H. (2000). "The DNA methyltransferases of mammals." Hum Mol Genet **9**(16): 2395-2402.

Bozzoni, V., O. Pansarasa, L. Diamanti, G. Nosari, C. Cereda and M. Ceroni (2016). "Amyotrophic lateral sclerosis and environmental factors." Funct Neurol **31**(1): 7-19.

Branscombe, T. L., A. Frankel, J. H. Lee, J. R. Cook, Z. Yang, S. Pestka and S. Clarke (2001). "PRMT5 (Janus kinase-binding protein 1) catalyzes the formation of symmetric dimethylarginine residues in proteins." J Biol Chem **276**(35): 32971-32976.

Bruneteau, G., T. Simonet, S. Bauche, N. Mandjee, E. Malfatti, E. Girard, M. L. Tanguy, A. Behin, F. Khiami, E. Sariali, C. Hell-Remy, F. Salachas, P. F. Pradat, E. Fournier, L. Lacomblez, J. Koenig, N. B. Romero, B. Fontaine, V. Meininger, L. Schaeffer and D. Hantai (2013). "Muscle histone deacetylase 4 upregulation in amyotrophic lateral sclerosis: potential role in reinnervation ability and disease progression." Brain **136**(Pt 8): 2359-2368.

Buratti, E., A. Brindisi, M. Giombi, S. Tisminetzky, Y. M. Ayala and F. E. Baralle (2005). "TDP-43 binds heterogeneous nuclear ribonucleoprotein A/B through its C-terminal tail: an important region for the inhibition of cystic fibrosis transmembrane conductance regulator exon 9 splicing." J Biol Chem **280**(45): 37572-37584.

Chestnut, B. A., Q. Chang, A. Price, C. Lesuisse, M. Wong and L. J. Martin (2011). "Epigenetic regulation of motor neuron cell death through DNA methylation." J Neurosci **31**(46): 16619-16636.

Chio, A., G. Borghero, M. Pugliatti, A. Ticca, A. Calvo, C. Moglia, R. Mutani, M. Brunetti, I. Ossola, M. G. Marrosu, M. R. Murru, G. Floris, A. Cannas, L. D. Parish, P. Cossu, Y. Abramzon, J. O. Johnson, M. A. Nalls, S. Arepalli, S. Chong, D. G. Hernandez, B. J. Traynor, G. Restagno and C. Italian Amyotrophic Lateral Sclerosis Genetic (2011). "Large proportion of amyotrophic lateral sclerosis cases in Sardinia due to a single founder mutation of the TARDBP gene." Arch Neurol **68**(5): 594-598.

Chio, A., G. Logroscino, O. Hardiman, R. Swingler, D. Mitchell, E. Beghi, B. G. Traynor and C. Eurals (2009). "Prognostic factors in ALS: A critical review." Amyotroph Lateral Scler **10**(5-6): 310-323.

Choudhary, C., C. Kumar, F. Gnad, M. L. Nielsen, M. Rehman, T. C. Walther, J. V. Olsen and M. Mann (2009). "Lysine acetylation targets protein complexes and co-regulates major cellular functions." Science **325**(5942): 834-840.

Cleary, J. D. and L. P. Ranum (2014). "Repeat associated non-ATG (RAN) translation: new starts in microsatellite expansion disorders." Curr Opin Genet Dev **26**: 6-15.

Cohen, T. J., J. L. Guo, D. E. Hurtado, L. K. Kwong, I. P. Mills, J. Q. Trojanowski and V. M. Lee (2011). "The acetylation of tau inhibits its function and promotes pathological tau aggregation." Nat Commun **2**: 252.

Cohen, T. J., A. W. Hwang, C. R. Restrepo, C. X. Yuan, J. Q. Trojanowski and V. M. Lee (2015). "An acetylation switch controls TDP-43 function and aggregation propensity." Nat Commun **6**: 5845.

Cudkowicz, M. E., P. L. Andres, S. A. Macdonald, R. S. Bedlack, R. Choudry, R. H. Brown, Jr., H. Zhang, D. A. Schoenfeld, J. Shefner, S. Matson, W. R. Matson, R. J. Ferrante, A. L. S. Northeast and V. A. A. L. S. R. C. National (2009). "Phase 2 study of sodium phenylbutyrate in ALS." Amyotroph Lateral Scler **10**(2): 99-106.

DeJesus-Hernandez, M., I. R. Mackenzie, B. F. Boeve, A. L. Boxer, M. Baker, N. J. Rutherford, A. M. Nicholson, N. A. Finch, H. Flynn, J. Adamson, N. Kouri, A. Wojtas, P. Sengdy, G. Y. Hsiung, A. Karydas, W. W. Seeley, K. A. Josephs, G. Coppola, D. H. Geschwind, Z. K. Wszolek, H. Feldman, D. S. Knopman, R. C. Petersen, B. L. Miller, D. W. Dickson, K. B. Boylan, N. R. Graff-Radford and R. Rademakers (2011). "Expanded GGGGCC hexanucleotide repeat in noncoding region of C9ORF72 causes chromosome 9p-linked FTD and ALS." Neuron **72**(2): 245-256.

Deng, H., K. Gao and J. Jankovic (2014). "The role of FUS gene variants in neurodegenerative diseases." Nat Rev Neurol **10**(6): 337-348.

Dhalluin, C., J. E. Carlson, L. Zeng, C. He, A. K. Aggarwal and M. M. Zhou (1999). "Structure and ligand of a histone acetyltransferase bromodomain." Nature **399**(6735): 491-496.

Dormann, D., T. Madl, C. F. Valori, E. Bentmann, S. Tahirovic, C. Abou-Ajram, E. Kremmer, O. Ansorge, I. R. Mackenzie, M. Neumann and C. Haass (2012). "Arginine methylation next to the PY-NLS modulates Transportin binding and nuclear import of FUS." EMBO J **31**(22): 4258-4275.

Dunlop, R. A., P. A. Cox, S. A. Banack and K. J. Rodgers (2013). "The non-protein amino acid BMAA is misincorporated into human proteins in place of L-serine causing protein misfolding and aggregation." PLoS One **8**(9): e75376.

Falkenberg, K. J. and R. W. Johnstone (2014). "Histone deacetylases and their inhibitors in cancer, neurological diseases and immune disorders." Nat Rev Drug Discov **13**(9): 673-691.

Feng, H. L., Y. Leng, C. H. Ma, J. Zhang, M. Ren and D. M. Chuang (2008). "Combined lithium and valproate treatment delays disease onset, reduces neurological deficits and prolongs survival in an amyotrophic lateral sclerosis mouse model." Neuroscience **155**(3): 567-572.

Ferraiuolo, L., J. Kirby, A. J. Grierson, M. Sendtner and P. J. Shaw (2011). "Molecular pathways of motor neuron injury in amyotrophic lateral sclerosis." Nat Rev Neurol **7**(11): 616-630.

Freibaum, B. D., R. K. Chitta, A. A. High and J. P. Taylor (2010). "Global analysis of TDP-43 interacting proteins reveals strong association with RNA splicing and translation machinery." J Proteome Res **9**(2): 1104-1120.

Gallinari, P., S. Di Marco, P. Jones, M. Pallaoro and C. Steinkuhler (2007). "HDACs, histone deacetylation and gene transcription: from molecular biology to cancer therapeutics." Cell Res **17**(3): 195-211.

Galvez, A. F., L. Huang, M. M. Magbanua, K. Dawson and R. L. Rodriguez (2011). "Differential expression of thrombospondin (THBS1) in tumorigenic and nontumorigenic prostate epithelial cells in response to a chromatin-binding soy peptide." Nutr Cancer **63**(4): 623-636.

Goldberg, A. D., C. D. Allis and E. Bernstein (2007). "Epigenetics: a landscape takes shape." Cell **128**(4): 635-638.

Goransson, M., M. K. Andersson, C. Forni, A. Stahlberg, C. Andersson, A. Olofsson, R. Mantovani and P. Aman (2009). "The myxoid liposarcoma FUS-DDIT3 fusion oncoprotein deregulates NF-kappaB target genes by interaction with NFKBIZ." Oncogene **28**(2): 270-278.

Gupta, S., S. Y. Kim, S. Artis, D. L. Molfese, A. Schumacher, J. D. Sweatt, R. E. Paylor and F. D. Lubin (2010). "Histone methylation regulates memory formation." J Neurosci **30**(10): 3589-3599.

Hebron, M. L., I. Lonskaya, K. Sharpe, P. P. Weerasinghe, N. K. Algarzae, A. R. Shekoyan and C. E. Moussa (2013). "Parkin ubiquitinates Tar-DNA binding protein-43 (TDP-43) and promotes its cytosolic accumulation via interaction with histone deacetylase 6 (HDAC6)." J Biol Chem **288**(6): 4103-4115.

Jackrel, M. E. and J. Shorter (2014). "Potentiated Hsp104 variants suppress toxicity of diverse neurodegenerative disease-linked proteins." Dis Model Mech **7**(10): 1175-1184.

Jin, B., Y. Li and K. D. Robertson (2011). "DNA methylation: superior or subordinate in the epigenetic hierarchy?" Genes Cancer **2**(6): 607-617.

Kamel, F., D. M. Umbach, R. S. Bedlack, M. Richards, M. Watson, M. C. Alavanja, A. Blair, J. A. Hoppin, S. Schmidt and D. P. Sandler (2012). "Pesticide exposure and amyotrophic lateral sclerosis." Neurotoxicology **33**(3): 457-462.

Kazantsev, A. G. and L. M. Thompson (2008). "Therapeutic application of histone deacetylase inhibitors for central nervous system disorders." Nat Rev Drug Discov **7**(10): 854-868.

Khare, S. P., F. Habib, R. Sharma, N. Gadewal, S. Gupta and S. Galande (2012). "HIstome--a relational knowledgebase of human histone proteins and histone modifying enzymes." Nucleic Acids Res **40**(Database issue): D337-342.

Kim, S. H., N. P. Shanware, M. J. Bowler and R. S. Tibbetts (2010). "Amyotrophic lateral sclerosis-associated proteins TDP-43 and FUS/TLS function in a common biochemical complex to co-regulate HDAC6 mRNA." J Biol Chem **285**(44): 34097-34105.

Knoll, J. H., R. D. Nicholls, R. E. Magenis, J. M. Graham, Jr., M. Lalande and S. A. Latt (1989). "Angelman and Prader-Willi syndromes share a common chromosome 15 deletion but differ in parental origin of the deletion." Am J Med Genet **32**(2): 285-290.

Koshland, D. and A. Strunnikov (1996). "Mitotic chromosome condensation." Annu Rev Cell Dev Biol **12**: 305-333.

Kwiatkowski, T. J., Jr., D. A. Bosco, A. L. Leclerc, E. Tamrazian, C. R. Vanderburg, C. Russ, A. Davis, J. Gilchrist, E. J. Kasarskis, T. Munsat, P. Valdmanis, G. A. Rouleau, B. A. Hosler, P. Cortelli, P. J. de

Jong, Y. Yoshinaga, J. L. Haines, M. A. Pericak-Vance, J. Yan, N. Ticozzi, T. Siddique, D. McKenna-Yasek, P. C. Sapp, H. R. Horvitz, J. E. Landers and R. H. Brown, Jr. (2009). "Mutations in the FUS/TLS gene on chromosome 16 cause familial amyotrophic lateral sclerosis." Science **323**(5918): 1205-1208.

Lagger, G., D. O'Carroll, M. Rembold, H. Khier, J. Tischler, G. Weitzer, B. Schuettengruber, C. Hauser, R. Brunmeir, T. Jenuwein and C. Seiser (2002). "Essential function of histone deacetylase 1 in proliferation control and CDK inhibitor repression." EMBO J **21**(11): 2672-2681.

Lagier-Tourenne, C., M. Polymenidou and D. W. Cleveland (2010). "TDP-43 and FUS/TLS: emerging roles in RNA processing and neurodegeneration." Hum Mol Genet **19**(R1): R46-64.

Landgrave-Gomez, J., O. Mercado-Gomez and R. Guevara-Guzman (2015). "Epigenetic mechanisms in neurological and neurodegenerative diseases." Front Cell Neurosci **9**: 58.

Lau, A. T., S. Y. Lee, Y. M. Xu, D. Zheng, Y. Y. Cho, F. Zhu, H. G. Kim, S. Q. Li, Z. Zhang, A. M. Bode and Z. Dong (2011). "Phosphorylation of histone H2B serine 32 is linked to cell transformation." J Biol Chem **286**(30): 26628-26637.

Lee, E. B., V. M. Lee and J. Q. Trojanowski (2011). "Gains or losses: molecular mechanisms of TDP43-mediated neurodegeneration." Nat Rev Neurosci **13**(1): 38-50.

Ling, S. C., C. P. Albuquerque, J. S. Han, C. Lagier-Tourenne, S. Tokunaga, H. Zhou and D. W. Cleveland (2010). "ALS-associated mutations in TDP-43 increase its stability and promote TDP-43 complexes with FUS/TLS." Proc Natl Acad Sci U S A **107**(30): 13318-13323.

Lister, R., M. Pelizzola, R. H. Dowen, R. D. Hawkins, G. Hon, J. Tonti-Filippini, J. R. Nery, L. Lee, Z. Ye, Q. M. Ngo, L. Edsall, J. Antosiewicz-Bourget, R. Stewart, V. Ruotti, A. H. Millar, J. A. Thomson, B. Ren and J. R. Ecker (2009). "Human DNA methylomes at base resolution show widespread epigenomic differences." Nature **462**(7271): 315-322.

Liu, R., J. S. Althaus, B. R. Ellerbrock, D. A. Becker and M. E. Gurney (1998). "Enhanced oxygen radical production in a transgenic mouse model of familial amyotrophic lateral sclerosis." Ann Neurol **44**(5): 763-770.

Lo, W. S., R. C. Trievel, J. R. Rojas, L. Duggan, J. Y. Hsu, C. D. Allis, R. Marmorstein and S. L. Berger (2000). "Phosphorylation of serine 10 in histone H3 is functionally linked in vitro and in vivo to Gcn5-mediated acetylation at lysine 14." Mol Cell **5**(6): 917-926.

Logroscino, G., B. J. Traynor, O. Hardiman, A. Chio, D. Mitchell, R. J. Swingler, A. Millul, E. Benn, E. Beghi and Eurals (2010). "Incidence of amyotrophic lateral sclerosis in Europe." J Neurol Neurosurg Psychiatry **81**(4): 385-390.

Mahadevan, L. C., A. C. Willis and M. J. Barratt (1991). "Rapid histone H3 phosphorylation in response to growth factors, phorbol esters, okadaic acid, and protein synthesis inhibitors." Cell **65**(5): 775-783.

Martin, L. J. and M. Wong (2013). "Aberrant regulation of DNA methylation in amyotrophic lateral sclerosis: a new target of disease mechanisms." Neurotherapeutics **10**(4): 722-733.

McDonald, K. K., A. Aulas, L. Destroismaisons, S. Pickles, E. Beleac, W. Camu, G. A. Rouleau and C. Vande Velde (2011). "TAR DNA-binding protein 43 (TDP-43) regulates stress granule dynamics via differential regulation of G3BP and TIA-1." Hum Mol Genet **20**(7): 1400-1410.

McGuire, D., L. Garrison, C. Armon, R. J. Barohn, W. W. Bryan, R. Miller, G. J. Parry, J. H. Petajan and M. A. Ross (1997). "A brief quality-of-life measure for ALS clinical trials based on a subset of items from the sickness impact profile. The Syntex-Synergen ALS/CNTF Study Group." J Neurol Sci **152 Suppl 1**: S18-22.

Meyer, T., A. Schwan, J. S. Dullinger, J. Brocke, K. T. Hoffmann, C. H. Nolte, A. Hopt, U. Kopp, P. Andersen, J. T. Epplen and P. Linke (2005). "Early-onset ALS with long-term survival associated with spastin gene mutation." Neurology **65**(1): 141-143.

Miller, K. M., J. V. Tjeertes, J. Coates, G. Legube, S. E. Polo, S. Britton and S. P. Jackson (2010). "Human HDAC1 and HDAC2 function in the DNA-damage response to promote DNA nonhomologous end-joining." Nat Struct Mol Biol **17**(9): 1144-1151.

Myung, N. H., X. Zhu, Kruman, II, R. J. Castellani, R. B. Petersen, S. L. Siedlak, G. Perry, M. A. Smith and H. G. Lee (2008). "Evidence of DNA damage in Alzheimer disease: phosphorylation of histone H2AX in astrocytes." Age (Dordr) **30**(4): 209-215.

Neumann, M., D. M. Sampathu, L. K. Kwong, A. C. Truax, M. C. Micsenyi, T. T. Chou, J. Bruce, T. Schuck, M. Grossman, C. M. Clark, L. F. McCluskey, B. L. Miller, E. Masliah, I. R. Mackenzie, H. Feldman, W. Feiden, H. A. Kretzschmar, J. Q. Trojanowski and V. M. Lee (2006). "Ubiquitinated TDP-43 in frontotemporal lobar degeneration and amyotrophic lateral sclerosis." Science **314**(5796): 130-133.

Niwa, J., S. Yamada, S. Ishigaki, J. Sone, M. Takahashi, M. Katsuno, F. Tanaka, M. Doyu and G. Sobue (2007). "Disulfide bond mediates aggregation, toxicity, and ubiquitylation of familial amyotrophic lateral sclerosis-linked mutant SOD1." J Biol Chem **282**(38): 28087-28095.

Pablo, J., S. A. Banack, P. A. Cox, T. E. Johnson, S. Papapetropoulos, W. G. Bradley, A. Buck and D. C. Mash (2009). "Cyanobacterial neurotoxin BMAA in ALS and Alzheimer's disease." Acta Neurol Scand **120**(4): 216-225.

Paez-Colasante, X., C. Figueroa-Romero, S. A. Sakowski, S. A. Goutman and E. L. Feldman (2015). "Amyotrophic lateral sclerosis: mechanisms and therapeutics in the epigenomic era." Nat Rev Neurol **11**(5): 266-279.

Petri, S., M. Kiaei, K. Kipiani, J. Chen, N. Y. Calingasan, J. P. Crow and M. F. Beal (2006). "Additive neuroprotective effects of a histone deacetylase inhibitor and a catalytic antioxidant in a transgenic mouse model of amyotrophic lateral sclerosis." Neurobiol Dis **22**(1): 40-49.

Piepers, S., J. H. Veldink, S. W. de Jong, I. van der Tweel, W. L. van der Pol, E. V. Uijtendaal, H. J. Schelhaas, H. Scheffer, M. de Visser, J. M. de Jong, J. H. Wokke, G. J. Groeneveld and L. H. van den Berg (2009). "Randomized sequential trial of valproic acid in amyotrophic lateral sclerosis." Ann Neurol **66**(2): 227-234.

Prasad, D. D., M. Ouchida, L. Lee, V. N. Rao and E. S. Reddy (1994). "TLS/FUS fusion domain of TLS/FUS-erg chimeric protein resulting from the t(16;21) chromosomal translocation in human myeloid leukemia functions as a transcriptional activation domain." Oncogene **9**(12): 3717-3729.

Qiu, H., S. Lee, Y. Shang, W. Y. Wang, K. F. Au, S. Kamiya, S. J. Barmada, S. Finkbeiner, H. Lui, C. E. Carlton, A. A. Tang, M. C. Oldham, H. Wang, J. Shorter, A. J. Filiano, E. D. Roberson, W. G. Tourtellotte, B. Chen, L. H. Tsai and E. J. Huang (2014). "ALS-associated mutation FUS-R521C causes DNA damage and RNA splicing defects." J Clin Invest **124**(3): 981-999.

Reed, S. M. and D. E. Quelle (2014). "p53 Acetylation: Regulation and Consequences." Cancers (Basel) **7**(1): 30-69.

Renthal, W. and E. J. Nestler (2008). "Epigenetic mechanisms in drug addiction." Trends Mol Med **14**(8): 341-350.

Renton, A. E., A. Chio and B. J. Traynor (2014). "State of play in amyotrophic lateral sclerosis genetics." Nat Neurosci **17**(1): 17-23.

Renton, A. E., E. Majounie, A. Waite, J. Simon-Sanchez, S. Rollinson, J. R. Gibbs, J. C. Schymick, H. Laaksovirta, J. C. van Swieten, L. Myllykangas, H. Kalimo, A. Paetau, Y. Abramzon, A. M. Remes, A. Kaganovich, S. W. Scholz, J. Duckworth, J. Ding, D. W. Harmer, D. G. Hernandez, J. O. Johnson, K.

Mok, M. Ryten, D. Trabzuni, R. J. Guerreiro, R. W. Orrell, J. Neal, A. Murray, J. Pearson, I. E. Jansen, D. Sondervan, H. Seelaar, D. Blake, K. Young, N. Halliwell, J. B. Callister, G. Toulson, A. Richardson, A. Gerhard, J. Snowden, D. Mann, D. Neary, M. A. Nalls, T. Peuralinna, L. Jansson, V. M. Isoviita, A. L. Kaivorinne, M. Holtta-Vuori, E. Ikonen, R. Sulkava, M. Benatar, J. Wuu, A. Chio, G. Restagno, G. Borghero, M. Sabatelli, I. Consortium, D. Heckerman, E. Rogaeva, L. Zinman, J. D. Rothstein, M. Sendtner, C. Drepper, E. E. Eichler, C. Alkan, Z. Abdullaev, S. D. Pack, A. Dutra, E. Pak, J. Hardy, A. Singleton, N. M. Williams, P. Heutink, S. Pickering-Brown, H. R. Morris, P. J. Tienari and B. J. Traynor (2011). "A hexanucleotide repeat expansion in C9ORF72 is the cause of chromosome 9p21-linked ALS-FTD." Neuron **72**(2): 257-268.

Robertson, K. D. (2005). "DNA methylation and human disease." Nat Rev Genet **6**(8): 597-610.

Rodriguez-Paredes, M. and M. Esteller (2011). "Cancer epigenetics reaches mainstream oncology." Nat Med **17**(3): 330-339.

Rogakou, E. P., W. Nieves-Neira, C. Boon, Y. Pommier and W. M. Bonner (2000). "Initiation of DNA fragmentation during apoptosis induces phosphorylation of H2AX histone at serine 139." J Biol Chem **275**(13): 9390-9395.

Rosen, D. R., T. Siddique, D. Patterson, D. A. Figlewicz, P. Sapp, A. Hentati, D. Donaldson, J. Goto, J. P. O'Regan, H. X. Deng and et al. (1993). "Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis." Nature **362**(6415): 59-62.

Rossetto, D., A. W. Truman, S. J. Kron and J. Cote (2010). "Epigenetic modifications in double-strand break DNA damage signaling and repair." Clin Cancer Res **16**(18): 4543-4552.

Rothstein, J. D., L. J. Martin and R. W. Kuncl (1992). "Decreased glutamate transport by the brain and spinal cord in amyotrophic lateral sclerosis." N Engl J Med **326**(22): 1464-1468.

Rotunno, M. S. and D. A. Bosco (2013). "An emerging role for misfolded wild-type SOD1 in sporadic ALS pathogenesis." Front Cell Neurosci **7**: 253.

Rouaux, C., N. Jokic, C. Mbebi, S. Boutillier, J. P. Loeffler and A. L. Boutillier (2003). "Critical loss of CBP/p300 histone acetylase activity by caspase-6 during neurodegeneration." EMBO J **22**(24): 6537-6549.

Rouaux, C., I. Panteleeva, F. Rene, J. L. Gonzalez de Aguilar, A. Echaniz-Laguna, L. Dupuis, Y. Menger, A. L. Boutillier and J. P. Loeffler (2007). "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." J Neurosci **27**(21): 5535-5545.

Rutter-Locher, Z., M. R. Turner, P. N. Leigh and A. Al-Chalabi (2016). "Analysis of terms used for the diagnosis and classification of amyotrophic lateral sclerosis and motor neuron disease." Amyotroph Lateral Scler Frontotemporal Degener: 1-5.

Ryu, H., K. Smith, S. I. Camelo, I. Carreras, J. Lee, A. H. Iglesias, F. Dangond, K. A. Cormier, M. E. Cudkowicz, R. H. Brown, Jr. and R. J. Ferrante (2005). "Sodium phenylbutyrate prolongs survival and regulates expression of anti-apoptotic genes in transgenic amyotrophic lateral sclerosis mice." J Neurochem **93**(5): 1087-1098.

Saha, R. N. and K. Pahan (2006). "HATs and HDACs in neurodegeneration: a tale of disconcerted acetylation homeostasis." Cell Death Differ **13**(4): 539-550.

Sambrook, J., D. W. Russell and J. Sambrook (2006). The condensed protocols from Molecular cloning : a laboratory manual. Cold Spring Harbor, N.Y., Cold Spring Harbor Laboratory Press.

Schwartz, J. C., C. C. Ebmeier, E. R. Podell, J. Heimiller, D. J. Taatjes and T. R. Cech (2012). "FUS binds the CTD of RNA polymerase II and regulates its phosphorylation at Ser2." Genes Dev **26**(24): 2690-2695.

Seto, E. and M. Yoshida (2014). "Erasers of histone acetylation: the histone deacetylase enzymes." Cold Spring Harb Perspect Biol **6**(4): a018713.

Sharma, S., T. K. Kelly and P. A. Jones (2010). "Epigenetics in cancer." Carcinogenesis **31**(1): 27-36.

Soppe, W. J., Z. Jasencakova, A. Houben, T. Kakutani, A. Meister, M. S. Huang, S. E. Jacobsen, I. Schubert and P. F. Fransz (2002). "DNA methylation controls histone H3 lysine 9 methylation and heterochromatin assembly in Arabidopsis." EMBO J **21**(23): 6549-6559.

Sreedharan, J., I. P. Blair, V. B. Tripathi, X. Hu, C. Vance, B. Rogelj, S. Ackerley, J. C. Durnall, K. L. Williams, E. Buratti, F. Baralle, J. de Belleroche, J. D. Mitchell, P. N. Leigh, A. Al-Chalabi, C. C. Miller, G. Nicholson and C. E. Shaw (2008). "TDP-43 mutations in familial and sporadic amyotrophic lateral sclerosis." Science **319**(5870): 1668-1672.

Strahl, B. D. and C. D. Allis (2000). "The language of covalent histone modifications." Nature **403**(6765): 41-45.

Sugai, F., Y. Yamamoto, K. Miyaguchi, Z. Zhou, H. Sumi, T. Hamasaki, M. Goto and S. Sakoda (2004). "Benefit of valproic acid in suppressing disease progression of ALS model mice." Eur J Neurosci **20**(11): 3179-3183.

Sun, S., S. C. Ling, J. Qiu, C. P. Albuquerque, Y. Zhou, S. Tokunaga, H. Li, H. Qiu, A. Bui, G. W. Yeo, E. J. Huang, K. Eggan, H. Zhou, X. D. Fu, C. Lagier-Tourenne and D. W. Cleveland (2015). "ALS-causative mutations in FUS/TLS confer gain and loss of function by altered association with SMN and U1-snRNP." Nat Commun **6**: 6171.

Sutedja, N. A., K. Fischer, J. H. Veldink, G. J. van der Heijden, H. Kromhout, D. Heederik, M. H. Huisman, J. J. Wokke and L. H. van den Berg (2009). "What we truly know about occupation as a risk factor for ALS: a critical and systematic review." Amyotroph Lateral Scler **10**(5-6): 295-301.

Takanashi, K. and A. Yamaguchi (2014). "Aggregation of ALS-linked FUS mutant sequesters RNA binding proteins and impairs RNA granules formation." Biochem Biophys Res Commun **452**(3): 600-607.

Tamaru, H. and E. U. Selker (2001). "A histone H3 methyltransferase controls DNA methylation in Neurospora crassa." Nature **414**(6861): 277-283.

Tang, L., E. Nogales and C. Ciferri (2010). "Structure and function of SWI/SNF chromatin remodeling complexes and mechanistic implications for transcription." Prog Biophys Mol Biol **102**(2-3): 122-128.

Tiwari, A. and L. J. Hayward (2005). "Mutant SOD1 instability: implications for toxicity in amyotrophic lateral sclerosis." Neurodegener Dis **2**(3-4): 115-127.

Trojsi, F., M. R. Monsurro and G. Tedeschi (2013). "Exposure to environmental toxicants and pathogenesis of amyotrophic lateral sclerosis: state of the art and research perspectives." Int J Mol Sci **14**(8): 15286-15311.

Urdinguio, R. G., J. V. Sanchez-Mut and M. Esteller (2009). "Epigenetic mechanisms in neurological diseases: genes, syndromes, and therapies." Lancet Neurol **8**(11): 1056-1072.

Valle, C., I. Salvatori, V. Gerbino, S. Rossi, L. Palamiuc, F. Rene and M. T. Carri (2014). "Tissue-specific deregulation of selected HDACs characterizes ALS progression in mouse models: pharmacological characterization of SIRT1 and SIRT2 pathways." Cell Death Dis **5**: e1296.

Van Deerlin, V. M., J. B. Leverenz, L. M. Bekris, T. D. Bird, W. Yuan, L. B. Elman, D. Clay, E. M. Wood, A. S. Chen-Plotkin, M. Martinez-Lage, E. Steinbart, L. McCluskey, M. Grossman, M. Neumann, I. L. Wu, W. S. Yang, R. Kalb, D. R. Galasko, T. J. Montine, J. Q. Trojanowski, V. M. Lee, G. D. Schellenberg and C. E. Yu (2008). "TARDBP mutations in amyotrophic lateral sclerosis with TDP-43 neuropathology: a genetic and histopathological analysis." Lancet Neurol **7**(5): 409-416.

Verdone, L., E. Agricola, M. Caserta and E. Di Mauro (2006). "Histone acetylation in gene regulation." Brief Funct Genomic Proteomic **5**(3): 209-221.

Vergara, X., G. Mezei and L. Kheifets (2015). "Case-control study of occupational exposure to electric shocks and magnetic fields and mortality from amyotrophic lateral sclerosis in the US, 1991-1999." J Expo Sci Environ Epidemiol **25**(1): 65-71.

Vucic, S. and M. C. Kiernan (2009). "Pathophysiology of neurodegeneration in familial amyotrophic lateral sclerosis." Curr Mol Med **9**(3): 255-272.

Vucic, S., J. D. Rothstein and M. C. Kiernan (2014). "Advances in treating amyotrophic lateral sclerosis: insights from pathophysiological studies." Trends Neurosci **37**(8): 433-442.

Waddington, C. H. (1959). "Canalization of development and genetic assimilation of acquired characters." Nature **183**(4676): 1654-1655.

Wang, W. Y., L. Pan, S. C. Su, E. J. Quinn, M. Sasaki, J. C. Jimenez, I. R. Mackenzie, E. J. Huang and L. H. Tsai (2013). "Interaction of FUS and HDAC1 regulates DNA damage response and repair in neurons." Nat Neurosci **16**(10): 1383-1391.

Wang, X., S. Arai, X. Song, D. Reichart, K. Du, G. Pascual, P. Tempst, M. G. Rosenfeld, C. K. Glass and R. Kurokawa (2008). "Induced ncRNAs allosterically modify RNA-binding proteins in cis to inhibit transcription." Nature **454**(7200): 126-130.

Wils, H., G. Kleinberger, J. Janssens, S. Pereson, G. Joris, I. Cuijt, V. Smits, C. Ceuterick-de Groote, C. Van Broeckhoven and S. Kumar-Singh (2010). "TDP-43 transgenic mice develop spastic paralysis and neuronal inclusions characteristic of ALS and frontotemporal lobar degeneration." Proc Natl Acad Sci U S A **107**(8): 3858-3863.

Yang, X. J. and E. Seto (2008). "Lysine acetylation: codified crosstalk with other posttranslational modifications." Mol Cell **31**(4): 449-461.

Yoo, Y. E. and C. P. Ko (2011). "Treatment with trichostatin A initiated after disease onset delays disease progression and increases survival in a mouse model of amyotrophic lateral sclerosis." Exp Neurol **231**(1): 147-159.

Yoshimura, A., R. Fujii, Y. Watanabe, S. Okabe, K. Fukui and T. Takumi (2006). "Myosin-Va facilitates the accumulation of mRNA/protein complex in dendritic spines." Curr Biol **16**(23): 2345-2351.

Zarei, S., K. Carr, L. Reiley, K. Diaz, O. Guerra, P. F. Altamirano, W. Pagani, D. Lodin, G. Orozco and A. Chinea (2015). "A comprehensive review of amyotrophic lateral sclerosis." Surg Neurol Int **6**: 171.

Zetterstrom, P., H. G. Stewart, D. Bergemalm, P. A. Jonsson, K. S. Graffmo, P. M. Andersen, T. Brannstrom, M. Oliveberg and S. L. Marklund (2007). "Soluble misfolded subfractions of mutant superoxide dismutase-1s are enriched in spinal cords throughout life in murine ALS models." Proc Natl Acad Sci U S A **104**(35): 14157-14162.

Zinellu, A., S. Sotgia, V. De Murtas, P. Cossu-Rocca, M. R. De Miglio, M. R. Muroni, A. Mura, M. G. Uras, M. Contini, L. Deiana and C. Carru (2011). "Evaluation of methylation degree from formalin-fixed paraffin-embedded DNA extract by field-amplified sample injection capillary electrophoresis with UV detection." Anal Bioanal Chem **399**(3): 1181-1186.

This research was partially supported by Fondazione Banco di Sardegna (2013 Grant, project title "Epigenetic modifications in the pathogenesis of Amyotrophic Lateral Sclerosis").

For the same reason, I would like to thank AriSLA foundation, who partially supported my PhD thesis with the 2014 grant "ALSHDCA1 – HDAC1 TDP43 interaction: implication for ALS".

Finally, I thank my supervisor, prof. Claudia Crosio, for her constant suppor and teaching, the Molecular biology Lab in Biomedical Sciences Department and everyone who collaborated to this work.