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RNA-binding proteins and RNA metabolism: a new scenario in the pathogenesis of Amyotrophic Lateral Sclerosis

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ABSTRACT

Several RNA-processing genes have been implicated in the pathogenesis of Amyotrophic lateral sclerosis (ALS). In particular, causative mutations in the genes encoding for two DNA/RNA binding proteins, TAR DNA binding protein-43 (TDP-43) and fused in sarcoma/translocated in liposarcoma (FUS/TLS), were recently identified in ALS patients. These genetic findings and the presence of abnormal aggregates of these two RNA-binding proteins in ALS affected tissues suggest that molecular mechanisms regulating RNA metabolism are implicated in ALS pathogenesis through common pathways. In this review similarities and differences between TDP-43 and FUS/TLS proteins and their activities in physiological and pathological conditions will be discussed.

Key words

TAR DNA binding protein-43 (TDP-43) • Fused in sarcoma/translocated in liposarcoma (FUS/TLS) • RNA-binding protein (RBP) • Protein aggregates

Introduction

Amyotrophic lateral sclerosis (ALS) is an adult-onset and fatal neurodegenerative disease characterized by the selective loss of upper and lower motor neurons in the cerebral cortex, brainstem and spinal cord. This neuronal degeneration leads to a progressive skeletal muscle atrophy and death by respiratory failure after 2-5 years from symptoms onset. The disease, presenting in middle age, has an incidence of 1-2 per 100,000 person-year. It is currently an incurable disease and treatment is largely limited to supportive care (Rowland and Shneider, 2001).

The familial ALS forms (fALS) represent only 10% of cases, while the sporadic forms (sALS) are the majority of cases, which are mostly phenotypically indistinguishable from fALS, suggesting the existence of common pathways at the basis of neuronal death.

The identification of copper/zinc superoxide dismutase 1 (*SOD1*) as the first causative gene, accounting for ~20% of fALS and ~3% of sALS cases, initiated the understanding of ALS pathogenesis (Rosen, 1993). In all these years the development and the extensive study of different mutant SOD1 cell and animal models were instrumental in unraveling the pathogenic mechanisms and pathways involved in motor neuron (MN) degeneration (Peviani et al., 2010). Indeed, several pathogenic mechanisms have been demonstrated to induce neuronal death in ALS, from oxidative stress and mitochondrial impairment to glutamate excitotoxicity, growth fac-

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tor deficiency, neuro-inflammation and defective axonal transport (Rothstein, 2009), although the primary or secondary role of each of these events in triggering MN degeneration still need to be determined. Several drugs and compounds were tested using such experimental models, although so far no therapy has been found to be effective when translated into clinical trials (Cudkowicz et al., 2010). Among the possible causes of this failure there is the fact that they proved to be effective in SOD1-linked fALS experimental models, while the majority of ALS cases are sporadic.

Recently, a new scenario about ALS pathogenesis emerged with the identification of a DNA/RNA binding protein, the TAR DNA binding protein 43 (TDP-43), as the major component of the ubiquitinated inclusions occurring in all sALS and in a sub-population of frontotemporal lobar dementia with ubiquitin inclusions (FTLD-U) patients (Arai et al., 2006; Neumann et al., 2006). Interestingly, causative mutations in TDP-43-encoding gene *TARDBP* and, more recently, in a second gene encoding another DNA/RNA binding protein, fused in sarcoma/translocated in liposarcoma (*FUS/TLS*), were identified in fALS as well as in sALS patients (Sreedharan et al., 2008; Kwiatkowski et al., 2009; Vance et al., 2009). Extensive mutational analyses on different ethnic groups, including Italian ALS patients referring to our clinical Centre, showed that *TARDBP* and *FUS/TLS* genes are responsible for about 4-5% of fALS and 1-2% of sALS cases each (Corrado et al., 2009; 2010; Lagier-Tourenne et al., 2010).

These genetic findings, together with anatomopathological observations showing that these two DNA/RNA-binding proteins abnormally aggregate in ALS affected tissues, redirected research studies on the comprehension of the complex molecular mechanisms regulating gene expression at post-transcriptional level as potential pathogenic clues. Interestingly, TDP-43 and FUS/TLS are not the only RNA-processing proteins implicated in ALS pathogenesis. Indeed, four other RNA-processing genes have been shown to be genetically associated with ALS, including the causative genes senataxin (*SETX)* and angiogenin *(ANG)*, as well as the risk factors elongator protein 3 (*Elp3*) and survival motor neuron (*SMN*) (van Blitterswijk and Landers, 2010).

Structure of TDP-43 and FUS/TLS: differences and similarities

TDP-43 and FUS/TLS are DNA/RNA binding proteins which are both structurally related to the heterogeneous ribonucleoproteins (hnRNPs) family, a group of predominantly nuclear RBPs with different cellular functions that form molecular complexes binding heterogeneous nuclear RNAs (Krecic and Swanson, 1999).

TDP-43 is a 414-aminoacids long protein identified for the first time in 1995 as a factor able to bind the long terminal repeat transactive response (TAR) element of the human immunodeficiency virus type 1 (HIV-1) DNA (Ou et al., 1995). Subsequent studies have shown that it is capable to bind also RNA, being involved in the exon splicing of several genes (Buratti et al., 2001; 2004; Wang et al., 2004; Mercado et al., 2005; Ayala et al., 2006; Bose et al., 2008). Like the other hnRNPs, TDP-43 contains two RNA recognition motifs (RRM1 and RRM2) that are evolutionary conserved and involved in RNA and DNA binding, but also in protein-protein interactions. The RRM1 domain is necessary for binding to single stranded RNA with at least five UG repeats (Buratti et al., 2001; Ayala et al., 2005), while the RRM2 is supposed to play an important role in chromatin organization (Ayala et al., 2008) and in TDP-43 dimerization (Kuo et al., 2009; Shiina et al., 2010). The C-terminal domain of TDP-43 contains a glycine-rich region important for the interaction with other proteins, in particular with hnRNP A1 and hnRNP A2/B1 (Buratti et al., 2005), and for the cystic fibrosis transmembrane regulator (CFTR) exon-skipping activity (Wang et al., 2004; Ayala et al., 2005). Interestingly, nearly all the mutations identified in *TARDBP* gene in ALS patients are localized in exon 6, coding for the C-terminal domain (Lagier-Tourenne and Cleveland, 2009). Moreover, the deletion of the C-terminal domain has been demonstrated to decrease TDP-43 shuttling between nucleus and cytoplasm, establishing also an important role of this region in TDP-43 cellular localization (Ayala et al., 2008). The N-terminal domain, containing two nuclear localization signals (NLS) and three putative caspase-3 cleavage consensus sites, is required for a correct CFTR exon 9 skipping activity and for TDP-43 homodimerization (Kuo et al.,

2009; Zhang et al., 2009). TDP-43 cleavage by caspase-3 is known to produce different C-terminal fragments, including the 25 kDa one which tends to accumulate, in a hyperphosphorylated state, in the cytoplasmic inclusions of ALS affected tissues (Neumann et al., 2006; Zhang et al., 2007; Zhang et al., 2009).

The FUS/TLS protein was first identified in 1993 as a chromosomal translocation-mediated fusion protein in human myxoid liposarcoma (Crozat et al., 1993; Rabbitts et al., 1993). Subsequently it was demonstrated to bind RNA and single or double stranded DNA (Cassiday and Maher, 2002). FUS/TLS is a 526-aminoacids long nuclear protein, also called hnRNP P2 (Calvio et al., 1995), and belongs to the TET or FET protein family, sharing a similar structure with two other members of this family, the Ewing's sarcoma protein and the TATA-binding protein-associated factor (Andersson et al., 2008). FUS/TLS protein structure is characterized by a N-terminal domain composed of sequences enriched in glutamine, glycine, serine and tyrosine residues (QGSY-region), a glycine-rich region, a RRM domain, two multiple Arginine-Glycine-Glycine (RGG) repeats flanking a zinc finger motif, and a C-terminal 18-residues long NLS region (Iko et al., 2004). FUS/TLS has been demonstrated to bind to RNA targets in specific GGUG sequences recognized by the zinc finger domain, while the RGG and RRM domains mediate the specificity of this interaction (Lerga et al., 2001; Iko et al., 2004). Although a GGUG consensus sequence has been identified in most of the RNA molecules recognized by FUS/TLS using an *in vitro* selection procedure (Lerga et al., 2001), the protein has been found also to interact with the 3'untranslated region (3'-UTR) of the actin stabilizing protein Nd1-L mRNA in which a GGUG-type motif was absent (Fujii and Takumi, 2005). Moreover, the RGG repeats go through dimethylation events that play a regulatory role in various cellular processes including transcription, transduction and protein sorting (Rappsilber et al., 2003). The NLS-containing C-terminal region of the protein, in which the majority of ALS mutations occur similarly to TDP-43, has been shown to be implicated in the translocation of FUS/TLS from the nucleus to the cytoplasm (Gal et al., 2010).

TDP-43 and FUS/TLS functions: differences and similarities

TDP-43 and FUS/TLS take part in multiple steps of the RNA processing pathway, which includes transcription and post-transcriptional regulatory processes of gene expression, such as splicing and RNA editing, but also mRNA stabilization and transport, translation and RNA degradation (Fig. 1). Consequently, the involvement of these two RNA-binding proteins (RBPs) in ALS has raised the hypothesis that the disease is caused by defects in (neuronal) RNA metabolism. Moreover, since TDP-43 and FUS/TLS have strongly related biological functions, this suggests that the two proteins might share common RNA targets selectively affecting MN survival.

Transcription

Both TDP-43 and FUS/TLS are involved in transcription regulation. In particular, acting as a transcriptional repressor, TDP-43 was initially identified to bind the TAR DNA sequence of the HIV-1 (Ou et al., 1995) and subsequently was also shown to bind the promoter of the mouse SP-10 gene involved in spermatogenesis (Abhyankar et al., 2007). Indeed, in different cell lines and in mammalian neurons, TDP-43 is mainly localized in perichromatin fibrils, nuclear sites of transcription and splicing, where it associates with actively transcribed genes (Ayala et al., 2008; Casafont et al., 2009).

The regulation of transcriptional processes exerted by FUS/TLS occurs through its association with the transcriptional machinery and its inclusion in the pre-initiation complex. Here it directly interacts with RNA polymerase II and the transcription factor II D influencing transcription initiation and promoter selection (Bertolotti et al., 1998; Yang et al., 2000). In addition, FUS/TLS interacts with several transcriptional regulators such as Spi-1/PU.1, YB-1 and NF-kB (Hallier et al., 1998; Uranishi et al., 2001), and with several nuclear hormone receptors, including steroid, thyroid hormone, and retinoid receptors (Powers et al., 1998). FUS/TLS has been recently shown to repress also the transcription mediated by RNA Polymerase III (Tan and Manley, 2010) and to be a key transcriptional regulatory sensor of DNA damage signals. Indeed, following its RNA-dependent allosteric modulation, FUS/TLS is able to specifically bind and inhibit CREB-binding protein

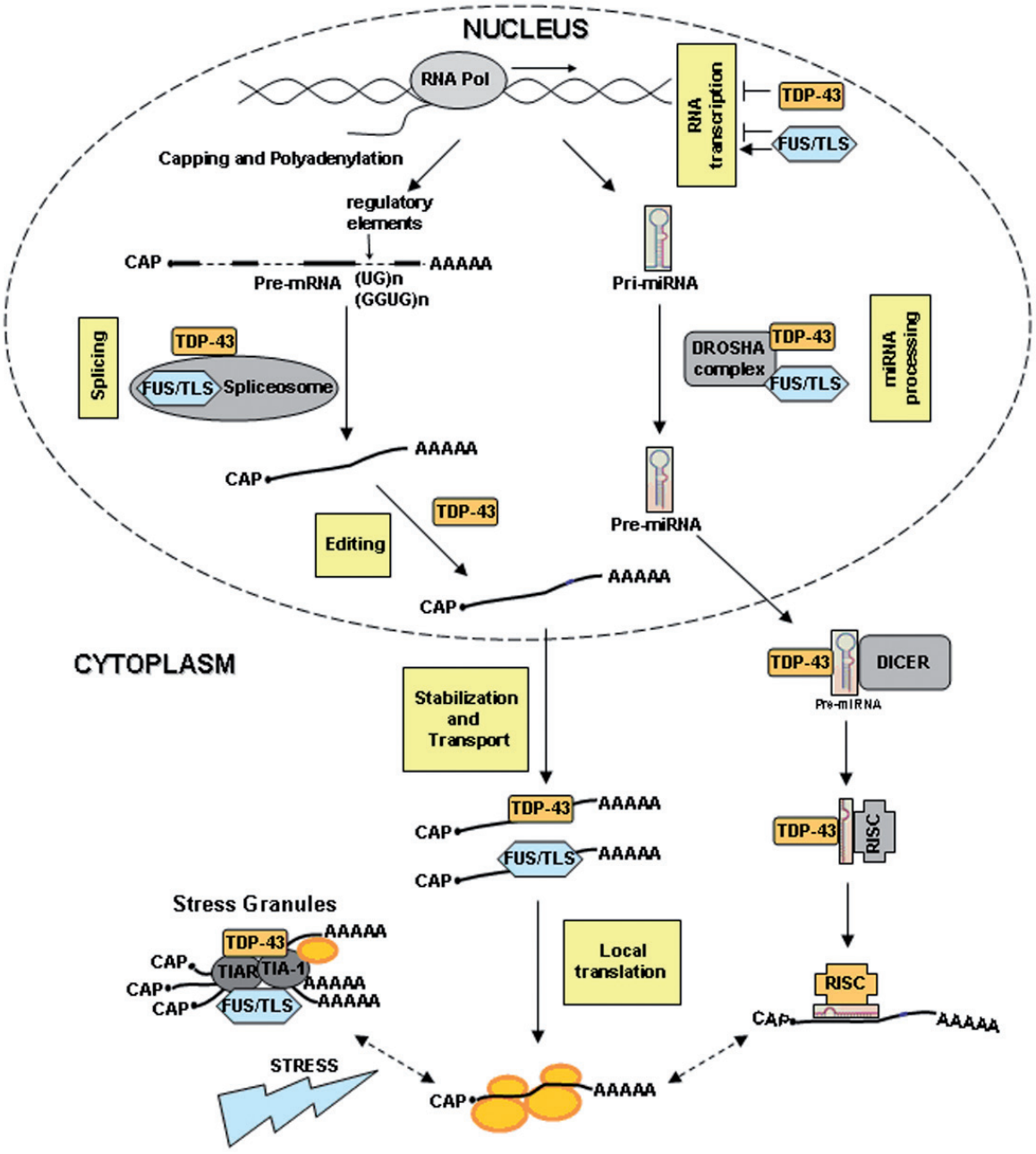

Fig. 1. - TDP-43 and FUS/TLS RBPs in RNA metabolism. A schematic representation of the different steps of RNA processing in which TDP-43 and/or FUS/TLS are involved is shown. These two RBPs show common regulatory mechanisms in RNA metabolism, from transcription to post-transcriptional processes in the nucleus and in the cytoplasm.

(CBP) and p300 histone acetyltransferase activities, leading to the repression of the cyclin D1 (CCND1) gene target in human cell lines (Wang et al., 2008).

Splicing

Even though very few RNA targets have been identified so far, it is well recognized that TDP-43 and FUS/TLS act as splicing factors, whose depletion or over-expression can affect the splicing pattern of specific targets (Lerga et al., 2001; Wang et al., 2004; Ayala et al., 2005; Buratti and Baralle, 2008; Camats et al., 2008). TDP-43 is known to regulate the alternative splicing of CFTR, Apolipoprotein A-II (Apo A-II) and SMN transcripts. In particular,

it recognizes and binds the intronic UG_n sequence in CFTR and Apo A-II pre-mRNAs by its glycine-rich region in the C-terminal domain, promoting exon 9 and exon 3 skipping, respectively (Buratti et al., 2001; Mercado et al., 2005). TDP-43 has been suggested to promote not only exon skipping, but also exon inclusion. In fact it is able to enhance inclusion of exon 7 during the maturation of human SMN2 pre-mRNA, leading to an increase of full-length SMN mRNA level in neurons (Bose et al., 2008). Moreover, TDP-43 associates with other splicing factors, including the serine/arginine-rich spliceosomal protein SC-35 (Wang et al., 2002; Buratti et al., 2005; Freibaum et al., 2010), by its C-terminal glycine-rich domain and the lack of this region, as mentioned above, has been demonstrated to affect CFTR exon skipping activity (Wang et al., 2004; Ayala et al., 2005).

Also FUS/TLS regulates alternative splicing interacting with other splicing factors, including YB-1 (Chansky et al., 2001; Rapp et al., 2002), Spi-1/PU.1 (Hallier et al., 1998), polypyrimidine tract-binding protein (Meissner et al., 2003), hnRNP A1 and C1/C2 (Zinszner et al., 1994), SG35 and other serine/arginine proteins (Yang et al., 2000; Meissner et al., 2003), even if its role in splicing regulation is not well understood yet. FUS/TLS is also a protein of the spliceosome machinery (Hartmuth et al., 2002; Rappsilber et al., 2002; Zhou et al., 2002) and, in association with the splicing complex, it binds indirectly the 5' splice sites (Kameoka et al., 2004) and directly the 3' splice sites (Wu and Green, 1997) of pre-mRNAs.

mRNA editing

The mRNA editing includes a variety of post-transcriptional processes leading to the modification of nucleotide sequences of RNA transcripts in different organisms (Simpson and Emeson, 1996). An efficient mRNA editing of the GluR2 subunit of glutamate AMPA receptors, which converts a glutamine (Q) to an arginine (R) in a transmembrane region of the protein thus regulating the overall calcium permeability of the receptor (Sommer et al., 1991), has been demonstrated to be important for the survival of MNs (Higuchi et al., 2000). In particular, a defective RNA editing at the GluR2 Q/R site was observed specifically in spinal MNs of patients with sALS (Kawahara et al., 2004). TDP-43 seems to be involved in this post-transcriptional mechanism since a very recent study suggests a molecular link between the reduced activity of adenosine deaminase acting on RNA 2 (ADAR2), the enzyme that specifically catalyzes GluR2 Q/R site-editing, and TDP-43 pathology (Aizawa et al., 2010).

miRNA processing

TDP-43 and FUS/TLS have been described to be associated also to Drosha enzyme in multi-protein complexes (Gregory et al., 2004). Drosha is a RNase type III enzyme involved in the first step of microRNA (miRNA) maturation (Kim et al., 2009) and converts pri-miRNA to pre-miRNA molecules (about 70-nucleotides long), which are exported to the cytoplasm where they are then processed in mature miRNA by the Dicer complex (Rana, 2007). miRNA molecules are able to bind mRNA targets and negatively regulate their expression level or their translation (Nilsen, 2007; Pillai et al., 2007; Standart and Jackson, 2007). TDP-43 was also found to associate with proteins involved in the cytoplasmic cleavage of pre-miRNA mediated by the Dicer enzyme (Freibaum et al., 2010). In different human cells, including neuroblastoma cell lines, changes in the total miRNA population were observed upon TDP-43 depletion (Buratti et al., 2010). In particular let-7b and miR-663 expression levels were down- and up-regulated, respectively, and both miRNAs were capable of binding directly to TDP-43. These TDP-43-miRNA interactions have been also shown to affect the expression levels of candidate cellular transcripts, such as the cyclin-dependent kinase 6 (Cdk6), which was previously found to be up-regulated following TDP-43 gene silencing (Ayala et al., 2008).

Regulation of mRNA fate in neuronal cell: transcript stabilization and transport

Regulation of mRNA fate is known to play an important role during the development of the nervous system and for the maintenance of neural activities in the adult brain. Post-transcriptional RBP-mediated regulatory mechanisms allow a precise spatio-temporal control of mRNA translation, associated to transport and subcellular compartmentalization of mRNAs in dendrites and axons (Besse and Ephrussi, 2008), so that disruption of such activities is supposed to severely impair neuronal cell metabolism. Messenger RNA transport into neurites is particularly relevant in highly polarized cells, such as MNs,

where an efficient metabolism and a fast response to stimuli are guaranteed by local protein synthesis at synapses and along axons (Yoo et al., 2010).

Although TDP-43 and FUS/TLS are ubiquitously expressed RBPs with main nuclear localization and activities, these proteins were recently demonstrated to participate also in the regulation of mRNA fate in neuronal cells, such as transcript stabilization, activity-dependent mRNA transport to dendrites and local translation in association to synaptic plasticity processes. In fact, a shuttling activity from the nucleus to the cytoplasm has been observed for both FUS/TLS and TDP-43 RBPs, and seems to be a common feature of these proteins in neuronal cells. Inhibition of RNA polymerase II by Actinomycin D leads to the accumulation of TDP-43 in the cytoplasm and suggest that a continuous RNA synthesis is necessary for TDP-43 import (Ayala et al., 2008). Regarding FUS/TLS its cytoplasmic localization in association with mRNA has been demonstrated after inhibition of RNA polymerase II transcription (Zinszner et al., 1997) as well as arginine residues dymethylation (Tan and Manley, 2009) and tyrosine phosphorylation by the fibroblast growth factor receptor 1 (FGFR-1) kinase (Klint et al., 2004).

The evidence of a biological role of TDP-43 and FUS/TLS in mRNA transport and cytoplasmic localization in neurons has emerged from their identification in RNA granules whose translocation to dendritic spines occurs after different stimuli (Kanai et al., 2004; Belly et al., 2005; Fujii et al., 2005; Elvira et al., 2006; Wang et al., 2008).

Primarily, FUS/TLS was found to be associated in RNA granules, thus partecipating in mRNA sorting to the dendritic spines, following the metabotropic glutamate receptor mGluR5 activation, and in regulating spine morphology (Fujii et al., 2005). Indeed, in hippocampal pyramidal neurons from FUS/TLS knock-out mice, a lower density and an abnormal morphology of spines were observed (Hicks et al., 2000; Fujii et al., 2005), being FUS/TLS probably involved in actin cytoskeleton reorganization in spines through its binding to the Nd1-L actin-stabilizing protein (Fujii and Takumi, 2005). After post-synaptic activation by the mGluR5 receptor, FUS/TLS accumulates in mRNA/protein complexes, called also ribonucleoprotein complexes (mRNPs), in dendritic spines (Fujii et al., 2005), facilitated also by the interaction with two actin-based motor, myosin Va (Yoshimura et al., 2006) and myosin VI (Takarada et al., 2009). Finally, FUS/TLS was found to localize with other RBPs and RNA molecules in the "spreading initiation centers" (de Hoog et al., 2004; Andersson et al., 2008), that are mRNPs existing only in early stages of adhering cell spreading, thus reinforcing the role of this protein in the regulation of local translation. FUS/TLS partecipates in the regulation of mRNA local translation at excitatory synapses by associating also with N-Methyl-D-aspartate (NMDA) receptor-adesion protein (Husi et al., 2000; Belly et al., 2005; Selamat et al., 2009).

Like FUS/TLS, also TDP-43 has been recently shown to be involved in dendritic formation, but its role in the regulation of local translation is less well known. Depletion of TDP-43 affects Drosophila MN synaptic formation and decreases dendritic branching (Feiguin et al., 2009; Lu et al., 2009). TDP-43 was found to be localized in the dendrites of rat hippocampal neurons, functioning in the regulation of neuronal plasticity (Wang et al., 2008). In particular, TDP-43 behaves as a neuronal activity-responsive factor co-localizing in RNA granules with the post-synaptic PSD-95 protein and with the beta-actin and the calcium calmodulin dependent kinase II alpha (CaMK) mRNAs (Wang et al., 2008). Furthermore, TDP-43 interacts with several proteins of the translation machinery (Freibaum et al., 2010). In hippocampal neurons, but not in NSC-34 motor neuronal cell line, it has been localized in processing bodies (P-bodies), constitutive RNP complexes where both mRNA degradation and mRNA-mediated translational arrest take place (Wang et al., 2008; Colombrita et al., 2009). In fact, TDP-43 behaves as a translational repressor and, after stimuli by KCl, it greatly associates with fragile mental retardation protein (FMRP) and Staufen 1, two RBPs known to regulate mRNA transport and local translation in neurons (Wang et al., 2008). Interestingly, TDP-43 has been found to bind the low molecular weight neurofilament (NFL) mRNA at the UG motifs present in its 3'UTR and this binding was demonstrated to stabilize the NFL transcript (Strong et al., 2007; Volkening et al., 2009; Volkening et al., 2010).

Translation and stress granules

A direct involvement of TDP-43 and FUS/TLS in the control of mRNA translation has been demonstrated since both RBPs were found to be recruited

into stress granules (SGs) (Andersson et al., 2008; Colombrita et al., 2009; Moisse et al., 2009; Bosco et al., 2010; Dormann et al., 2010; Freibaum et al., 2010; Gal et al., 2010; Nishimoto et al., 2010), cytoplasmic foci where, after a sub-lethal environmental stress induced *in vitro*, there is an immediate block of the translation machinery with sequestration of the actively-translating mRNAs (Anderson and Kedersha, 2008). SGs represent a protective mechanism to bypass the cellular insult as the majority of mRNAs is silenced in these macromolecular structures in stalled 48S ribosomal complexes, while only specific and essential transcripts (i.e. Hsp70) are maintained in active translation (Kedersha and Anderson, 2002). During stress, SGs are in dynamic equilibrium among polysomes and P-bodies. It is experimentally proven that once the insult is removed, these RNP complexes soon disaggregate in favour of a parallel polysome reassembly and mRNA translation re-initiation (Thomas et al., 2010).

TDP-43 has been demonstrated to be recruited to SGs after different environmental stressors, but in contrast to other SG markers it is neither an essential component of SGs nor a neuroprotective factor in stress conditions (Colombrita et al., 2009). The presence of TDP-43 in SGs *in vivo* was found in axotomized MNs and these cytoplasmic foci were shown to dissolve after neuronal recovery (Moisse et al., 2009; Sato et al., 2009). These results suggest that TDP-43-positive SGs mediate the stabilization and transport of the NFL mRNA to the injury site for local translation of NFL protein required for axonal repair (Moisse et al., 2009). Regarding FUS/TLS, since the majority of fALS-associated mutations occur within the NLS region, the role of this domain in SG formation has been extensively explored. In particular, deletions or mutations within the NLS region caused cytoplasmic mislocalization of FUS/TLS and SG formation (Bosco et al., 2010; Gal et al., 2010). Differently from TDP-43, in response to oxidative stress or heat shock conditions, only the ALS-linked FUS/TLS mutants, and not wild-type FUS/TLS, were shown to assemble into SGs both *in vitro* and *in vivo* (Bosco et al., 2010). The nuclear import of FUS/TLS has been demonstrated to be dependent on Transportin, and interference with this transport pathway leads to cytoplasmic redistribution and FUS/TLS recruitment into SGs (Dormann et al., 2010). Moreover, proteins known to be stress granule markers, such as the poly-A binding protein (PABP-1), co-localized with FUS/TLS inclusions in FUS/TLS-mutated fALS patients but were absent in cells expressing wild-type FUS/TLS, implicating that SG formation may represent a pathogenetic mechanism in ALS (Dormann et al., 2010; Gal et al., 2010). Of note, TDP-43-positive cytoplasmic inclusions, which are present in sALS and in SOD1-negative fALS MNs, do not contain SG markers suggesting a different pathogenetic mechanism for TDP-43 in ALS (Colombrita et al., 2009).

TDP-43 and FUS/TLS cytoplasmic mislocalization and inclusions: loss or gain of function?

The presence of abnormal protein aggregates is a pathological feature of the majority of neurological disorders, including ALS (Aguzzi and O'Connor, 2010). Recently, TDP-43 has been found as the major protein component of the intracellular inclusions observed in the neuronal tissues of patients affected from both apparently sporadic and SOD1-negative familial forms of ALS and in a subset of FTLD cases in a sort of clinical continuum (Mackenzie et al., 2007). TDP-43-positive inclusions were also found in other neurodegenerative disorders including Alzheimer and Parkinson's disease (Chen-Plotkin et al., 2010). Interestingly, FUS/TLS aggregates/inclusions not immunoreactive for TDP-43 were found in neurons and glial cells of post-mortem brain and spinal cord tissue from ALS patients carrying *FUS/TLS* mutations (Kwiatkowski et al., 2009; Vance et al., 2009; Suzuki et al., 2010; Tateishi et al., 2010). Such inclusions also represent the pathological hallmark of a subset of tau-, ubiquitin- and TDP-43-negative FTLD cases (Munoz et al., 2009; Neumann et al., 2009; Woulfe et al., 2010), indicating that neurodegenerative processes determined by FUS/TLS mislocalization can be independent of TDP-43. Moreover, FUS/TLS has been found to be associated to the intranuclear polyglutamine inclusions in Huntington disease and in other polyglutamine disorders, such as spinocerebellar ataxia type 1, 2, 3, and dentatorubral-pallidoluysian atrophy (Doi et al., 2008; 2010).

The impact of TDP-43 and FUS/TLS in the neurodegeneration field has been so pervasive that disease

nomenclatures are currently being modified in "TDP-43 and FUS/TLS proteinopathies" to better reflect the new clinical and pathological findings originating from recent research. At the moment, disease mechanisms related to these two RBPs are not clear. Since in ALS affected MNs both TDP-43 and mutant FUS/TLS RBPs are mislocalized in the cytoplasm where they appear to be recruited into aggregates (Kwiatkowski et al., 2009; Nonaka et al., 2009; Vance et al., 2009; Barmada et al., 2010), their altered localization may play a pivotal role in neurodegeneration as it may result in the loss of their proper function in the nucleus (loss-of-function effects) and/or in their potential toxicity in the cytoplasm (gain-of-function effects). The observation that in the inclusion-bearing cells of ALS patients these RBPs are absent from nuclei (Kwiatkowski et al., 2009; Vance et al., 2009) raised the hypothesis that toxicity might be caused by aggregation and sequestration from their normal nuclear function. In this view, the sequestration of TDP-43 and FUS/TLS in pathological aggregates is supposed to determine a loss of function of the protein with severe consequences on mRNA metabolism and post-transcriptional regulation of gene expression.

On the contrary, aggregates might have a toxic gain-of-function and their formation would trigger the neurodegeneration process, independently on the physiological cellular activities of these proteins. Interestingly, both TDP-43 and FUS/TLS missense mutations causative of ALS disease predominantly affect the C-terminal domains of the protein containing motifs for protein-protein interactions and nuclear localization, respectively, and seem to promote aggregates formation and cell toxicity *in vitro* (Pesiridis et al., 2009; Gal et al., 2010).

Consistently with the "gain-of-function" hypothesis, increased cytoplasmic localization and the associated formation of TDP-43 intracellular aggregates have also been found in different animal models, including transgenic mice for wild-type TDP-43 (Wils et al., 2010; Xu et al., 2010), rats with adenovirus-mediated wild-type TDP-43 over-expression (Tatom et al., 2009) and Drosophila (Li et al., 2010). Also *in vitro*, in different cell models, the over-expression of TDP-43 mutants in the NLS domain or of C-terminal fragments (CTFs) determined the cytoplasmic redistribution of the protein (Winton et al., 2008; Nonaka et al., 2009; Barmada et al., 2010). Particularly, in primary rat cortical neurons, cell toxicity was associated to the total amount of mutant TDP-43 in the cytoplasm, suggesting that mislocalized cytoplasmic TDP-43 exhibits a toxic gain-of-function and induces cell death (Barmada et al., 2010). Similar to TDP-43, *in vitro* experiments have shown that in different cell lines cytoplasmic mislocalization of mutant FUS/TLS resulted in aggregate formation and MN toxicity (Kwiatkowski et al., 2009; Vance et al., 2009; Bosco et al., 2010; Dormann et al., 2010; Gal et al., 2010).

TDP-43 can also be ubiquitinated, hyperphosphorylated and abnormally cleaved to generate CTFs, which are released in the cytoplasm and can be potentially toxic (Arai et al., 2006; Neumann et al., 2006; Kwong et al., 2008), but there is no evidence that hyperphosphorylated or cleaved forms of FUS/TLS protein are present in the aggregates (Kwiatkowski et al., 2009; Neumann et al., 2009; Vance et al., 2009). In cell cultures, an increase of cytoplasmic TDP-43 localization and intracellular aggregates have been shown after the inhibition of either the autophagic or the ubiquitin-proteasome system (UPS) (Colombrita et al., 2009; Urushitani et al., 2010; Wang et al., 2010), suggesting that both these two degradation systems may be involved in TDP-43 aggregate formation. In line with this hypothesis, TDP-43 depletion has been demonstrated to reduce the expression levels of the histone deacetylase 6 (HDAC6), a protein implicated in the autophagic degradation of poly-ubiquitinated protein aggregates (Kawaguchi et al., 2003; Boyault et al., 2007; Lee et al., 2010), and to increase the polyglutamine-mediated citotoxicity in a cellular model of spinocerebellar ataxia (Fiesel et al., 2010). Indeed, the accumulation of ubiquitinated protein aggregates in association to neurodegeneration is observed in HDAC6 knockout Drosophila and mice animal models (Lee et al., 2010). Moreover, TDP-43 was recently described to associate with ubiquilin1, another protein binding to ubiquitinated proteins, which promotes their autophagosome- as well as their proteosome-mediated degradation (Kim et al., 2009).

While the role of ubiquitination of TDP-43 in the pathogenesis of ALS is not well understood yet, even if it seems to be a late event (Mori et al., 2008; Giordana et al., 2010), the phosphorylation process of TDP-43 and of its CTFs has been more investigated. However, whether this represents a primary or secondary event in the disease is still unclear. Using phosphospecific antibodies, which only recognize abnormal TDP-43 aggregates, it was possible to

identify the serine residues 409/410 as the main sites of TDP-43 phosphorylation (Hasegawa et al., 2008; Inukai et al., 2008; Kadokura et al., 2009; Neumann et al., 2009). Also the 25 kDa CTFs, originated from TDP-43 caspase-mediated proteolitic cleavage, were found to be hyperphosphorylated in serines 409/410 (Hasegawa et al., 2008; Inukai et al., 2008; Kadokura et al., 2009; Neumann et al., 2009) and to accumulate in insoluble fractions of nervous system tissues derived from ALS and FTLD patients (Arai et al., 2006; Neumann et al., 2006). When such CTFs are transfected in cells, they enhance cytoplasmic accumulation, insolubility, phosphorylation, polyubiquitination and toxicity, recapitulating pathological features of the TDP-43 proteinopathy (Igaz et al., 2009; Zhang et al., 2009). However, phosphorylation of TDP-43 and/or CTFs is not necessary for inducing aggregation and cytotoxicity (Dormann et al., 2009; Zhang et al., 2009). Interestingly, while the full-length TDP-43 is more present in the spinal cord inclusions of ALS and FTLD patients, CTFs seem to preferentially accumulate in affected cortical and hippocampal regions (Igaz et al., 2008). TDP-43 cytoplasmic mislocalization and aggregation and the presence of CTFs were demonstrated also in *in vivo* and *in vitro* experiments after downregulation or loss-of-function condition of the growth factor progranulin, another protein implicated in a specific subset of FTLD cases (Baker et al., 2006; Cruts et al., 2006; Zhang et al., 2007). Finally, in support of its pathogenic role, the 25 kDa CTF was demonstrated to increase during disease progression in transgenic mice expressing mutant TDP-43 (Wils et al., 2010). Another fragment of 35 kDa was found to accumulate in lymphoblastoid cell lines from TDP-43 mutated ALS patients (Kabashi et al., 2008; Rutherford et al., 2008). This soluble TDP-43 fragment is considered non pathogenic and seems to be an alternative isoform produced from a different in-frame translation start-site located downstream of the natural initiation codon (Nishimoto et al., 2010).

Summary

ALS is a complex neurodegenerative disorder and, although multiple mechanisms were found to be involved in the disease process, the exact cause of the selective MN degeneration still remains elusive. To date, increasing evidence supports that altered RNA metabolism contributed to the pathogenesis of a wide spectrum of neurological diseases and, in particular, several RNA-processing genes have been implicated in the pathogenesis of ALS.

In this review we focused our attention on the role of TDP-43 and FUS/TLS, two RBPs that were recently discovered to participate in multiple steps of the RNA processing pathway, in ALS. They are structurally similar proteins and have strongly related biological functions, being involved both in transcription and post-transcriptional regulatory processes of gene expression, such as splicing but also mRNA stabilization and transport, translation and RNA degradation. Although TDP-43 and FUS/TLS are ubiquitously expressed RBPs, these proteins were demonstrated to be involved in the regulation of mRNA fate in neuronal cells, suggesting that their dysfunction may trigger MN death in ALS.

The identification of causative mutations in the genes encoding for this two DNA/RNA binding proteins in ALS patients combined to the observation that TDP-43 and FUS/TLS abnormally aggregate in disease affected tissues emphasizes the role played by RNA metabolism and post-transcriptional regulatory pathways in neurodegeneration and represents an important advance in the understanding of the potential pathogenic clues for this MN disorder. Although disease mechanisms related to TDP-43 and FUS/TLS RBPs are not well-known yet, elucidating the physiological functions of these proteins in the central nervous system and characterizing their intermolecular interactions are crucial steps in clarifying disease pathways. At the same time, since these two nuclear RBPs are mislocalized in the cytoplasm where they form aggregates in ALS affected tissues, their redistribution to the cytoplasm may play a pivotal role in neurodegeneration, resulting in the loss of their proper function in the nucleus (loss-of-function effects) and/or in their potential toxicity in the cytoplasm (gain-of-function effects). The "loss-" versus the "gain-of-function" hypotheses represent another important issue to be addressed.

In the last two years different ALS animal models for TDP-43, including Drosophila (Feiguin et al., 2009; Lu et al., 2009; Hanson et al., 2010; Li et al., 2010), zebrafish (Kabashi et al., 2010), transgenic mice (Wegorzewska et al., 2009; Kraemer et al., 2010; Stallings, 2010; Wils et al., 2010; Xu, 2010)

and rats (Tatom et al., 2009; Zhou et al., 2010) were established. Even though the emerging data opened controversial discussions, such TDP-43 animal models do represent valuable tools in the understanding of the neurodegenerative processes observed in ALS patients and will be of fundamental importance for future drug-development prospects and preclinical approaches.

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