SAPIENZA
Università di Roma
Facoltà di Scienze Matematiche Fisiche e Naturali

DOTTORATO DI RICERCA
IN GENETICA E BIOLOGIA MOLECOLARE

XXVI Ciclo
(A.A. 2012/2013)

Analysis of FUS/TLS involvement in Amyotrophic Lateral Sclerosis

Stefano Dini Modigliani

Docenti guida
Dott.ssa Mariangela Morlando
Prof.ssa Irene Bozzoni

Tutore
Prof.ssa Maria Teresa Carrì

Coordinatore
Prof.ssa Irene Bozzoni

INDEX

GLOSSARY

ALS	Amyotrophic Lateral Sclerosis
cap	7-methyl-guanosine
CTD	Carboxy-terminal domain
dsRNABD	double-stranded RNAs Binding Domain
FALS	Familiar Amyotrophic Lateral Sclerosis
FUS/TLS	Fused in sarcoma/translocated in liposarcoma
hnRNP	heterogeneous nuclear ribonucleoproteins
miRNA	microRNA
MN	motoneuron
MRE	microRNA Response Element
NES	Nuclear Export Signal
NII	Neuronal Intranuclear Inclusions
NLS	Nuclear Localization Signal
NMD	nonsense-mediated mRNA decay
QGSY	Gln-Gly-Ser-Tyr-rich region
RGG	Arg-Gly-Gly-rich motif
RNAPII	RNA polymerase II
RNAPIII	RNA polymerase III
ROS	Reactive oxygen species
RRM	RNA recognition motif
SALS	Sporadic Amyotrophic Lateral Sclerosis
snRNA	small nuclear RNAs
snRNP	small nuclear ribonucleoprotein
SOD1	Superoxide dismutase 1
SR protein	serine/arginine-rich protein
TAF15	TATA-binding protein associated factor 15
TBP	TATA binding protein
TDP43	TAR DNA-binding protein 43
TFIID	Transcription Factor II D
TUTase	Terminal Uridil Transferases
UTR	untranslated region

SUMMARY

FUS is a multifunctional protein involved in almost all step of RNA metabolism, from transcription, to splicing and RNA transport and translation. FUS mutations have been associated to Amyotrophic Lateral Sclerosis (ALS) onset, a lethal neurodegenerative disease that leads to specific degeneration of upper and lower motoneurons. In this research project I demonstrated that FUS is involved in microRNA (miRNAs) biogenesis, a family of small RNAs that participate in post-transcriptional regulation of gene expression by repressing mRNA translation. In particular I demonstrated that FUS is important for the biogenesis of a group of miRNAs, including those with a pivotal role in neuronal differentiation and synaptogenesis. I showed that FUS is able to participate in miRNAs biogenesis facilitating the processing of precursor molecules (pri-miRNAs). Furthermore, I demonstrated that FUS is able to activate two feed-forward regulatory loops important for the maintenance of the correct cellular level of the FUS protein. Increased amount of FUS has been described, indeed, in ALS patients, suggesting that the overdose of FUS becomes toxic for the cellular homeostasis. In particular, a strong increase of FUS protein has been described in ALS patients carrying mutations in the 3'UTR of FUS mRNA. Even though, in this case, the protein is wild type, an ALS phenotype still occurs, and this may be due to the failure of some regulatory mechanisms that control FUS levels. I showed the existence of two mechanisms able to control FUS levels: on one side FUS induces the skipping of the exon 7 of its own pre-mRNA, leading to the formation of an out-of-frame mRNA predicted to be degraded by nonsense-mediated decay; on the other side FUS is able to upregulate miR-141 and miR-200a, which in turn repress FUS synthesis. Therefore when FUS levels increase, these two feed-forward regulatory loops, acting on pre-mRNA splicing and on mRNA translation, are able to restore the physiological levels of FUS. The failure of these mechanisms might contribute to the ALS

pathogenesis, where the uncontrolled increase of FUS results toxic for the cell. Notably, one mutation found in the 3'UTR of FUS in two ALS patients, is localized in the binding site for miR-141 and miR-200a, and I demonstrated that this mutation affects the ability of these miRNAs to target FUS mRNA. So, in these patients, this regulatory process probably fails in controlling FUS protein levels, and this may be one of the mechanisms leading to ALS pathogenesis.

1 – INTRODUCTION

1.1 - Amyotrophic Lateral Sclerosis

Amyotrophic Lateral Sclerosis (ALS) is a devastating late-onset disorder that leads to specific degeneration of upper and lower motor neurons. Loss of these neurons leads to muscle atrophy, weakness, fasciculations and spasticity.
The age of clinical onset of ALS is variable, but the majority of the patients develop the disease after the fourth decade of life; juvenile ALS forms also exist, but are less common (Robberecht *et al*., 2013). Most patients with ALS die within 3 to 5 years after symptoms onset due to respiratory insufficiency, but the variability in clinical disease duration is large and ranges from months to decades (Logroscino *et al*., 2008). Large differences in survival and age of onset are a hallmark of this disease, even between patients carrying the same mutation and belonging to the same family, suggesting the presence of other factors that influence the phenotype (Regal *et al*., 2006). Large differences exist also in the incidence of ALS among different populations. In Caucasians the incidence of ALS is about 2 cases per 100′000 per year and the prevalence is approximately 6–8 per 100′000 (Logroscino *et al*., 2010). Incidence rates increase with age, with a peak between 70 and 80 years, and men are more frequently affected than women.
The first characterization of ALS was performed in 1869 by the French medical doctor Jean-Martin Charcot, who deduced the relationship between the clinical signs and the autopsy findings (Charcot, 1869; Charcot *et al*., 1874). Ten years later, in 1880, the Canadian medical doctor William Osler reported the first familiar form of ALS with autosomal dominant inheritance, described in a family from Vermont, which, more recently, has been shown to have a mutation in SOD1 gene (Osler, 1880; Roulea *et al*., 2007). In 1993 mutations in the gene coding for the superoxide dismutase 1

(SOD1) were associated, for the first time, with the onset of one of the familiar forms of ALS (Rosen *et al*., 1993).
The relevant pathological feature of ALS is the progressive injury and cell death of lower motor neuron groups in the spinal cord and brain stem, and of upper motor neurons in the motor cortex. The disease is clinically characterized by progressive muscle weakness, atrophy and spasticity resulting in the end in complete paralysis of voluntary muscles, leading to death by respiratory insufficiency. Almost all the muscle functions became impaired with the exception of those controlling the bladder, the sphincters and the eye movement, which are affected only in the very late stage of the disease (Kandel *et al*., 1991).
ALS is conventionally classified in two categories depending on the history of the patients. If patients have affected relatives, they are classified as familial ALS (FALS) cases, otherwise they are considered as sporadic ALS (SALS) ones. FALS accounts for 5-10% of all cases and is predominantly autosomal dominant inherited; lots of genes have been identified that are mutated and are responsible for the onset of FALS cases (Verma *et al*., 2013). On the other side the causes of SALS are still not known. Clinically, FALS and SALS are very similar, and this is really encouraging, because the study of the molecular and cellular basis of FALS might provide understanding into the pathogenesis of SALS.
Different environmental risk factors has been suggested as causative of ALS onset, and the most significant associations are with advancing age and exposure to tobacco smoke, but currently there are also other evidences supporting the contribution of other environmental risk factors (Table 1) (Nelson *et al*., 2000; Wijesekera *et al*., 2009)

1.1.1 - Molecular and cellular pathways in ALS

The pathogenesis of ALS is not clear, and the exact mechanism that leads to cell death is currently not known. A lot of evidences described in patients suggest that the neurodegenerative processes occurring in ALS could be the results of a complex interplay between multiple mechanisms. These include genetic factors,

Table 1. Exogenous risk factors implicated in sporadic ALS:

- Age at menopause (females)
- Dietary factors
- Electrical injury
- Family history of non-ALS neurodegenerative disease (Parkinson's or Alzheimer's disease)
- Geographical residence (rural, suburban or urban)
- Gulf war service (Male veterans)
- Maternal age, Number of births (in females) & Birth order, Loss of child
- Occupation
- Physical activity
- Playing football professionally
- Previous poliomyelitis infection
- Race/ethnicity
- Smoking
- Toxin exposure (agricultural chemicals, lead)
- Trauma (e.g. Head injury)
- Years of education

(from Wijesekera *et al.*, 2009)

oxidative stress, excitotoxicity, protein aggregation, damage to critical cellular processes, including axonal transport, and to organelles, such as mitochondria (Shaw, 2005; Figure 1). Due to the specificity of ALS clinical and cellular features, and due to the large spectrum of genetic/risk factors and pathway altered in ALS, it has been proposed that all these different elements lead to ALS pathology through a downstream common pathway yet undiscovered. Nevertheless the description and the characterization of all the altered regulatory networks in ALS might help its identification.

Oxidative stress and mitochondrial dysfunction. Oxidative stress arises when the levels of Reactive Oxygen Species (ROS) exceed the amounts required for normal redox signaling. Notably high levels of ROS have been detected in the cerebrospinal fluid and in

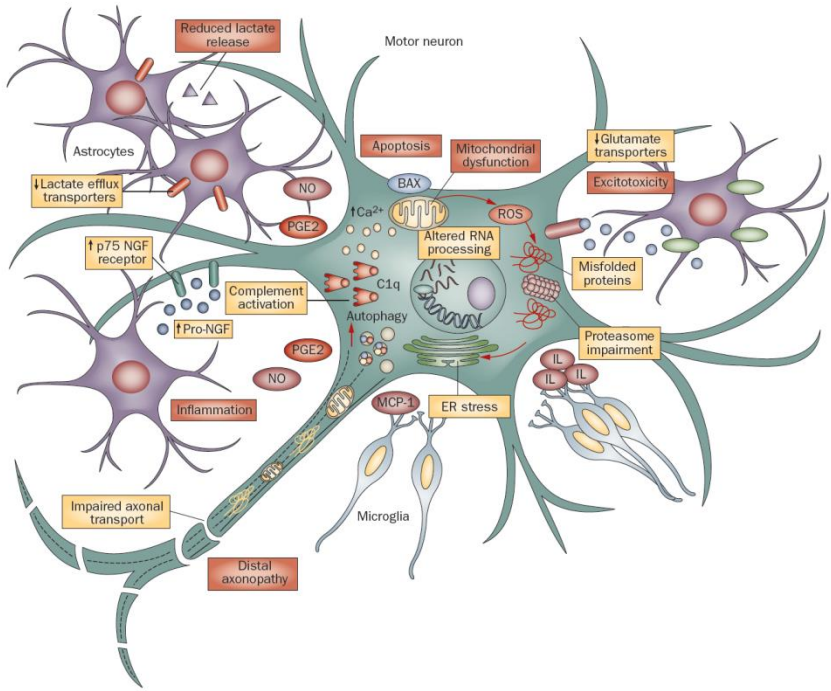


Figure 1. Molecular and cellular pathways in Amyotrophic Lateral Sclerosis. Schematic representation of ALS-associated pathway. ALS is a complex disease involving activation of several cellular pathways in motor neurons, and deregulated interaction with neighboring glial cells (Ferraiuolo *et al.*, 2011).

the spinal cord of SALS patients (Tohgi *et al*., 1999). ROS cause permanent oxidative damage to major cellular components such as proteins, DNA, lipids, and cell membranes (Bogdanov *et al*., 2000; Girotti 1998; Shaw *et al*., 1995). The oxidative stress has been particularly studied in ALS, also because in about 20% of FALS cases the causative mutation resides in the SOD1 gene, which encodes for a cellular antioxidant defense protein.

Even though increased levels of markers of oxidative stress have been consistently observed in ALS, the origin of oxidative stress and the exact role of ROS in disease processes are still not clear (Barber *et al*., 2010). It has been proposed that oxidative stress in

ALS may be caused by a misbalanced metabolism of iron (Carrì *et al*., 2003; Jeong *et al*., 2009). Neurons are non-dividing cells, and the effects of oxidative stress may be cumulative, and injury by free radical species is one of the causes of the age related deterioration in neuronal function that occurs in neurodegenerative diseases.
Age related deterioration of mitochondrial function is considered a potentially important factor that contributes to late onset of neurodegenerative diseases. There are a lot of evidences, from ALS patients and animal models, indicating mitochondrial function failure as a central issue in ALS pathogenesis. Mitochondria are both the major site of ROS formation and, at the same time, are particularly susceptible to oxidative stress. Therefore, when mitochondria are damaged, they release ROS, which, in turn, are able to induce additional mitochondrial damage (Lin *et al*., 2006).
When mitochondria are damaged in ALS, they have altered morphology and functions, and membrane permeability, leading to elevated calcium levels and decreased activity of respiratory chain complexes I and IV, implicating defective energy metabolism. The increased concentration of calcium in the cell induces the activity of several enzymes that generate toxic ROS, amplifying the ROS mediated toxicity (Adam-Vizi *et al*., 2010).

Protein aggregation and impairment of axonal transport. Aggregates of disease-linked mutant proteins are hallmarks of neurodegenerative diseases and, in particular, of ALS. These aggregates are frequently found in spinal motoneurons of all types of ALS patients and disturb normal protein homeostasis inducing cellular stress (Bendotti *et al*., 2013). They interfere with different cellular functions, including mitochondrial function and intracellular transport, and the derived stress leads to axonal retraction and cell death. These aggregates found in ALS patients contain ubiquitinated inclusions of many different proteins, some of which may have a known intrinsic tendency to aggregate (SOD1, TDP43, FUS, and OPTN). Interestingly, FUS and TDP43 ALS-associated mutations enhance the rate of aggregation of these proteins (Johnson *et al*., 2009; Sun *et al*., 2011).
Protein aggregates result toxic for motoneurons, because they may trap proteins with important functions for the cell, and, on the other

side, these aggregates may impair the axonal transport causing mechanical impedance. In SOD1 mutant mice, defects in axonal transport are well documented, and this defect clearly precedes ALS symptoms (Bilsland *et al*., 2010).

Excitotoxicity. Excitotoxicity consists on neuronal injury caused by excessive glutamate induced stimulation of the postsynaptic glutamate receptors. This excessive stimulation leads to a massive calcium influx in the cell that cause increased nitric oxide formation and thereby neuronal death (Shaw, 2005). This was one of the first theories proposed for motoneuron degeneration in ALS, based on the evidence that increased levels of glutamate were observed in the cerebrospinal fluid of patients (Rothstein *et al*., 1992). Later, an association between motoneuron degeneration in ALS and the impairment of the astroglial glutamate transporter EAAT2 has been demonstrated, but seems to be a secondary effect rather than a primary one in ALS onset (Bendotti *et al*., 2001).

Contribution of non-neuronal cells and inflammatory dysfunction. Although motoneurons are the main impaired cells in ALS pathogenesis, there is extensive evidence that non-neuronal cells and inflammatory dysfunction contribute to this disease. Glial cells play an important role in the pathology onset.
Recent evidence revealed that, in particular, oligodendrocytes activity contributes to ALS onset. In murine models of ALS, oligodendrocytes degenerate but are constantly replaced; however, these replaced cells appear to be insufficient in terms of metabolic and trophic support, contributing to the motor neuron loss in ALS (Lee *et al*., 2012). Studies of mutant SOD1 mice have shown that motoneuron death in ALS is non-cell autonomous, since other cells contribute to disease onset, as astrocytes and microglia (Ilieva *et al*., 2009). In ALS patients, these cells are progressively activated as the disease progresses, initiating the process known as neuroinflammation. This process has a dual role: on one side, it has a protective role through the modulation of the helpful inflammatory response thus slowing disease progression (Beers *et al*., 2012). On the other side, at later stage, this persistent activation

becomes toxic. In this inflammatory condition, motoneurons continue to release factors that activate microglia, that in turn starts being proinflammatory and neurotoxic, enhancing motoneuron damage (Appel *et al*., 2011). Furthermore, astrocytes expressing mutated SOD1 release factors that are selectively toxic to motoneurons and induce wild type motoneurons degeneration *in vivo* (Papadeas *et al*., 2011).

1.1.2 - Genetics of ALS

A lot of genes have been associated with ALS onset (Table 2). The mutations of these genes cause the onset of the pathology, and the inheritance is almost always autosomal dominant. The first link between an ALS-associated gene and chromosome 21 was discovered in 1991 (Siddique *et al*., 1991). Only two years later the gene associated with FALS was identified as SOD1, coding for a copper-zinc superoxide dismutase (Rosen *et al.*, 1993). Mutations in this gene have been found in 12–23% of FALS (Sabatelli *et al*., 2013). Even though a decrease of dismutase activity in ALS patients was immediately observed (Deng *et al*., 1993), it has been well demonstrated that this lack of dismutase activity is not the primary cause of ALS, since not all the mutations affect normal enzymatic activity. Therefore it has been shown that mutations in SOD1 confer a toxic additional function to the protein, probably linked to the intracellular localization of the mutant enzyme (Carrì *et al*., 2012). Mutant SOD1 causes neurodegeneration by affecting mitochondria homeostasis, neurofilaments and axonal transport, functions of endoplasmic reticulum and proteasome (Pasinelli *et al*., 2006). Since SOD1 identification, many other genes have been linked to ALS disease and they are the cause of about 70-80% of FALS cases (Table 2). In 2006 TDP43 (TAR DNA-binding protein 43) has been identified as the main component of the ubiquitinated aggregates found in some FALS patients (Arai *et al*., 2006; Neumann *et al*., 2006). In these patients a reduction of the amount of TDP43 protein in the nucleus was observed, suggesting that in this patients the pathogenesis of ALS could be due to a loss of

Table 2: Genes mutated in ALS

Mutated gene	Gene locus	Inheritance	Estimated % of FALS
SOD1	21q22.1	Dominant	20%
TDP43	1p36.2	Dominant	1–5%
FUS/TLS	16p11.2	Dominant	1–5%
TAF15	17q11.1–q11.2	Unknown	Unknown
EWSR1	Unknown	Unknown	Unknown
ANG	14q11.2	Dominant	<1%
SETX	9q34	Dominant	Unknown
C9ORF72	9p21.3–p13.3	Dominant	40–50%
ATXN2	12q24	Dominant	<1%
UBQLN2	Xp11	Dominant	<1%
OPTN	10p15–p14	Dominant	<1%
SQSTM1	5q35	Dominant	Unknown
VCP	9p13	Dominant	<1%
CHMP2B	3p11	Dominant	Unknown
FIG4	6q21	Dominant	Unknown
DAO	12q24	Dominant	<1%
VAPB	20q13.3	Dominant	<1%
Peripherin	12q13.12	Sporadic	Unknown
DCTN1	2p13	Dominant	Unknown
NFH	22q12.2	Dominant?	Unknown
PFN1	17p13.2	Dominant	Unknown
Spatacsin	15q21.1	Recessive	Unknown
Alsin	2q33.2	Recessive	<1%
Awaits identification	18q21	Dominant	Unknown
Awaits identification	20ptel–p13	Dominant	Unknown
Awaits identification	15q15.1–q21.1	Recessive	Unknown

function of TDP43 in this cellular compartment (Neumann *et al*., 2006; Van Deerlin *et al*., 2008). TDP-43 is a multifunctional RNA binding protein, manly localized in the nucleus, involved in transcription, RNA splicing and transport. As a consequence of mutations, TDP43 delocalizes in the cytoplasm, and forms stress granule-based aggregates, causing ALS onset (Johnson *et al*., 2009). Another gene particularly interesting for ALS pathogenesis, is FUS/TLS (or FUS). In 2003 ALS was correlated to a locus in chromosome 16 (16q12.1-16q12.2) (Abalkhail *et al*., 2003), and in 2009 were identified a lot of mutations in FUS gene as responsible of ALS onset (Kwiatkowski *et al*., 2009; Vance *et al*., 2009). FUS is mutated in about 4% of patients affected by FALS and about 1% of patients affected by SALS (Thomas *et al*., 2013). Notably FUS is another RNA binding protein, and, as TDP-43, is involved in different aspects of RNA metabolism, from transcription, to splicing and RNA transport. The identification of ALS-causing mutations in TDP43 and FUS leads to the idea that aberrant RNA metabolism contributes to ALS pathogenesis and the findings that a surprising number of proteins linked to ALS are directly or indirectly involved in RNA processing supported this hypothesis.

1.1.3 - Diagnosis and therapies

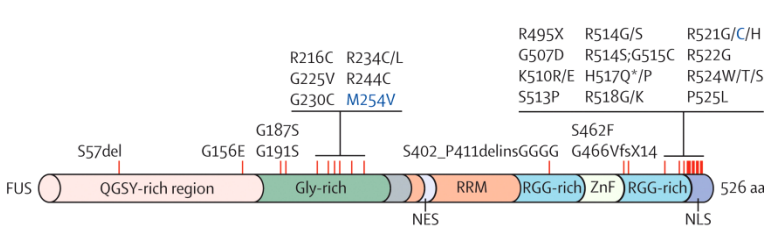
Despite advances in investigative medicine, diagnosis of ALS is based on the presence of very specific clinical features, and is based on examinations necessary also to rule out other diseases with similar symptoms (e.g. Cervical radiculomyelopathy and multifocal motor neuropathy). The diagnosis of ALS is primarily based on the symptoms and signs observed in the patient, through neurologic examination, in order to investigate whether symptoms such as muscle weakness, atrophy of muscles, hyperreflexia and spasticity have a progression compatible with a neurodegenerative disorder. Appropriate tests necessary to correlate the symptoms specifically to ALS must be conducted, like electromyography (EMG), a test that measures nerve conduction velocity (NCV) and brain and spinal magnetic resonance imaging (MRI). The diagnosis, when performed by an experienced clinician, is accurate 95% of the times (Rowland *et al*., 2010).

There is no cure for ALS yet, so the clinical care is finalized in maintaining the quality of life and prolonging life as much as possible. Current therapies are directed to symptomatic relief or replacement of neurotransmitters. However, most of these therapies are not able to reduce the neurodegenerative progression (Katsuno *et al*., 2012). This is due mainly to the lack of knowledge of the exact causative pathway of neurodegeneration, the lack of animal models that are able to exactly recapitulate the human pathogenesis and the lack of knowledge about pre-symptomatic phase. The only drug available for ALS patients is riluzole (6-(trifluoromethoxy) benzothiazol-2-amine), that is able only to extend patients survival of only 2-3 months (Miller *et al.*, 2012). Beyond riluzole, a lot of different drugs have been tested, but no benefits for patients has been demonstrated (Zinman *et al*., 2011).

1.2 - FUS/TLS

The gene FUS/TLS (fused in sarcoma/translocated in liposarcoma, or FUS) encodes for a 526 aminoacid protein, widely expressed in most of the human tissues, and mainly localized in the nucleus, even though cytoplasmic localization has been detected in many cell types (Andersson *et al*., 2008). Moreover, FUS carries out some of its functions by shuttling between the nucleus and the cytoplasm (Zinszner *et al*., 1997). FUS belongs to FET protein family, that includes Ewing's sarcoma protein (EWS) and TATA-binding protein associated factor 15 (TAFII68/TAF15). All proteins belonging to this family have a similar structure characterized by a N-terminal QGSY-rich domain, a highly conserved RNA recognition motif, multiple repetitions of RGG-rich domain implicated in RNA binding and a C-terminal zinc finger motif (Iko *et al*., 2004). In particular FUS is characterized by a Nuclear Localization Signal (NLS) at the extreme C-terminus, and by a N-terminal degenerated and repeated SYGQQS sequence which, when fused to transcriptional factor, functions as a strong transcriptional activator as it happens in liposarcomas and myeloid leukaemia (Prasad *et al*., 1994; Figure 2A).
FUS carries out many functions involved in RNA metabolism (Figure 2B). Different *in vitro* analysis demonstrated that FUS is able to bind both RNA and DNA molecules (Lagier-Tourenne *et al*., 2010) and in particular is able to bind preferentially RNA molecules enriched in the GGUG sequence (Iko *et al*., 2004).
FUS is involved in RNA transcriptional regulation. FUS has been found associated with the transcriptional machinery by direct interaction with RNA polymerase II and the transcription factor II D (TFIID), thus influencing transcription initiation and promoter selection (Bertolotti *et al*., 1998; Yang *et al*., 2000). FUS is also able to interact with specific transcription factors such as NF-Kb e Spi-1 (Uranishi *et al*., 2001; Hallier *et al*., 1998) thus influencing the transcription of the target genes. Recently it has been demonstrated that FUS also represses transcription of RNA Polymerase III (Tan *et al*., 2010).

A



Mutations in the 3'UTR of FUS
48 G>A
59 G>A
108 C>T
110 G>A

B

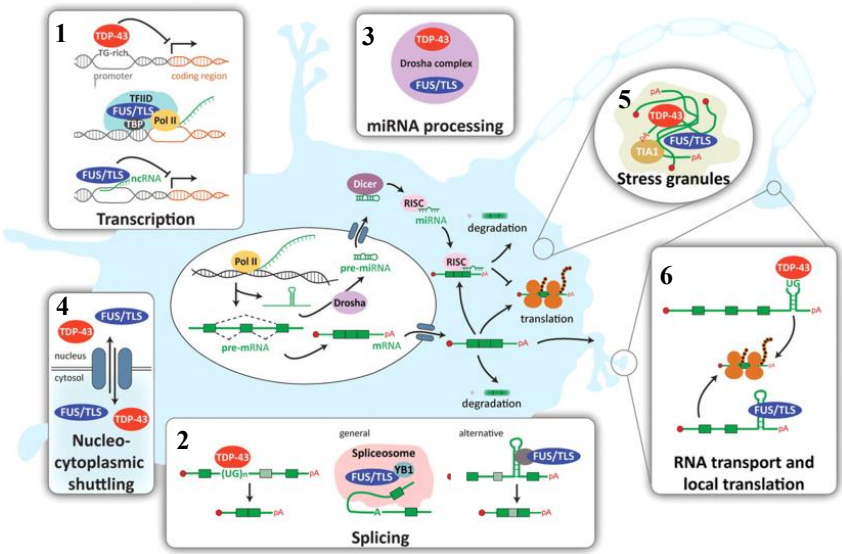


Figure 2. (**A**) Schematic overview of protein domains of FUS and identified gene mutations associated with ALS. NES=nuclear export signal. NLS=nuclear localisation signal. QGSY=Gln-Gly-Ser-Tyr-rich region. RGG=Arg-Gly-Gly-rich motif. RRM=RNA recognition motif (adapted from Mackenzie *et al.*, 2010). (**B**) Proposed physiological roles of FUS/TLS and TDP-43. (1) FUS associates with TBP suggesting its role in transcription. (2) FUS was identified as a part of the spliceosome. (3) Both proteins were found in a complex with Drosha, suggesting their role in miRNA processing. (4) Both TDP-43 and FUS/TLS shuttle between the nucleus and the cytoplasm and (5) are incorporated in stress granules. (6) Both proteins are involved in the transport of mRNAs to dendritic spines (adapted from Lagier-Tourenne *et al.*, 2010).

FUS has an important role also in splicing. It has been demonstrated that it is able to influence the splicing of reporter genes, interacting with several splicing factor (e.g. Yb1, SR proteins, hnRNP A1 and C1/C2) (Lagier-Tourenne *et al*., 2010). Recent experiments of "individual-nucleotide resolution Cross-Linking and ImmunoPrecipitation" (iCLIP) carried out in mouse brain, revealed that FUS associates with the precursor transcripts of mRNA by binding preferentially GGUG enriched sequences and regulating the splicing process (Rogelj *et al*., 2012). Finally, it has been demonstrated that FUS is able to regulate the splicing of RNA binding protein coding genes, including snRNP70, which contains intronic sequences highly conserved that are bound by FUS (Nakaya *et al*., 2013).
In addition FUS is involved in the regulation of nucleo-cytoplasmic shuttling of RNA molecules (Zinszner *et al*., 1996). FUS has been found in RNA transport granules, and is able to bind the Nd1-L transcripts and transport them in spines of mouse ippocampal neurons, where local translation takes place (Fuji *et al*., 2005). Nd1-L encodes for an actin-stabilizing protein, and FUS-null mice neurons display abnormal spine morphology, suggesting that FUS may be involved in spine morphology and in actin reorganization in spines (Fuji *et al*., 2005).

1.2.1 - FUS mutations and ALS: the importance of being regulated

Mutations in FUS gene associated with ALS onset were described for the first time in 2009 (Figure 2A; Kwiatkowski *et al*., 2009; Vance *et al*., 2009). Mutations in FUS account for about 4% of FALS and less than 1% of SALS (Rademakers *et al*., 2010).
Although the majority of patients carrying FUS mutations exhibits a classical ALS phenotype without cognitive impairment, FUS-ALS patients disclose some distinctive characteristics and, depending on the mutation, they show diverse clinical course of the disease. The age of onset is generally earlier than in other SALS and FALS patients, and some patients show juvenile onset and very aggressive course with survival significantly shorter than in other FALS (Sabatelli *et al*., 2013).

The majority of ALS-linked mutations are clustered in the extreme C-terminal region of the FUS protein, and almost all of them are missense changes (Figure 2A). Analysis of the brains and spinal cords of ALS patients with FUS mutations revealed that FUS is mislocalized and accumulated in the cytoplasm. Most of ALS-linked mutations in FUS disrupts the nuclear import and cause the mislocalization of the protein in the cytoplasm, where it associates with stress granules that may form inclusions (Dormann *et al*., 2010). When FUS is mislocalized, and the nucleus lacks the proper amount of this protein, FUS level may not be enough to carry out all its functions. On the other side, the accumulation of FUS in the cytoplasm could be toxic for the cell. The mutated forms of FUS could acquire, indeed, a new, toxic function, both in the nucleus and in the cytoplasm, that could alter cellular homeostasis. FUS knock-out mice on an inbred background display perinatal death and exhibit abnormal lymphocytes and chromosomal instability (Hicks *et al*., 2000), whereas knock-out mice on an outbred background develop male sterility and exhibit increased sensitivity to ionizing irradiation (Kuroda *et al*., 2000). More recently it has been observed that FUS knock-out hippocampal neurons displayed abnormal spine morphology and a reduced number of spines (Fujii *et al*., 2005). Furthermore it has been recently demonstrated that FUS has a crucial role in the DNA damage response and DNA repair. FUS directly interacts with histone deacetylase 1 (HDAC1), and the recruitment of FUS to double-stranded break sites is important for proper DNA damage response signaling (Wang *et al*., 2013). On the other side transgenic mice overexpressing wild-type human FUS develop an aggressive phenotype with an early onset of several pathological features observed in human ALS patients (Mitchell *et al*., 2013).

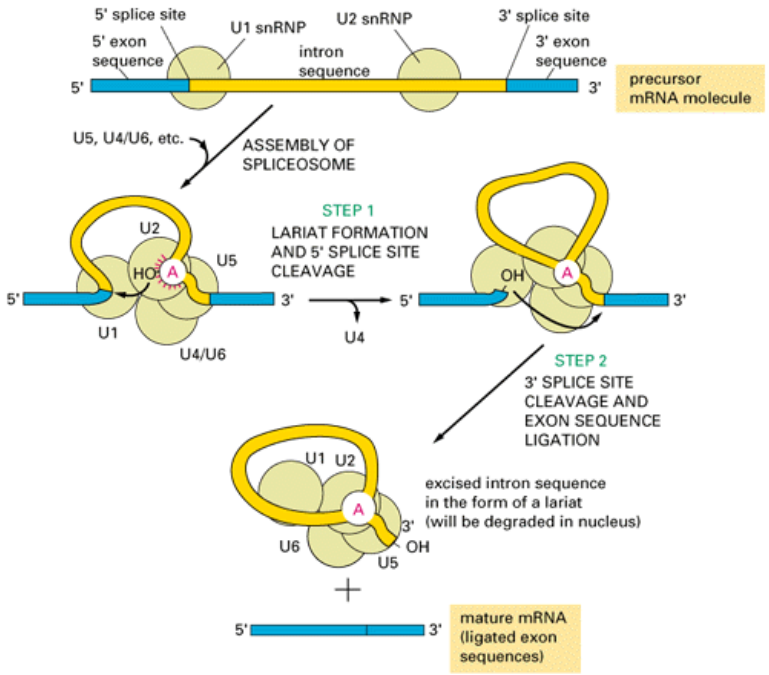
Recently, four different mutations in the 3' untranslated region (3'UTR) of FUS were identified in ALS patients. In fibroblasts coming from these patients, it has been observed a strong increase of the FUS protein and, in particular, a strong accumulation in the cytoplasm (Figure 2A; Sabatelli *et al*., 2013). The 3'UTR is a region of mRNA where different RNA binding proteins or microRNAs (miRNAs) may exert their regulatory role. The

observation that mutations affecting only the regulatory region of the gene, but not the coding sequence, are causative of ALS, underlines the importance of FUS to be finely regulated.
Therefore, when some of the regulatory mechanisms fail, FUS levels may result altered and damages for the cell homeostasis occur with the establishment of an ALS phenotype.

1.3 - Splicing

The expression of protein-coding genes in eukaryotes starts in the nucleus where RNA polymerase II (RNAPII) transcribes genomic coding DNA sequence into a precursor messenger RNA (pre-mRNA). The pre-mRNA must undergo a number of processing steps, highly regulated, to yield a mature and functional messenger RNA (mRNA), which is then ready to be exported and used by the translational machinery in the cytoplasm (Maniatis *et al*., 2002). In higher eukaryotes, most protein-coding genes contain long sequences (named introns) that are transcribed in the pre-mRNA and must be removed in a process called splicing, leaving the protein coding sequences (exons) appropriately aligned and jointed together into the mRNA.
Splicing consists of two *trans*-esterification reactions that are driven by the spliceosome, a large ribonucleoprotein complex containing five small nuclear RNAs (snRNAs; U1, U2, U4, U5, and U6) and approximately 200 additional proteins (Montes *et al.*, 2012) (Figure 3A). Spliceosome assembly occurs in an ordered step-wise manner and leads to the identification of the *cis*-sequence elements that define the exon-intron boundaries (the 5′ and 3′ splice sites) and the associated 3′ sequences for intron excision (the polypyrimidine tract, and the branch point sequence). The two *trans*-esterification reactions are carried out with the involvement of five small nuclear ribonucleoprotein (snRNPs), and a lot of different proteins, catalyzing the intron excision and exon–exon ligation reactions. Exons can be excluded or included in the final mRNA molecule, depending on regulatory *cis* elements and the protein factors that can be involved, leading to the formation of different mature mRNAs.
Alternative splicing, the event by which the same pre-mRNA can be spliced in different ways leading to the formation of different mature mRNAs, contributes significantly to the diversity of the cell and to the tissue specific protein expression profiles (Maniatis *et al*., 2002) (Figure 3B). Through alternative splicing, one single gene may give rise to many different protein isoforms, often with distinct

A



B

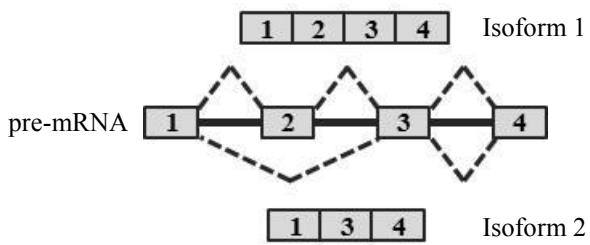


Figure 3. Pre-mRNA splicing. (**A**) Upper panel: schematic representation of a metazoan pre-mRNA, with two exons at the 5' and 3' ends, separated by an intron. Lower panel: schematic representation of the two *trans*-esterification reactions driven by the spliceosome that lead to the formation of the mature mRNA. (**B**) Schematic representation of an alternative splicing event. From the same pre-mRNA, through the inclusion of different exons in the mature mRNA, can be produced different isoforms, with different function.

functions. From deep-sequencing based expression analyses, emerged that more than 90% of multi-exonic human genes undergo alternative splicing (Wang *et al*., 2008). In *Homo Sapiens*, the brain shows the highest level of alternative spliced genes, with more than 40% of genes being alternatively spliced (Yeo *et al*., 2004).
Interestingly, the suboptimal arrangement and poor conservation of the *cis*-elements are an essential property of eukaryotes, in which the sequences are sensitive to the combinatorial regulation exerted by different splicing factors, that in different conditions can influence the alternative splicing events (Izquierdo *et al*., 2006).
There are other *cis*-elements that are able to regulate alternative splicing, known as exonic and intronic splicing silencers or enhancers (ISS, ISE, ESS, and ESE). These elements are recognized by specific RNA-binding proteins that include heterogeneous nuclear ribonucleoproteins (hnRNPs) and serine/arginine-rich (SR) proteins (Wu *et al*., 1993). These are positive and negative regulators of splicing that, in combination with a lot of additional auxiliary regulators, produce a huge number of potentially different combinations of elements that vary among different tissues and distinct homeostatic environments (Lin *et al*., 2007).
It is widely accepted that transcription and splicing are physically and functionally related and they can be reciprocally influenced. When RNAPII starts transcribing, the carboxy-terminal domain (CTD) and transcription elongation factors play central roles in recruiting splicing factors on pre-mRNA (Maniatis *et al*., 2002). This coupling implies that splicing occurs co-transcriptionally, with only few exceptions in which this process can also be post-transcriptional. The transcriptional rate can influence pre-mRNA splicing. Subcanonical splicing sequences are more efficiently recognized by splicing machinery when RNAPII is slow, while when RNAPII is transcribing fast, canonical splicing sequences are the most easily recognised by the splicing machinery (Kornblihtt *et al*., 2004).

1.3.1 - Splicing and disease

Most of the hereditary diseases are caused by point mutations, and more than 80% of them are reported as missense or nonsense

mutations. Notably, about 50% of the mutations results in aberrant splicing (Lopez-Bigas *et al*., 2005). Alternative spicing, or aberrant splicing lead to deletions or substitutions of protein domains, to frameshift, or to premature stop codon formation. When mutations lead to the formation of a truncated isoform of a protein, this could be pathological for two reasons: on one side, there is no more production of the full-length protein; on the other side, this smaller isoform could have a new dominant-negative function, pathogenic for the cell. Alternative spicing, or aberrant splicing, can also lead to exon skipping, causing a frameshift and inducing the formation of a premature stop codon. Transcripts like this are then recognized by the cell as non-functional, so they are degraded by nonsense-mediated mRNA decay (NMD), reducing the amount of the full-length transcript, and so the amount of the protein in the cell.
Splicing can be altered by both mutations in *cis* elements, and alterations in *trans* factors involved in this processes. One example regards CTRF gene, whose mutations are associated with cystic fibrosis. About 25% of synonymous variants resulted in altered splicing inducing exon 12 skipping (Pagani *et al*., 2005). So even without changing the coding properties of a gene, point mutations can alter the correct splicing leading to the alteration of the functionality of the final protein product and then to pathology onset.

1.3.2 – FUS, splicing and ALS

Recent experiments of "high-throughput sequencing of immunoprecipitated and cross-linked RNA" (HITS–CLIP) revealed that FUS is one of the proteins involved in splicing regulation. FUS is able to regulate the splicing process of a lot of coding genes, and FUS knockdown altered the expression of more than 1000 exons in the mouse neuronal transcriptome (Nakaya *et al*., 2013). Interestingly, among the gene regulated by FUS, there are a lot of genes coding for RNA-binding proteins (RBP) and carrying highly conserved introns (Nakaya *et al*., 2013). The observation that FUS participates in an extensive network of cross-regulation of other RBPs by targeting their conserved introns, suggest that perturbations of FUS in ALS may lead to changes in the

transcriptome, as a result of direct effects of FUS on bound transcripts and of secondary effects through other RBPs regulated by FUS.
Notably, FUS is also able to bind its own pre-mRNA, in particular FUS is able to bind the 3'UTR and the region between exon 6 and exon 8 (Lagier-Tourenne *et al*., 2012). This region is particularly conserved and this is an unusual feature for intronic sequences, suggesting that this region may be involved in some regulatory events. FUS intracellular levels must be extremely regulated, and these evidence comes from the observations that increased amounts of FUS can lead to ALS pathogenesis (Sabatelli *et al.*, 2013). Moreover the overexpression of *wild type* FUS in mice causes the development of an aggressive phenotype with pathological features observed in human ALS patients (Mitchell *et al*., 2013). Notably, the exogenous overexpression of FUS in these mice induced a decrease of the endogenous FUS protein. These observations suggest that FUS could be involved in some autoregulatory mechanisms acting on its own pre-mRNA. So, it would be really interesting to elucidate in which way FUS can regulate itself, in order to understand which aspects of this regulatory mechanisms are altered in ALS patients.

1.4 - MicroRNAs

MicroRNAs (miRNAs) are a large class of small (about 18-23 nucleotides) non-coding RNAs involved in a variety of biological processes by regulating gene expression at the post-transcriptional level. The first miRNA identified is lin-4, discovered 20 years ago during the study of *C. elegans* larval development (Lee *et al*., 1993). Lin-4 gene was demonstrated to give rise to a small RNA of 22 nucleotides in length able to interact with the 3′-UTR of the lin-14 mRNA, leading the repression of lin-14 expression (Wightman *et al*., 1993). It took other 7 years to discover the second member of this class of RNAs, let-7 (Reinhart *et al*., 2000). Since then, research attention on miRNAs studies has increased exponentially in the years, leading the discovery that miRNAs are conserved among organisms (Pasquinelli *et al*., 2000) and are present in almost all eukaryotes (Bartel *et al*., 2004). In the human genome 1872 miRNA genes have been identified (as categorized by the release 20 of miRBase; Kozomara *et al*., 2013). MiRNAs are expressed in all human tissues, and lots of them are tissue specific and participate in the regulation of pivotal biological processes, like development, differentiation and stimuli response. It has been estimated that one miRNA is able to recognise and target up to hundreds of mRNAs, and, on the other side, a mRNA could be potentially regulated by different miRNAs (Brennecke *et al*., 2005). Bioinformatic analysis predicted that about 60% of protein-coding genes can be post-transcriptionally regulated by miRNAs (Friedman *et al*., 2009). These assessments underline the importance of the regulatory network in which miRNAs are involved and the importance of increasing our knowledge on miRNA field to better understand the regulatory mechanisms that are important for the cellular metabolism.

1.4.1 - miRNA biogenesis

miRNA biogenesis starts in the nucleus where RNAPII, or, in particular situations, RNAPIII, transcribe genes coding for miRNAs leading the formation of long precursor transcripts named primary miRNAs (pri-miRNAs) (Figure 4). Pri-miRNAs can contain one or

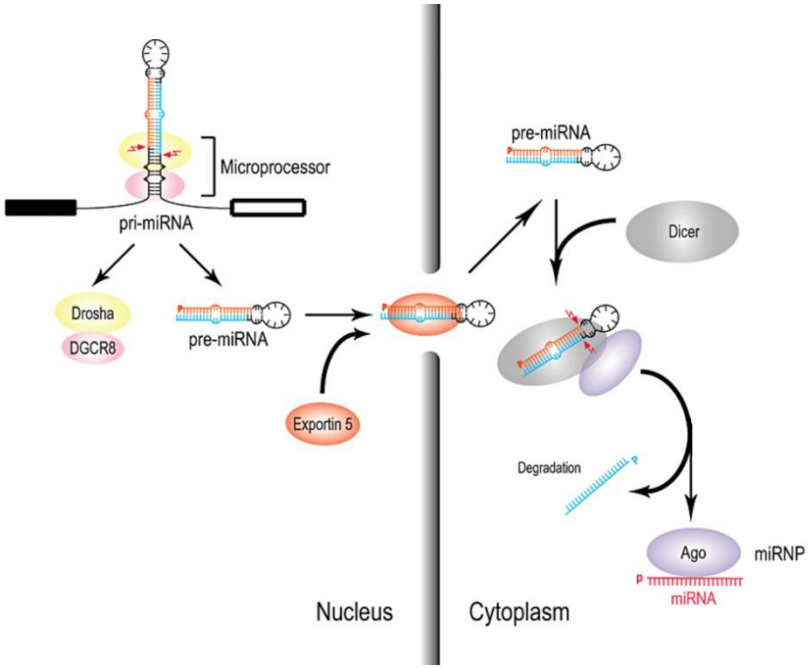


Figure 4. microRNA biogenesis. After transcription by RNA Polimerase II or III, the pri-miRNA is first cropped by the microprocessor into a ~70 nt hairpin pre-miRNA. The core components of the microprocessor are Drosha and DGCR8. The pre-miRNA is then exported by RanGTP and Exportin-5 to the cytoplasm. In the cytoplasm, another RNAse III endonuclease, Dicer, is responsible for dicing pre-miRNAs into short RNA duplexes termed miRNA duplexes. After Dicer processing, the miRNA duplex is unwound and the mature miRNA binds to an Argonaute (Ago) protein, while the other molecule is degraded. The miRNA/Ago ribonucleoprotein that is formed represents the core component of the effector complexes that mediate miRNA function and is known as miRNP (adapted from Liu *et al*., 2007).

several miRNAs embedded in characteristic stem loop hairpin structures. Many miRNA-coding sequences overlap with annotated genes for coding or non-coding RNAs, which are referred to as "host" genes. However, pri-miRNA sequences are not extensively characterized and when is localized within or near a known gene, it is often assumed that transcription of the host gene produces a transcript that ultimately gives rise to the miRNA. However, often occurs also that this miRNA has its own promoter, suggesting that it is independently transcribed and regulated regardless of the "host" gene.

In the canonical pathway, a pri-miRNA is cleaved by the Microprocessor complex, composed mainly of Drosha and its RNA-binding protein partner, DGCR8. The cleavage occurs often while the pri-miRNA is still associated with the chromatin (Morlando *et al*., 2008; Ballarino *et al.*, 2009). Drosha is a 160Kda protein belonging to the RNAse III family, with two RNAse III domains and a domain able to bind double-stranded RNAs molecules (dsRBD). The cleavage catalysed by Drosha leads to the formation of a 2-nt 3' overhang that is necessary for the recognition by the other factor involved in the following biogenesis process (Lee *et al*., 2003). DGCR8 is an essential cofactor that is able to recognise the pri-miRNAs, in particular it binds the junction between the ssRNA and the dsRNA of the stem-loop structure, recruiting Drosha to cleave the stem loop 11 nucleotides away from the ssRNA-dsRNA junction (Han *et al*., 2006). The Microprocessor activity leads to the formation of a precursor molecule of about 70 nucleotides, named pre-miRNA, that maintains the typical stem-loop structure (Mourelatos *et al*., 2002) and that is exported to the cytoplasm by Exportin-5 (Yi *et al*., 2003).

For miRNA genes localized in the intron of a host gene, the pri-miRNAs sequences are processed co-transcriptionally, when the host gene transcript is still not spliced (Kim *et al*., 2007). This is possible because the Microprocessor acts co-transcriptionally without altering the splicing of the host-gene (Morlando *et al*., 2008). Therefore, from the same transcript both the mRNA and the miRNA molecules can be generated.

In the cytoplasm the pre-miRNA is processed by Dicer, another endonuclease that belongs to the RNAse III family (Bernstein *et al*.,

2001). Dicer recognizes the 2-nt 3' overhang produced by Drosha cleavage, and with its two endonucleolytic domains Dicer cleaves the hairpin structure at the base of the apical loop, leading to the formation of another 2-nt 3' overhang and producing a miRNA duplex intermediate of about 22 base pairs (Zhang *et al*., 2004). These RNA duplexes are then separated and only one of the strands is selected as the mature miRNA, while the other is rapidly degraded. Mature miRNAs are incorporated into Argonaute 2 (Ago2) protein-containing effector complex, known as miRNP (miRNA-containing ribonucleoprotein complex). Ago2 is composed mainly of a PAZ domain, responsible for the binding of the 3'end of mature miRNAs, a MID domain, responsible for the binding of the 5'end, and PIWI domain with endonucleolytic activity (Mourelatos *et al*., 2002). MiRNPs contain also GW182, a protein rich in glycine and tryptophan aminoacids important for the recruitment of a large amount of factors involved in mRNA silencing (Chekulaeva *et al*., 2011).

1.4.2 - miRNA mechanisms of action

In mammals, miRNA sequences guide miRNP to the target mRNA by imperfect complementary to the miRNA response element (MRE), leading to translational repression and/or accelerated mRNA decay (Liu *et al.*, 2007). The sequences recognized in the mRNAs are almost always localized in the 3'UTR (Filipowicz *et al*., 2005). The only portion of the miRNA that generally binds perfectly to the MRE is localized between nucleotides 2 and 8 and is called "seed". It plays a pivotal role in target recognition (Doench *et al*., 2004), even though the other nucleotides of the miRNA can also influence the binding affinity (Grimson *et al*., 2007). After the interaction between miRNP and mRNA, this complex localizes in specific cytoplasmic regions named Processing bodies (P-bodies) (Liu *et al*., 2005). P-bodies are dynamic structures involved in mRNA degradation or in accumulation of mRNA translationally repressed. The localization of miRNP complexes in P-bodies could prevent ribosomal association thus contributing to translational repression. In particular conditions, repressed mRNAs can be subsequently reactivated and translated again after P-bodies

disassembly, as for instance, in response to oxidative stress (Brengues *et al*., 2005). A lot of effort has been dedicated to the study of the mechanism of action of miRNAs, and different models have been proposed. Protein synthesis is a step-wise process that requires the involvement of a variety of factors. First of all, eukaryotic translation initiation factor 4E (eIF4E) binds the 7-methyl-guanosine (cap) of the 5'end of mRNA. In addition eIF4E binds to the initiation factor 4G, that, in turn, recruits the 40S ribosomal subunit and interacts with poly(A) tail bind protein (PABP) at the 3'end of mRNA. All these interactions lead to the circularization of the mRNA by joining together the 5' and 3' ends of the molecule (Richter *et al*., 2005). A first model theorises that miRNA inhibition may occur at early step of translation in different ways. In *Drosophila* it has been observed that miRNAs are able to inhibit the recruitment of the ribosomal subunits through the action of EIF6, recruited by the miRNP complex (Thermann *et al*., 2007; Chendrimada *et al*., 2007). Furthermore Ago2 could compete with eIF4E for the binding with the cap, inhibiting the formation of the translation initiation complex (Mathonnet *et al*., 2007). Another theory states that miRNAs are able to inhibit mRNA translation either by co-translational degradation of the nascent polypeptide or by premature dissociation of ribosomal complexes after the translation initiation (Petersen *et al*., 2006). Lastly, another model supports the idea that miRNAs induce the deadenylation of the poli(A) tail at the 3' of the mRNA, leading to a destabilization and lastly to a degradation of the mRNA (Bagga *et al*., 2005; Giraldez *et al*., 2006). It is reasonable to assume that different miRNAs perform their function in different ways depending on the target transcripts and the cofactors recruited.

1.4.3 - Regulation of miRNAs biogenesis

The expression of miRNAs themselves is subjected to regulation through a variety of mechanisms that impact every step of their biogenesis. MiRNAs expression levels vary depending on cell type, cellular differentiation stage, and on stimuli that the cell or the tissue receive (Landgraf *et al*., 2007).

The first step that can be regulated, is the transcription of miRNAs. A lot of different transcriptional factors associated to RNAPII are able to modulate miRNA expression. For example, Miogenin and MyoD are able to bind promoter regions of miR-1 and miR-133 inducing their expression during myogenesis (Liu *et al*., 2007). MiRNAs often reside inside other genes, so in these situations the expression of the miRNA could be dependent on the expression of the host gene. Also epigenetic modifications can contribute to the regulation of miRNA expression. For example in T-cell lymphomas, miR-203 chromatin locus is highly methylated if compared with normal T-cells, leading the expression of the miRNA only in the latter situation (Bueno *et al*., 2008).
Recently, a lot of mechanisms that regulate miRNAs expression at post-transcriptional level have been characterized. For example, the factor Smad4 is able to contribute to miR-21 processing. After specific stimuli, Smad4 translocates into the nucleus, where binds to the Microprocessor complex through p68 factor and enhances pri-miR-21 processing (Hata *et al*., 2009). Another miRNA that is post-transcriptionally regulated is miR-18a. The loop sequence of pri-miR-18a is particularly conserved and is able to bind hnRNP A1 protein. This binding causes a relaxation of the structure that leads to a more efficient Drosha processing (Michlewski *et al*., 2008). In this paper the authors performed a bioinformatic analysis from which emerged that 14% of human pri-miRNAs has a conserved loop sequence. The authors suggested that this conservation might have a functional meaning: for an efficient processing of this pri-miRNAs the loop structure may serve as platform for the binding of cofactors that help Drosha processing.
On the other side, one miRNA whose processing is inhibited post-transcriptionally is let-7. It has been demonstrated that LIN-28 protein is able to bind let-7 loop sequence, inhibiting the efficient processing by the Microprocessor (Newman *et al*., 2008; Viswanathan *et al*., 2008). It has been proposed that LIN-28 is also able to inhibit let-7 pre-miRNA processing interfering with Dicer activity (Rybak *et al*., 2008). LIN-28 is, indeed, able to recruit a Terminal Uridil Transferases (TUTase) that adds an Uridin tale at the 3' end of the pre-miRNA. This modification blocks Dicer-

mediate processing and leads the pre-miRNA to degradation (Heo *et al*., 2009).
Recently emerged that also editing events are able to influence miRNA processing. The editing is a process in which RNA sequence is modified by ADAR enzymes, that are able to deaminate adenosines into inosines (Reenan, 2001). For example, two adenosines of miR-151 stem are deaminated by ADAR1. The deamination causes a complete inhibition of the processing mediated by Dicer, with an accumulation of the precursor molecules (Kawahara *et al*., 2007).
From these data is clear that the regulation of the expression of the miRNAs is very complex, and that the study of the factors involved in these regulative mechanisms is particularly interesting.
In 2004 Gregory laboratory has identified a lot of different cofactors associated with Drosha, and among them it has been found the protein FUS (Gregory *et al*., 2004). Since FUS is mutated in about 4% of FALS and less than 1% of SALS, it is really interesting to study whether FUS has a role in miRNA biogenesis and whether in miRNA levels are altered in ALS patients.

1.5 - FUS, miRNAs and neuronal differentiation

In the neuronal system, miRNAs are extremely abundant and regulate pivotal processes like differentiation, synaptogenesis and neuronal plasticity (Kosik *et al*., 2006). The deregulation of these control mechanisms carried out by miRNAs might lead to severe consequences in the cellular metabolism. There are a lot of diseases in which altered levels of miRNA expression have been observed, as in Tourette, in Fragile X syndrome and in brain tumours (Barbato *et al*., 2009). A miRNA particularly interesting for its role in neuronal differentiation is miR-9. miR-9 is able to inhibit translation of REST, a transcriptional repressor that is highly expressed in pluripotent stem cells while it decreases in neural precursors and in neuronal cells. Therefore, miR-9, inhibiting the expression of REST, contributes to a correct differentiation of neuronal cells. In several neurodegenerative diseases, a deregulation of the expression of REST has been described, underlying the importance of REST and the importance of its regulation for a

correct neuronal differentiation program (Packer *et al*., 2008). Due to the ability of FUS to bind DNA and RNA molecules (Lagier-Tourenne *et al.*, 2010), and to participate in the neuronal differentiation and neuronal activity (Cozzolino *et al.*, 2012), it turns out to be very interesting to study if this protein is involved in miRNA biogenesis. Its regulative effects could be both at transcriptional and post-transcriptional levels, and in the latter situation, FUS could act both in miRNA processing and transport.
Altered FUS localization has been observed not only in ALS, but also in other neurodegenerative diseases. In particular, it has been observed that FUS binds to Neuronal intranuclear inclusions (NIIs) in brain of patients with spinocerebellar ataxia type 1, 2, 3, and dentatorubralpallidoluysian atrophy (Doi *et al*., 2010). Furthermore, FUS is the major component of nuclear polyQ aggregates of a cellular model of Huntington disease, and in this context the soluble form of FUS is reduced (Doi *et al*., 2008). The change of FUS to an insoluble form may be a common process among the diseases with the formation of polyQ aggregates and ALS. Thus to understand FUS molecular mechanisms of action could help to understand the pathological phenotypes observed in those diseases in which FUS activity is compromised.

2 - AIMS

FUS is a protein implicated in a wide range of cellular processes, including transcription and mRNA processing. Recently, mutations in FUS gene were reported to be associated with familial forms of Amyotrophic Lateral Sclerosis (FALS) thus increasing the interest in this protein and suggesting a crucial function in neural cells.
FUS shows a predominant nuclear localization even though it is known to shuttle between the nucleus and the cytoplasm; however, ALS-linked mutations lead to predominance of cytoplasmic versus nuclear FUS localization. Even though the exact mechanism by which this protein becomes pathogenic in ALS remains uncertain, many evidences infer that the toxicity of FUS mutants is somehow related to this nucleus/cytoplasmic imbalance.
One interesting observation regarding FUS function was derived from data indicating the Drosha protein as a putative FUS interactor. Since Drosha is an essential component of the microprocessor complex, required for miRNA biogenesis, and its activity may be modulated by regulatory proteins, it has been suggested that FUS may regulate miRNA expression by modulating the activity of this processing enzyme.
In this thesis I analyzed the FUS mode of action in the control of miRNA biogenesis in neuronal cells. Then I aimed to clarify the impact of the mutations of FUS in this biological process and in ALS pathogenesis. miRNAs are small non-coding RNAs that are involved in the post-transcriptional regulation of gene expression, and their deregulation is involved on the onset of many diseases. The study of FUS impact on miRNA biogenesis and of the role of ALS-associated mutations, that could result in altered miRNA production, could provide a possible link between deregulation of miRNA expression and ALS pathogenesis.
On the other side I was interested in another aspect of FUS on ALS pathology: it is well documented that FUS levels are crucial for the cell homeostasis, and that increased amounts of FUS are associated

to ALS onset. More recently new ALS-associated mutations in the FUS gene were discovered in the 3'UTR , which are linked to increased protein levels (Sabatelli *et al.*, 2013). These mutations lead to ALS onset, even if the FUS protein is wild type. In this project I analysed the mechanisms that are involved in the maintenance of the correct cellular levels of FUS. I showed that FUS is able to bind its own pre-mRNA (intron 7), therefore I evaluated the role of FUS in the splicing process. In addition I assessed a potential role of miRNAs in controlling the expression of FUS by binding its 3'UTR. The study of the regulatory mechanisms that control FUS levels is crucial to understand which are the altered pathways that leads to FUS accumulation and ALS onset.

3 - RESULTS

3.1 - The expression of a subset of microRNAs is altered upon FUS knock down

miRNAs are particularly enriched in the nervous system, where they carry out a crucial role in neuronal differentiation, in synaptogenesis and in plasticity (Kosik *et al*., 2006). As a consequence of this, miRNA deregulation can lead to severe consequences in the correct differentiation and functioning of neural cells. For this reason I used the SK-N-BE cell line derived from human neuroblastoma as a model system for the study of FUS and miRNA expression during neuronal differentiation. SK-N-BE cells can be induced to differentiate into neuronal like cells by treatment with all-trans retinoic acid (RA).
Differentiated SK-N-BE cells were utilized to test the effect of FUS downregulation on miRNA expression. Cells treated with control (siScr) and anti-FUS siRNAs (siFUS) were analysed at 6 days after retinoic acid-induced differentiation. At this time point, most of the miRNAs playing a crucial role in neuronal differentiation reach the strongest up-regulation while the N-MYC protein, present only in proliferating cells, is downregulated (Laneve *et al.,* 2007; Figure 5). 6 independent experiments have been carried out and an average reduction of about 75% of FUS protein was obtained (Figure 6A). miRNA expression profiling was performed through high-throughput quantitative real time PCR: out of 377 miRNAs, 166 were deregulated more than 15%, with the majority (90%) being downregulated (Figure 6B). Among these, several miRNAs known to have a crucial role in neuronal function, differentiation and synaptogenesis (miR-9, miR-125b and miR-132; Laneve *et al.,* 2007 and 2010; Packer *et al.,* 2008; Edbauer *et al.,* 2010; Pathania *et al.,* 2012) were found. Notably, the protein levels of the microprocessor major components, Drosha and DGCR8, were

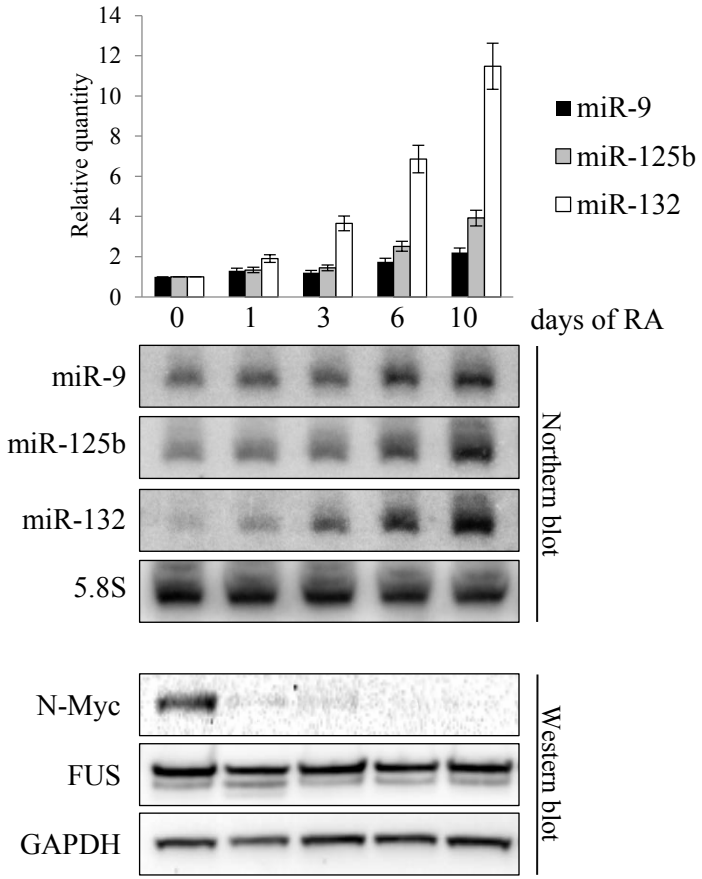


Figure 5. SK-N-BE cells *in vitro* differentiation. SK-N-BE cells were induced to differentiate with retinoic acid (RA) and incubated for the indicated times (0, 1, 3, 6 and 10 days). Upper Panel: miR-9, miR-125b and miR-132 were analyzed by Northern blot using corresponding specific oligonucleotides. 5.8S rRNA was used as internal control. The histogram indicates the relative levels normalized for the 5.8S signal. Lower panel: Western blot analysis of N-Myc and FUS proteins at the same time points. GAPDH was used as internal control.

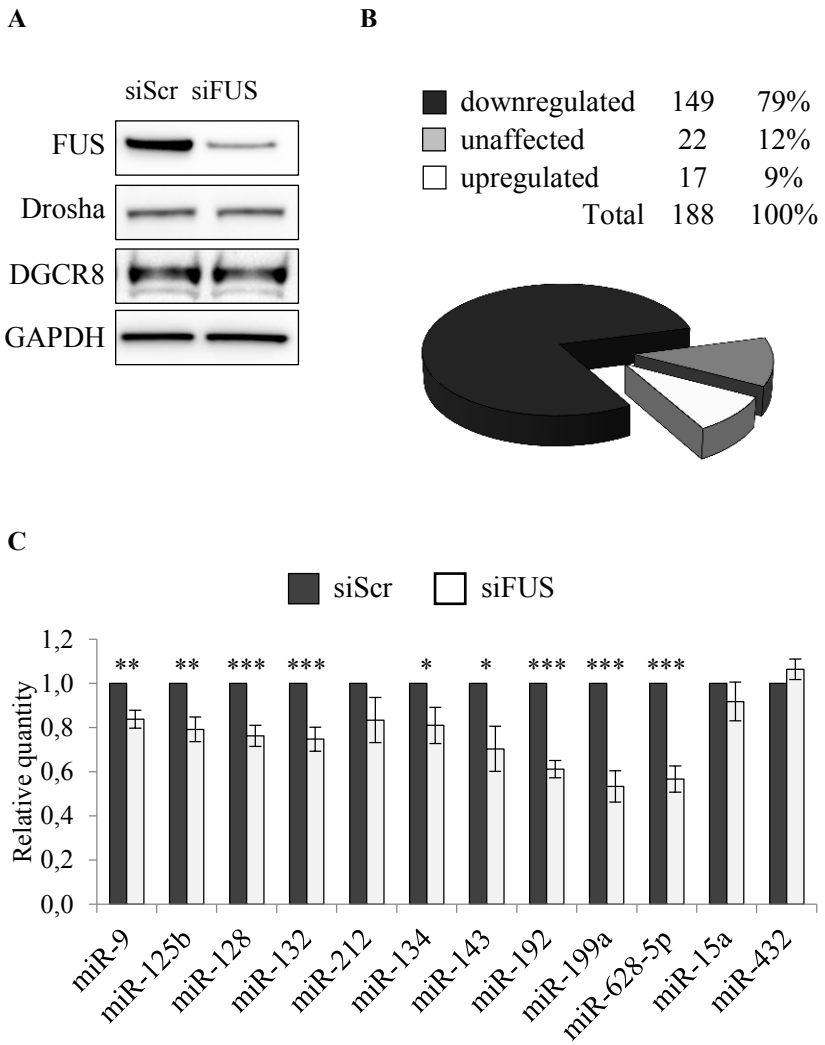


Figure 6. Knockdown of FUS in SK-N-BE cells. (**A**) SK-N-BE cells were treated with anti-FUS siRNA (siFUS) or control siRNA (siScr) and maintained in retinoic acid for 6 days. Levels of FUS, Drosha and DGCR8 were analyzed by Western blot. GAPDH was used as a loading control. (**B**) miRNA profiling in SK-N-BE cells treated with anti-FUS siRNA (siFUS) or with control siRNA (siScr), cultured in RA for 6 days. Pie charts and table show the percentage of miRNA derelegulated more than 15%. (**C**) miRNA levels from the same cells were analyzed by qRT-PCR, and normalized for the snoRNA-U25 internal control. Significance was assessed by Unpaired Student's t-test (*P<0.05, **P<0.01, ***P<0.001).

unaffected upon FUS downregulation (Figure 6A). Figure 6C shows qRT-PCR analysis on a selection of miRNAs derived from 6 independent experiments with similar FUS depletion (70-80%): even if the effect on accumulation was in some case small (18% for miR-9, 20% for miR-125b and 25% for miR-132), the values were very reproducible in the different experiments. Other species, not restricted to neuronal cells, were more affected, such as miR-192, miR-199a and miR-628-5p that decreased to approximately 50% of control value. In contrast, miR-15a and miR-432 levels were unaffected and they have been utilized as controls in the following experiments. Notably, several of the down-regulated miRNAs (such as the neuronal miR-9, miR-125b and miR-132) displayed altered expression even when FUS levels were decreased to only 45% (data not shown), indicating that even half the levels of FUS are sufficient to affect the accumulation of specific miRNAs.
The effects of FUS downregulation were also tested in HeLa cells, where RNAi provided 85% reduction (Figure 7A). Notably high-throughput analysis using a Taqman array real time PCR revealed that in HeLa cells a lower proportion of miRNA species were negatively affected with respect to neuronal cells (Figure 7B). In order to test the accumulation of neuronal specific miRNAs, expression cassettes under the control of the ubiquitous U1 snRNA promoter were produced and individually transfected. Figure 7C indicates that the accumulation of the neuronal-specific miRNAs is affected similarly to neuronal cells and in some cases at a higher level (miR-212 and miR-132). Similarly to SK-N-BE cells, the miR-15a and miR-432 endogenous controls were unaffected. These results indicate that FUS regulates specific miRNA levels independently from their promoters, acting at some post-transcriptional step in miRNA biogenesis.

3.2 - FUS binds specific pri-miRNA transcripts

A lot of factors that influences miRNA biogenesis are able to bind directly to pri-miRNAs molecules. To clarify in which way FUS is able to participate in miRNA biogenesis, I first tested the ability of FUS to bind to specific pri-miRNA molecules.

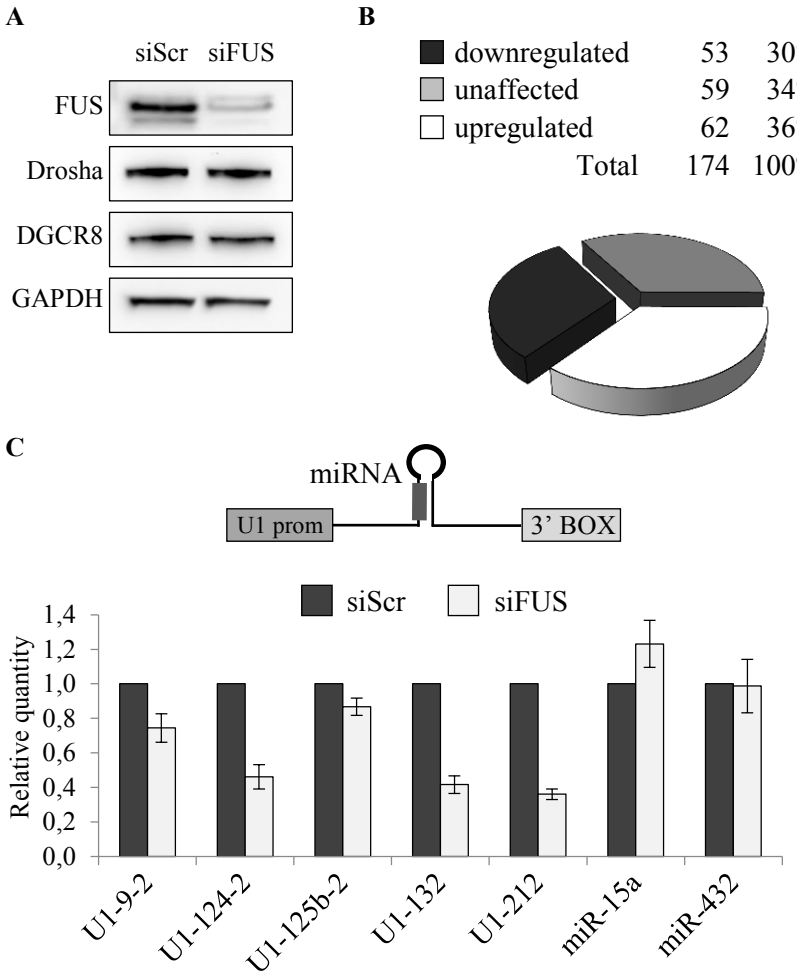


Figure 7. Knockdown of FUS in HeLa cells. (**A**) HeLa cells were treated with anti-FUS siRNA (siFUS) or with control siRNA (siScr). Levels of FUS, Drosha and DGCR8 were analyzed by Western blot. GAPDH was used as a loading control. (**B**) miRNA profiling in HeLa cells treated as in (A). Pie charts and table show the percentage of miRNA derelegulated more than 15%. (**C**) Plasmid constructs carrying different pri-miRNA sequences under the control regions of the U1snRNA gene were transfected in HeLa cells treated as in (A). Expression levels of mature microRNAs were analyzed by Northern blot (miR-9-2, miR-124 and miR-125b-2) or by qRT-PCR (miR-132, miR-212, miR-15a and miR-432). For miR-15a and miR-432 the endogenous levels were measured. Error bars represent s.e.m. from 3 independent experiments.

Binding of a recombinant GST-FUS protein to different labelled pri-miRNAs was tested by band shift analysis *in vitro*. Figure 8A shows that those miRNAs affected by FUS depletion are also able to interact with it, maintaining a considerable amount of binding even in the presence of 250-fold excess of cold tRNA competitor. Notably, the control miR-15a, unaffected by FUS depletion, does not show any specific interaction. The only exception, among the tested miRNAs, was pri-miR-628 that, even if affected by FUS depletion, did not show, in our experimental conditions, any specific binding. Moreover, titration of FUS protein in an *in vitro* binding assay revealed that pri-miR-9-2/FUS interaction is concentration dependent (Figure 8B).
Specificity of binding was also analyzed in extracts of SK-N-BE cells loaded on streptavidin columns pre-bound with *in vitro* transcribed biotinylated pri-miR-9-2 or pri-miR-15a. Figure 8C shows that FUS is strongly enriched in the bound fraction of pri-miR-9-2 at difference with pri-miR-15a.
Previous analyses on several pri-miRNA binding proteins indicated that the highly conserved terminal loops can act as platforms for trans-acting factors (Michlewski *et al.,* 2008 and 2010). In this regard, sequence comparison of the loops of the affected miRNAs did not show any obvious consensus. However, since the miR-9-2 loop contains a GU-rich sequence that was suggested to represent a FUS recognition element (Iko *et al.,* 2004), I tested the effect of its mutation on FUS binding. The three *G* residues of the loop were substituted by *C* nucleotides and the resulting construct (miR-9-2 *mut)* was tested for *in vitro* binding (Figure 9A). Such mutation produced a decrease of 50% in FUS interaction, indicating a consistent contribution of the terminal loop in binding specificity. However, it is possible that the stem provides the remaining binding specificity, as shown by FUS global RNA targets analysis (Hoell *et al.,* 2011). A similar feature was also demonstrated for HnRNP A1 where two binding regions were found: a primary one corresponding to the terminal loop of pri-miR-18a and a secondary site at the bottom of the stem (Michlewski *et al.,* 2008). Moreover, the existence of different apparently disparate binding motifs of FUS has been already observed and suggested to be due to multiple

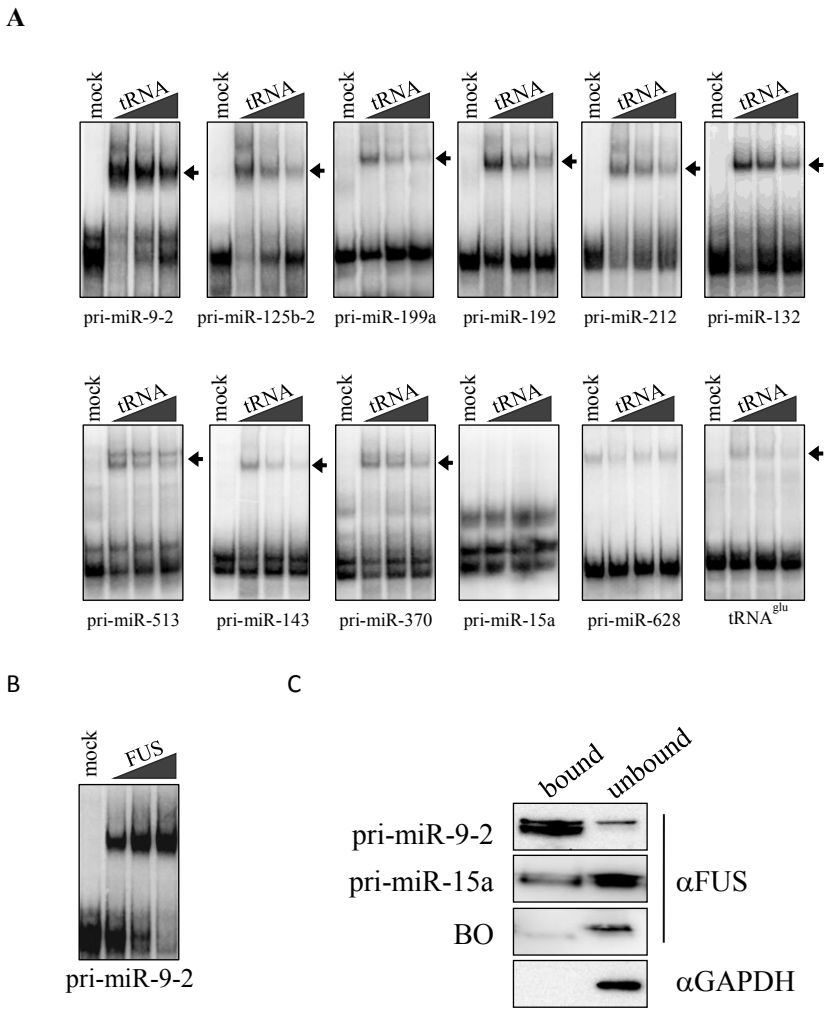


Figure 8. FUS binds *in vitro* specific pri-miRNA transcripts. (**A**) Band shift assays with recombinant GST-FUS using *in vitro* ^{32}P-labelled pri-miRNAs in the presence of increasing amounts of cold tRNA competitor (50, 100 and 250-fold molar excess). Mock samples with the GST peptide were used as control (lanes mock). The arrow points to the specific RNA-protein complex. (**B**) Band shift analysis with increasing amounts of GST-FUS (75, 150 and 300 ng) using pri-miR-9-2. (**C**) Streptavidin-conjugated magnetic beads bound to biotinylated pri-miRNA transcripts were loaded with nuclear extract from SK-N-BE cells. The bound and unbound fractions were tested for FUS binding by Western analysis. GAPDH detection and beads-only (BO) samples were used as negative controls.

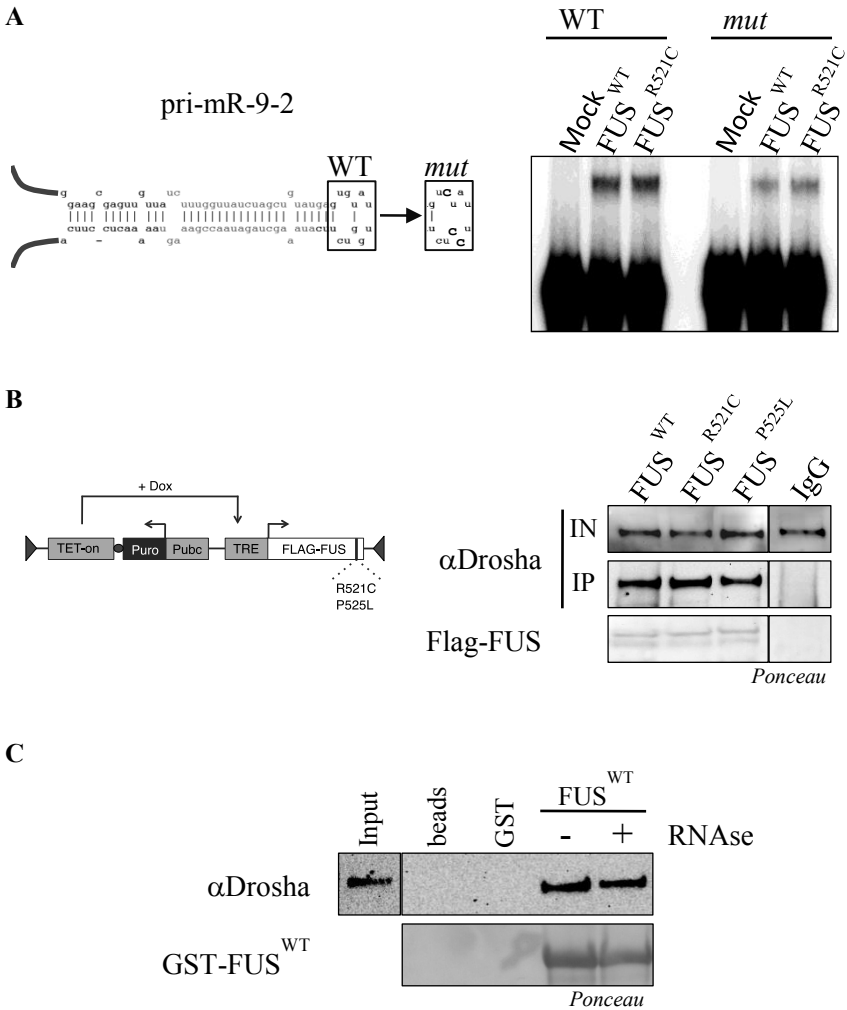


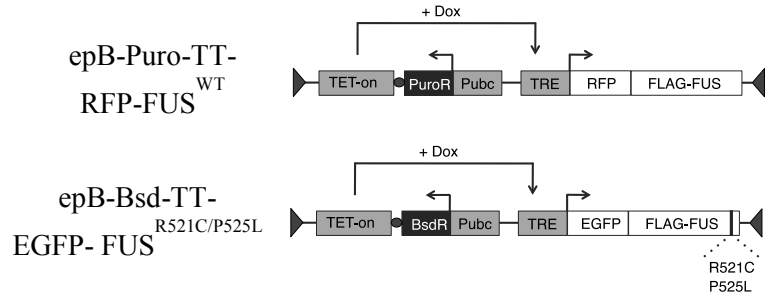
Figure 9. Mutations of FUS do not alter pri-miRNA and Drosha binding. (**A**) Schematic representation of pri-miR-9-2 wild type (WT) and the mutant derivative (*mut*). Right panel: band shift assay with GST peptide (Mock) or with 300 ng of wild type (FUS^{WT}) or mutant (FUS^{R521C}) GST-fusions using *in vitro* ^{32}P-labelled pri-miR-9-2. (**B**) Left panel: schematic representation of the constructs expressing flagged version of FUS wild type and of its mutant derivatives (R521C and P525L). Right panel: nuclear extracts from stable SK-N-BE cell lines expressing flagged FUS were immunoprecipitated and analyzed by Western blot for Drosha interaction (**C**) Pull down of GST-FUS incubated with SK-N-BE nuclear extracts treated (+) or not (-) with RNase A.

distinct nucleic acid-binding domains, which may function independently or in combination (Tan *et al.,* 2012).

3.3 - C-terminal mutations of FUS do not affect either miRNA or Drosha binding

I next tested the RNA binding ability of the FUS^{R521C} mutant derivative, one of the most common mutation linked to the ALS pathology shown to provide a severe phenotype (Belzil *et al.,* 2009; Kwiatkowski *et al.,* 2009). Recombinant GST-FUS^{R521C} was tested for pri-miR-9-2 binding (Figure 9A). Interestingly, this derivative provided the same binding activity of the wild type protein both on pri-miR-9-2 (WT) and on its mutant derivative (*mut*), indicating that the C-terminal region is not involved in miRNA recognition (Figure 9A).
Mutations in the C-terminal region have been described to produce cytoplasmic delocalization of the protein (Chiò *et al.,* 2009; Kwiatkowski *et al.,* 2009; Vance *et al.,* 2009; Dormann *et al.,* 2010). In fact, FUS^{R521C} as well as FUS^{P525L}, another common ALS-associated mutation, were shown to delocalize in the cytoplasm in HeLa transfected cells (Dormann *et al.,* 2010) as well as in post-mortem motor neurons, where they form aggregates (Vance *et al.,* 2009; Kwiatkowski *et al.,* 2009).
In order to test FUS localization in our cellular system, stable clones of SK-N-BE cells, expressing recombinant FUS^{R521C} and FUS^{P525L} fused to the Green Fluorescent Protein under a Doxycycline (Dox) inducible promoter, were generated. Each cell line contained also wild type FUS fused to the Red Fluorescent Protein (see schematic representation in Figure 10A). Figure 10B shows that both mutant proteins display altered cellular localization with respect to the WT form: EGFP-FUS^{P525L}, which corresponds to a very severe and juvenile form of ALS, is highly delocalized in the cytoplasm three days after Dox induction and produces a large number of aggregates. On the contrary, the cytoplasmic delocalization of EGFP-FUS^{R521C}, which is a more common mutation and correlates with an adult form of ALS, is less pronounced than EGFP-FUS^{P525L}. Notably, neither EGFP-FUS^{R521C} nor EGFP-FUS^{P525L} affected the

A



B

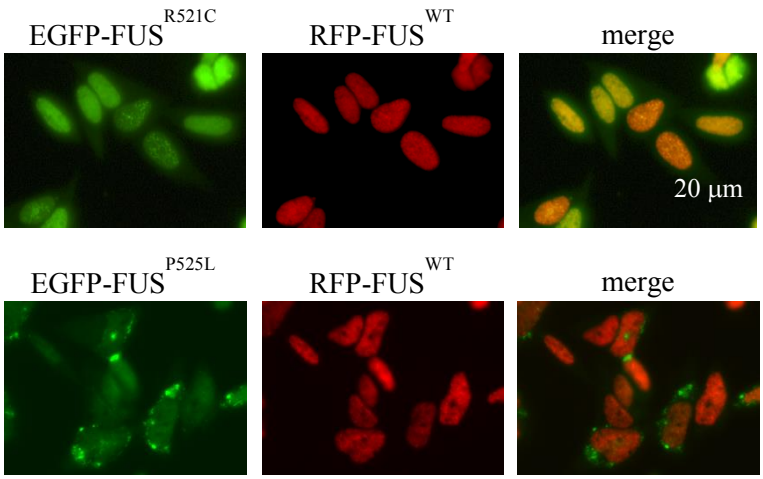


Figure 10. Intracellular localization of wild type and mutated FUS proteins. (**A**) Schematic representation of the epB-Puro-TT-RFP-FUS^{WT}, epB-Bsd-TT-EGFP-FUS^{R521C} and epB-Bsd-TT-EGFP-FUS^{P525L} constructs. Triangles indicate the 5' and 3' Terminal Repeats (TR) of the epiggyBac vector. (**B**) SK-N-BE cells were co-transfected with epB-Puro-TT-RFP-FUS^{wt} and epB-Bsd-TT-EGFP-FUS^{R521C} (top panels) or with epB-Puro-TT-RFP-FUS^{wt} and epB-Bsd-TT-EGFP-FUS^{P525L} (bottom panels), together with a plasmid encoding for the epiggyBac transposase. After selection, stably transfected cells were induced with Doxycyclin for 3 days.

cellular localization of the co-expressed RFP-FUS^{WT}, which remained confined to the nucleus.
Since FUS was described as a Drosha interactor, I next tested the ability of the two FUS mutants (FUS^{R521C} and FUS^{P525L}), to form complexes with Drosha in SK-N-BE cells expressing FLAG-tagged FUS constructs (see schematic representation in Figure 9B). Co-IP experiments indicated that both Flag-FUS^{R521C} and Flag-FUS^{P525L} are complexed with Drosha similarly to the wild type (Figure 9B). To further characterize the binding properties between FUS and Drosha, I performed a GST pull-down assay with or without RNAse treatment. The GST-Pull down assay demonstrated that FUS-Drosha interaction is resistant to RNase treatment (Figure 9C).
These data indicate that the C-terminal mutations of FUS do not affect either miRNA or Drosha binding. This, together with the finding that even 50% depletions of FUS alter miRNA biogenesis, suggests that the cytoplasmic delocalization observed with the FUS^{R521C} and FUS^{P525L} mutants could affect the cellular repertoire of miRNAs by decreasing the levels of the protein available in the nucleus.

3.4 - Exogenous FUS rescues miRNA accumulation in RNAi-FUS treated cells

I next checked to what extent wild type and mutant FUS proteins were able to rescue miRNA biogenesis in RNAi treated cells. SK-N-BE cell lines, carrying integrated copies of wild type or mutant Flag-FUS cDNAs with an unrelated 3'UTR and under the control of Doxycycline (Dox), were utilized. Upon treatment with siRNAs specific for the FUS 3'UTR, efficient depletion of the endogenous FUS protein was observed and, upon Dox induction, exogenous Flag-FUS expression was obtained (Figure 11A).
The experiments shown in Figure 11B indicate that miR-132, miR-9 and miR-192 levels are decreased in cells treated with siRNA against the 3'UTR of FUS in the absence of Dox and are rescued upon activation of the exogenous wild type FUS. The results with the two FUS mutants are consistent with their delocalization

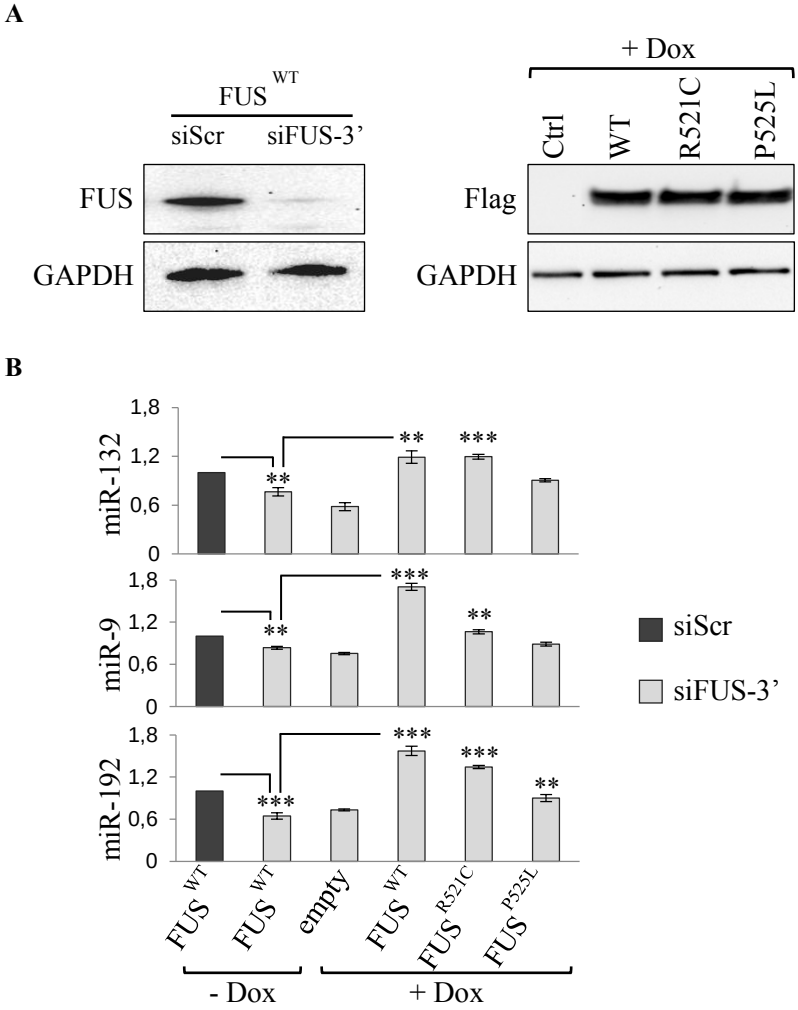


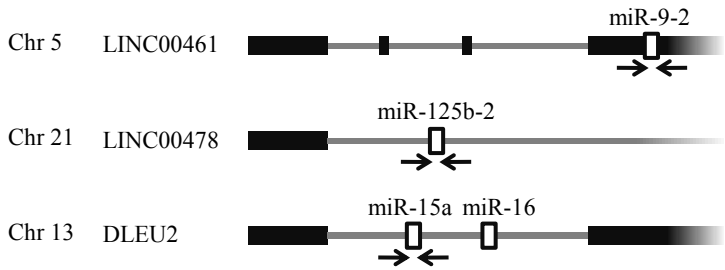
Figure 11. Exogenous FUS can rescue the effects of endogenous FUS depletion. (**A**) Western blot of samples from SK-N-BE cell lines carrying the constructs indicated in Figure 9B or a control construct (Ctrl). Cells were treated with siRNA against the 3'UTR of FUS (siFUS-3') or control siRNA (siScr) for 6 days in retinoic acid and in absence (left panel) or presence (right panel) of Doxycycline. Exogenous FLAG-FUS expression was tested using Flag antibodies while GAPDH was used as control. (**B**) The histogram show the miRNA levels from cells treated as described in (A), analyzed by qRT-PCR. Error bars represent s.e.m. from 3 independent measurements and the significance was assessed by Unpaired Student's t-test (**P<0.01, ***P<0.001).

phenotype: FUS^{R521C}, which displays only a slight cytoplasmic delocalization, is able to rescue miRNAs at levels similar to control, while FUS^{P525L}, which has a stronger delocalization phenotype, has a lower rescue activity. It is important to note that also FUS^{P525L} provides sufficient rescue activity since, due to the overexpression conditions utilized, considerable amount of protein is still present in the nucleus (see Figure 10B).
In conclusion, these experiments demonstrate a direct involvement of FUS on miRNA biogenesis and again indicate a direct correlation with the amount of FUS localized in the nucleus.

3.5 - FUS cooperates with co-transcriptional Drosha recruitment

Since it has been shown that the microprocessor complex acts co-transcriptionally (Morlando *et al.*, 2008; Ballarino *et al.,* 2009), I examined whether FUS is associated with the chromatin and whether it participates in Drosha recruitment. Chromatin immunoprecipitation (ChIP) assays were performed on chromatin from SK-N-BE cells treated with RA for 6 days.
Figure 12 shows that FUS is bound to the chromatin of miR-9-2 and miR-125b-2 coding loci, and that this association is lost after RNase treatment. Therefore, localization of FUS on miRNA chromatin loci is dependent on RNA, consistent with Microprocessor mechanism of action. Upon RNAi-mediated downregulation (Figure 13A), FUS association to the chromatin was consistently reduced (Figure 13B, panels FUS). Moreover, specific association was found on those pri-miRNA loci for which specific FUS binding was identified, whereas very low levels were detected on the pri-miR-15a locus. These findings suggest that chromatin recruitment of FUS at specific miRNA loci occurs during transcription and that it requires binding to nascent pri-miRNAs.
ChIP with Drosha antibodies indicated that this protein was present on all miRNA loci. Upon FUS depletion, even though Drosha cellular levels were unaffected (Figure 13A), its association was reduced on those miRNA loci where FUS-pri-miRNA interaction

A



B

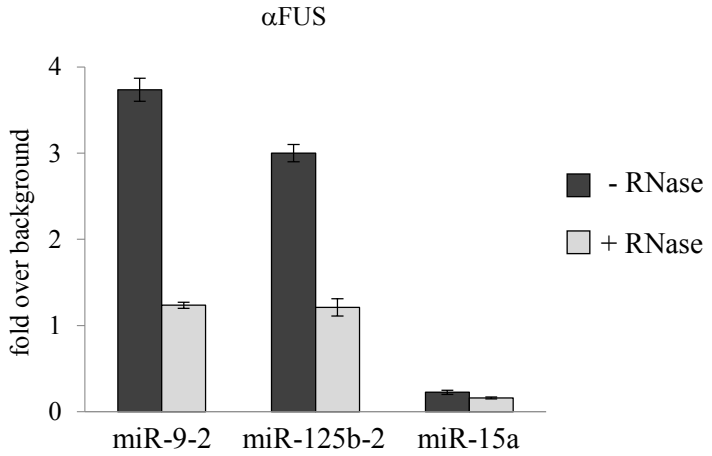


Figure 12. FUS is associated to the chromatin. (**A**) Schematic representation of miR-9-2, miR-125b-2 and miR-15a gene organization. Arrows indicate the positions of the PCR primers used. (**B**) ChIP analysis with anti-FUS antibodies using chromatin of SK-N-BE cells treated with retinoic acid (RA) for 6 days (black bars). Before immunoprecipitation half of the sample was treated with RNase (grey bars). Co-amplifications were carried out with miRNA- and tRNA-specific primers. The histograms show the values of FUS immunoprecipitation on miRNA loci normalized for the tRNA signal and expressed as enrichment over background (IgG). Error bars represent s.e.m. from 3 independent experiments.

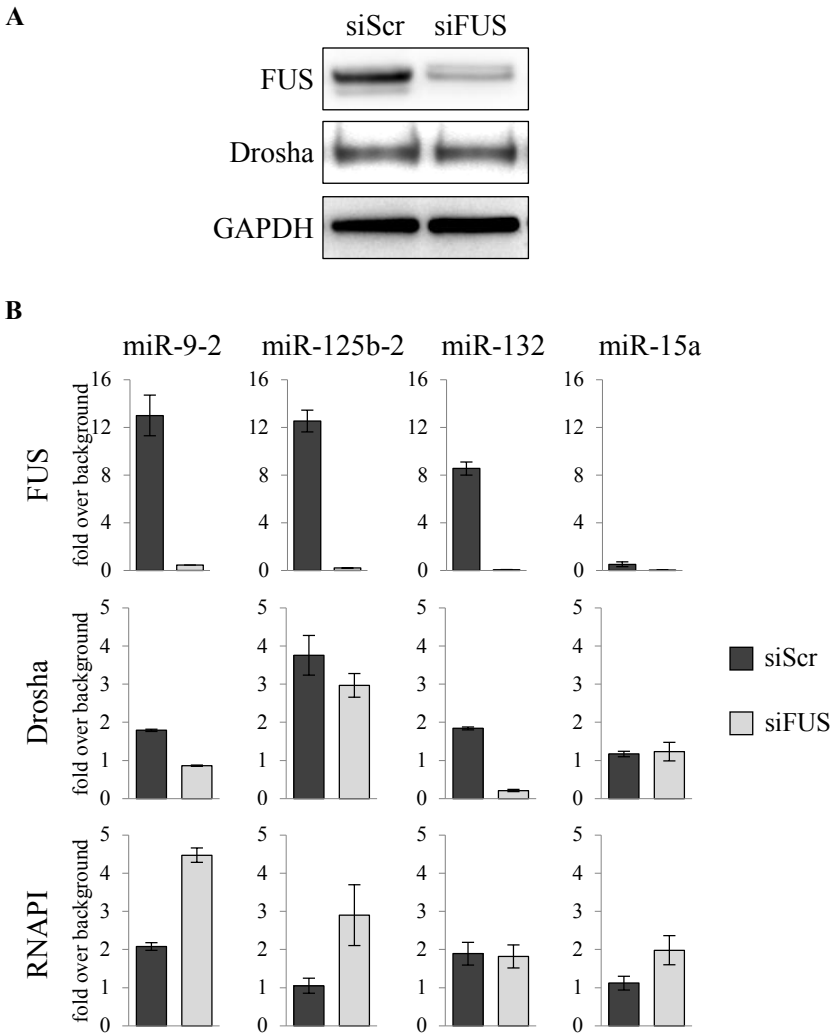


Figure 13. FUS affects co-transcriptional Drosha recruitment. SK-N-BE cells were treated with anti-FUS siRNA (siFUS) or control siRNA (siScr) and maintained in retinoic acid (RA) for 6 days. (**A**) Western blot analysis of FUS, Drosha and GAPDH. (**B**) ChIP analyses with antibodies against FUS, Drosha and Pol II. Co-amplifications were carried out with miRNA- (miR-9-2, miR-125b-2, miR-132 and miR-15a) and chromosome IV intergenic region-specific primers. The histograms show the IP values on miRNA loci normalized for the intergenic region and expressed as enrichment over background signals (IgG). Error bars represent s.e.m. from 3 independent experiments.

was found (Figure 13B, panels Drosha). Indees, Drosha recruitment was not affected in the case of miR-15a that neither binds FUS nor is affected by its depletion. The decrease of Drosha recruitment on FUS-dependent miRNA loci was not due to defects in transcription since no decrease in RNA polymerase II loading was detected (Figure 13B, panels RNAPII). Instead, a slight increase in RNA polymerase II recruitment was observed upon FUS depletion for both miR-9-2 and miR125b-2. In consideration of previous data on FUS affecting transcription, with both positive and negative effects (Wang *et al.,* 2008; Tan *et al.,* 2012), it cannot be excluded that the alterations of RNA polymerase II loading upon FUS depletion on miRNA loci could be due to a secondary effect of FUS on transcription elongation or polymerase release and recycling.
These data allowed me to conclude that FUS interaction is required for efficient recruitment of Drosha at specific pri-miRNA loci at early stages of transcription. These data, together with the observation that the FUS-Drosha interaction does not require RNA, allow me to suggest that the binding of FUS to nascent pri-miRNA molecules cooperates with efficient subsequent Drosha recruitment at the same sites (Figure 14).

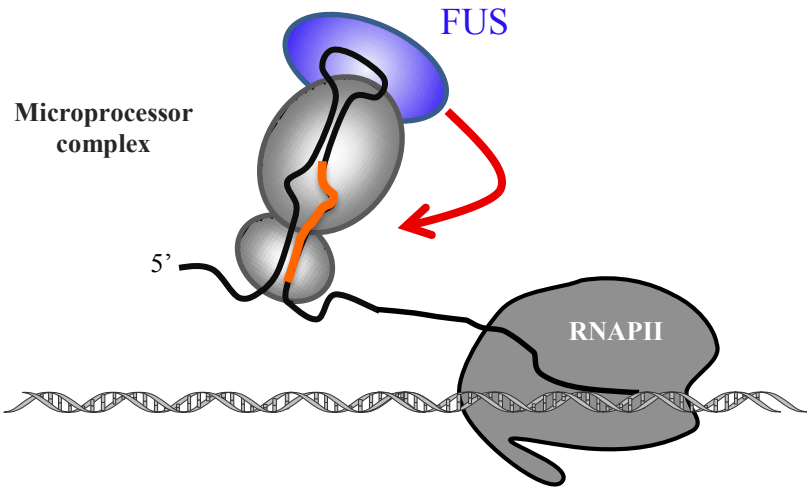


Figure 14. Model of FUS role in miRNAs biogenesis. My data allowed me to propose a model in which FUS participate in miRNA biogenesis by facilitating an efficient recruitment of Drosha at specific pri-miRNA sites. In particular FUS is able to recognize pri-miRNA transcripts when they are still associated with the chromatin at early stages of transcription. Since FUS-Drosha interaction does not require RNA, I suggest that the binding of FUS to nascent pri-miRNA molecules cooperates with efficient subsequent Drosha recruitment at the same sites.

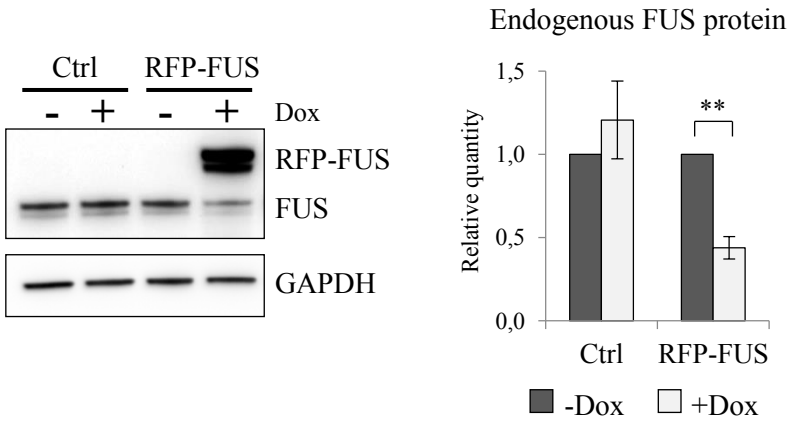
3.6 - FUS overexpression induces downregulation of the endogenous protein.

Increased amount of FUS protein has been described in a class of ALS patients, and this increase seems to be toxic for the cell homeostasis. Indeed, also the overexpression of human wild type FUS protein in mice causes the development of an aggressive phenotype with pathological features seen in human ALS patients (Mitchell *et al*., 2013). For this reason, I analyzed the pathways that are activated by FUS overexpression. At first I analyzed the effect of FUS, ectopically overexpressed, on the endogenous protein, in order to understand if any regulative mechanism was activated. SK-N-BE cells, carrying integrated copies of wild type RFP-FUS cDNA with an unrelated 3'UTR and under the control of Doxycycline (Dox), were utilized. Overexpression of RFP-FUS produced a strong accumulation of the cDNA-encoded form, alongside with a conspicuous reduction of the endogenous levels of FUS protein (Figure 15A). This suggested the existence of a feedback regulation exerted by the exogenous construct (devoid of introns and 3'UTR) on the expression of the endogenous FUS. I next analysed also the levels of endogenous FUS mRNA after induction of RFP-FUS construct. Intriguingly, also FUS mRNA was strongly downregulated (Figure 15B). These observations suggest that this regulative feedback influences some steps of the mRNA processing or stability and that the strong accumulation of the RFP-FUS fused protein from a construct devoid of the 3'UTR suggests a possible role also of this region in FUS regulation.

3.7 - FUS overexpression induces exon skipping in FUS pre-mRNA

Recent observations by Lagier-Tourenne *et al*., (2012), revealed that FUS has the ability to bind its own pre-mRNA, as observed by CLIP-seq analysis. Interestingly, FUS binding sites are enriched in the 3'UTR and in the highly conserved region between exon 6 and exon 8 (Figure 16A). The high conservation of an intronic region suggests a functional role for this sequence. By band-shift analysis I

A



B

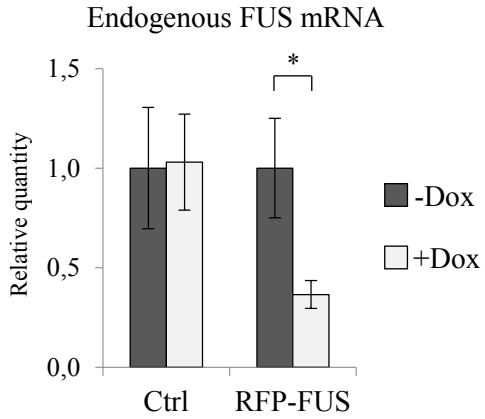


Figure 15. FUS overxpression induces the doenregulation of endogenous FUS. (**A**) Western blot analysis with FUS antibodies on total proteins extracted from SK-N-BE cells, carrying a RFP control construct (Ctrl) or the RFP-FUS expression cassette (as in Figure 10A, epB-Puro-TT-RFP-FUS^{WT}), grown for 48 hours in absence (-) or presence (+) of doxycycline (Dox). GAPDH was used as a loading control (left panel). A densitometric analysis of the endogenous FUS protein quantification is also shown (right panel). (**B**) Levels of endogenous FUS mRNA measured by qRT-PCR in cells treated as in (A). GAPDH mRNA was used as a loading control Error bars represent s.e.m. from 3 independent measurements and the significance was assessed by Unpaired Student's t-test (*P<0.05; **P<0.01).

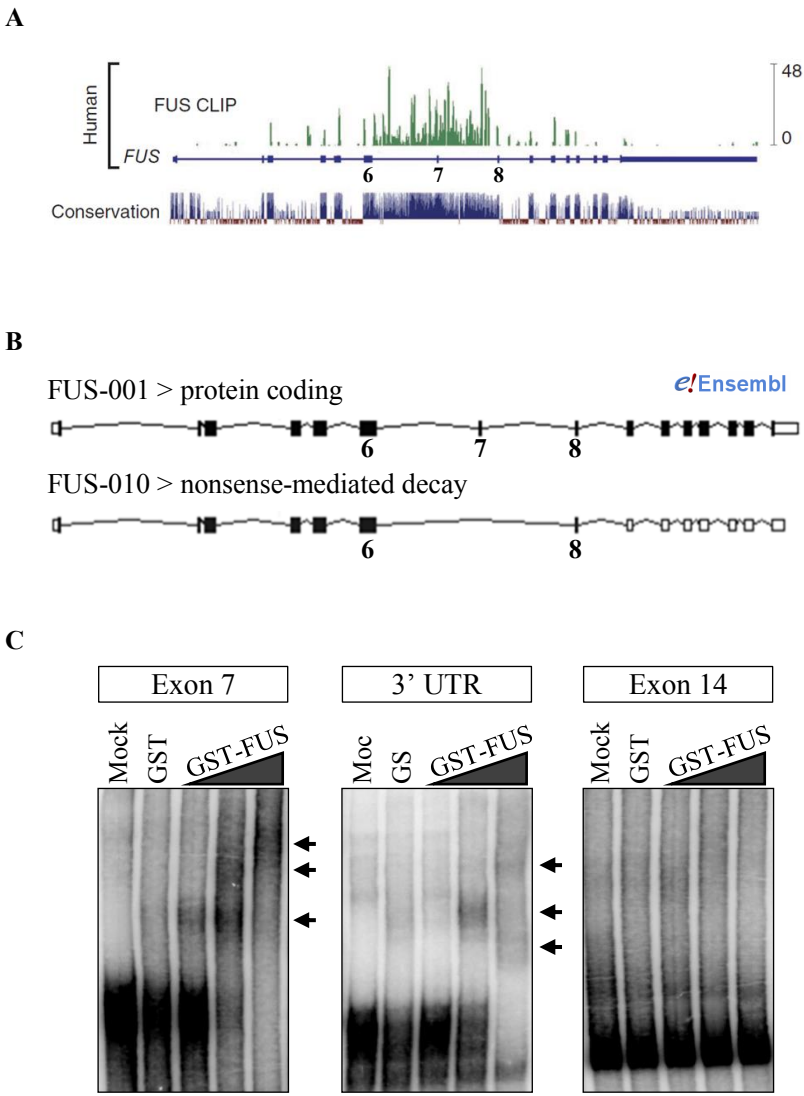


Figure 16. FUS binds directly exon 7 and 3'UTR of its own transcript. (**A**) Clip-Seq data from Lagier-Tourenne *et al.* (2012) showing the binding capacity of FUS on its own pre-mRNA. (**B**) Two isoforms annotated for FUS gene from Ensembl database. (**C**) Band-shift assay using *in vitro* ^{32}P-labelled transcripts with either GST or GST-FUS recombinant proteins. Exon 7 and flanking regions, and the 3'UTR transcripts were used for the assay. Exon 14 and flanking regions transcript was used as a negative control. The arrow points to the specific RNA-protein complex.

checked the ability of a recombinant GST-FUS protein to bind directly the 3'UTR of FUS and the exon 7 including the flanking sequences. Interestingly, FUS is able to bind directly both the exon 7 and the 3'UTR of FUS *in vitro*, but not the exon 14, used as negative control. Therefore, FUS binds directly to this conserved region, and probably is able to participate in regulatory feedback by taking part in the splicing of its own pre-mRNA.
Interestingly, among the different isoforms annotated in Ensembl database, beyond the 15-exons protein-coding one (FUS-001), there is an isoform lacking exon 7 (FUS-010; Figure 16B). This isoform is predicted to be degraded by nonsense-mediated decay because of the formation of a premature stop codon. Therefore, I tested whether FUS overexpression could affect the splicing of its own pre-mRNA. RFP-FUS fusion protein (the same constructs used in Figure 15A) was overexpressed in SK-N-BE cells. Through qRT-PCR using specific oligonucleotides that are able to recognize only the isoform lacking of exon 7 (Figure 17A), I observed that FUS overexpression induced a strong increase of the amount of this isoform (Figure 17B). Then, I measured the levels of the isoform lacking exon 7 in SK-N-BE cells depleted of FUS through RNAi (Figure 17C) and I observed a 90% decrease of this isoform. Notably, this decrease was stronger than the decrease measured for the total amount of FUS mRNA, due to RNAi (Figure 17C). Altogether these data describe a feed-forward feedback, in which the overexpression of FUS induces the skipping of exon 7 from the endogenous FUS pre-mRNA, producing an out-of-frame mRNA unable to make a functional protein, thus ensuring the correct level of the FUS protein (Figure 22).

3.8 - The 3'UTR of FUS has an important role for FUS regulation, altered by G48A mutation

To test whether in the FUS autoregulatory control also the 3'UTR is involved, I started with the characterization of this region. 3'RACE assay allowed the mapping of the 3' end of the FUS mRNA isoform expressed in SK-N-BE cells 164 nucleotides downstream of the termination codon (Figure 18A). To test the impact of the 3'UTR of

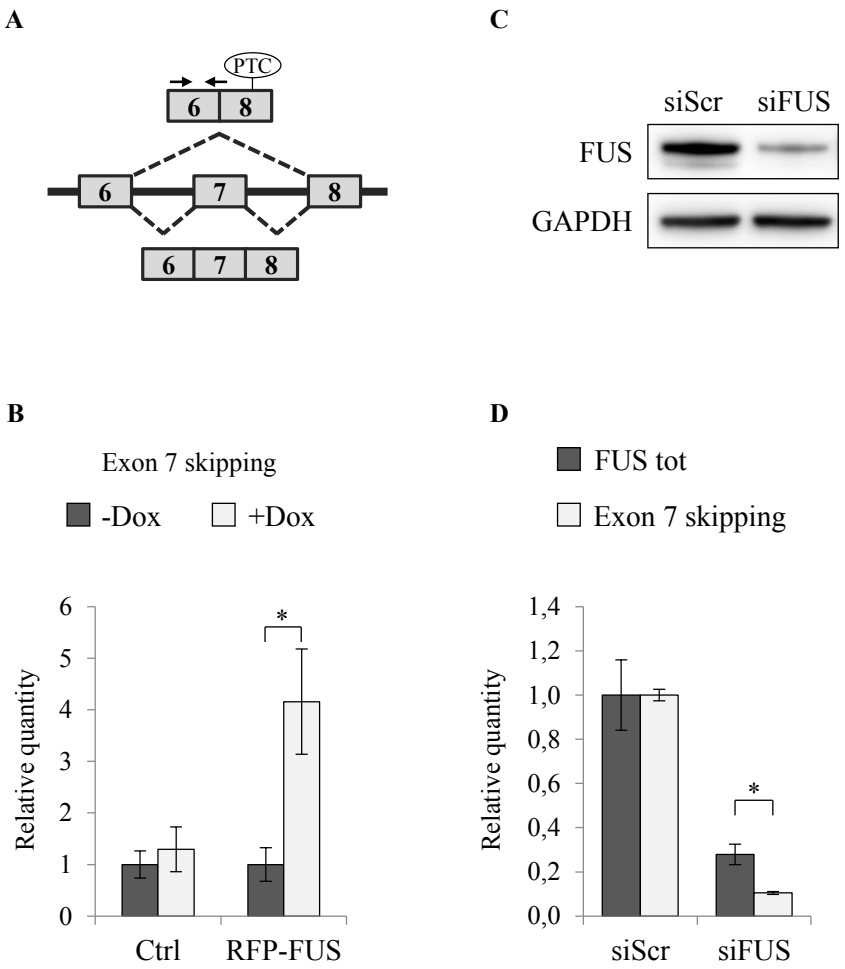


Figure 17. (**A**) Schematic representation of the exon 6-exon 8 portion of the FUS pre-mRNA, together with the two spliced isoforms deriving from the alternative use of exon 7. The premature stop codon (PTC) and the position of the oligonucleotides (arrows) used for qRT-PCR are indicated. (**B**) The histogram shows the levels of the exon 7 skipping measured by qRT-PCR in cells stably transfected and treated as in Figure 15A. (**C**) Western blot analysis with FUS antibodies of proteins from SK-N-BE cells treated with anti-FUS (siFUS) or control (siScr) siRNA. GAPDH was used as loading control. (**D**) The levels of all FUS isoforms (FUS tot) and exon 7 skipped mRNAs were measured by qRT-PCR in cells treated as in (C). Significance was assessed by Unpaired Student's t-test (*P<0.05, **P<0.01).

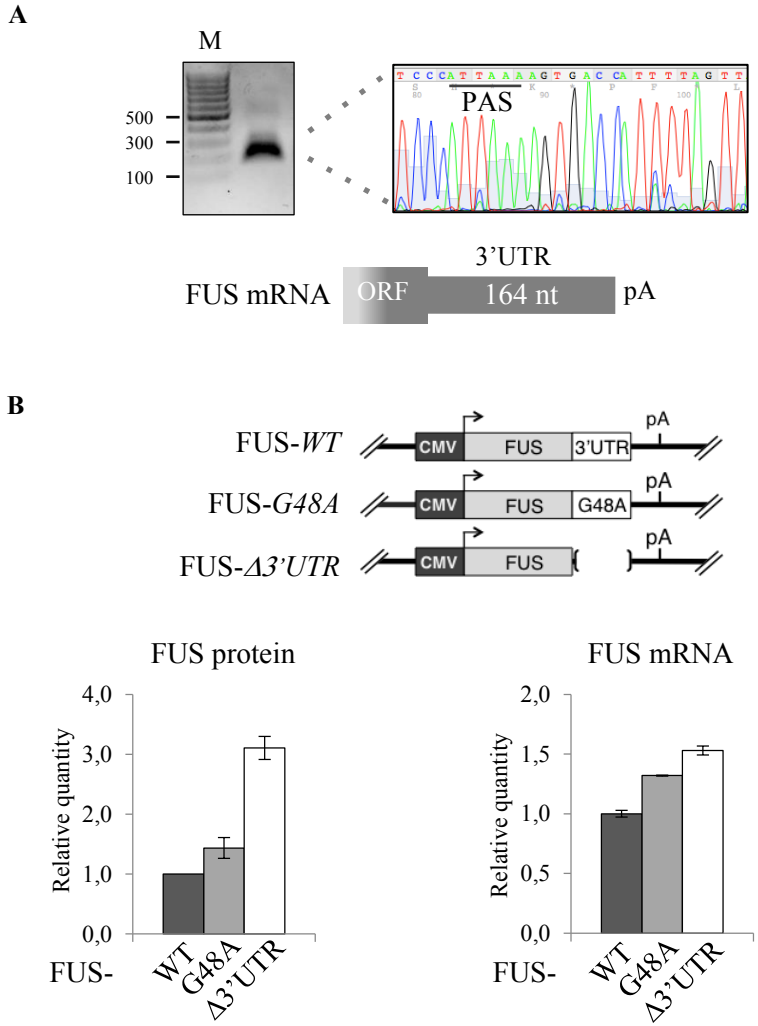


Figure 18. (**A**) Agarose gel showing the results of the 3'RACE assay performed on RNA from SK-N-BE cells. Part of the DNA sequence corresponding to the gel band is shown together with the underlined polyadenylation site (PAS) and a schematic representation of the FUS open reading frame (ORF) and 3'UTR. (**B**) FUS cDNA constructs used; FUS-*WT*, contains the wild-type 3'UTR, while the mutant derivatives contain the G48A substitution (FUS-*G48A*) or the deletion of the entire 3'UTR (FUS-*Δ3'UTR*). Histograms show the levels of FUS protein (left panel) and mRNA (right panel) obtained from SK-N-BE cells transfected with the different FUS constructs. FUS mRNA levels were normalised on the neomycin marker co-expressed from the same plasmid.

FUS on protein accumulation I prepared different plasmids containing FUS cDNA with or without the 3'UTR (FUS-*WT* and FUS-*Δ3'UTR*). Transfection of these plasmids in SK-N-BE cells, resulted in a strong accumulation of the FUS protein and mRNA derived from the constructs lacking of the 3'UTR (Figure 18B).
Bioinformatic research for miRNA responsive elements (MREs) in the FUS 3'UTR, revealed that in this region there is a predicted conserved binding site for miR-141 and miR-200a. These two miRNAs share the same seed sequence and belong to the same miRNA family. Interestingly, among the identified 3'UTR mutations associated to ALS (Sabatelli *et al.*, 2013; Figure 2A), two patients carried the G48A substitution (in one case of inherited type) that localizes the predicted binding site for miR-141 and miR-200a.
When the G48A mutation was tested in the context of a cDNA construct (FUS-*G48A*), the levels of FUS, as well as of its mRNA, were reproducibly higher with respect to those raised from the wild type construct (Figure 18B), indicating the contribution of this mutation on the control of FUS accumulation. Notably, the increase did not reach the levels observed with FUS-*Δ3'UTR*, suggesting the presence in the 3'UTR of additional regulatory elements important for controlling the homeostatic levels of FUS. This is in line with the observation that three other 3'UTR mutations, associated to severe ALS, are linked to high accumulation of the FUS protein (Sabatelli *et al*., 2013). However, none of these additional mutations appears to affect putative conserved miRNA binding sites, according to TargetScan and PicTar analysis.

3.9 - miR-141 and miR-200a target FUS mRNA, but not the G48A mutant derivative

In order to test if FUS is a real target of miR-141 and miR-200a, I performed a canonical luciferase assay. Furthermore, I checked also if the G48A mutation, associated with ALS, affects the miRNA-mediated repression.
Luciferase reporters harboring the 3'UTR of FUS (Luc-FUS-*WT*) or the deletion of the miR-141/200a seed site (Luc-FUS-*Δseed*) or the

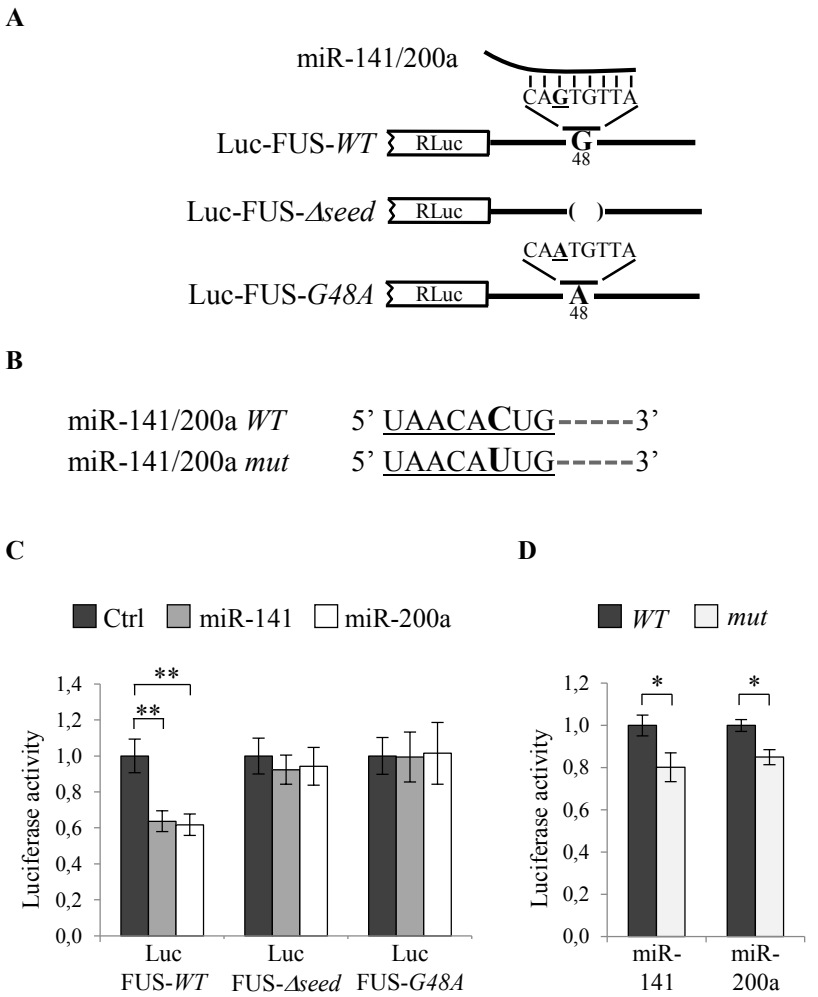


Figure 19. (**A**) Luciferase fusion constructs. (**B**) Seed sequence of miR-141 and miR-200a (miR-141/200a *WT*) and of their mutant derivatives (miR-141/200a *mut*) containing the complementary substitution to the G48A mutation. (**C**) Relative luciferase levels of Luc-FUS-*WT,* Luc-FUS-*Δseed* and Luc-FUS-*G48A* constructs co-transfected in SK-N-BE cells with en empty vector (Ctrl) or with miRNA (miR-141 or miR-200a) expressing plasmids. (**D**) Histograms indicate the relative luciferase activity of the Luc-FUS-*G48A* construct co-transfected in SK-N-BE cells with wild type (WT) or mutant (*mut*) miRNA expressing plasmids. Data were derived from three independent experiments; error bars represent s.e.m., significance was assessed by Unpaired Student's t-test (*P<0.05, **P<0.01).

G48A point mutation (Luc-FUS-*G48A*) were individually co-transfected in SK-N-BE cells with miR-141 and miR-200a expressing plasmids or with a control plasmid (Ctrl) (Figure 19A). Luciferase levels of Luc-FUS-*WT* were significantly reduced with each one of the two miRNAs, whereas Luc-FUS-*Δseed* lacked this effect (Figure 19C). Notably, the Luc-FUS-*G48A* construct, containing the G48A point mutation, was also insensitive to miR-141 or miR-200a repression (Figure 19C). Therefore, in patients with G48A mutations the regulation mediated by miR-141 and miR-200a might not succeed.
However, co-transfection of Luc-FUS-*G48A* with plasmid expressing miR-141 or miR-200a derivatives, containing a nucleotide substitution complementary to the G48A mutation (miR-141/200a *mut*), resulted in rescue of miRNA-dependent repression (Figure 19D). These data indicate the specificity of the G to A substitution for miR-141/200a recognition and function. Therefore, in ALS patients carrying G48A mutation the increased levels of the FUS protein might not be lowered by the action of miR-141/200a.

3.10 - FUS induces the expression of miR-141 and miR-200a

Since FUS, a well described Drosha interactor, was previously shown to enhance miRNA expression by direct binding to nascent pri-miRNAs on the chromatin and facilitating co-transcriptional processing, I tested these features on miR-141 and miR-200a. SK-N-BE cells, carrying integrated copies of wild type FRP-FUS cDNA under the control of Doxycycline (Dox), were utilized. Notably, upon FUS overexpression in SK-N-BE cells, the levels of miR-141 and miR-200a strongly increased (Figure 20). MiR-15a and miR-432 levels, previously shown to be unaffected by FUS modulation, did not change after FUS overexpression. To recapitulate the mechanisms of action of FUS on these two miRNAs, the binding of a recombinant GST-FUS protein to labelled pri-miRNAs was tested *in vitro*. By band-shift assay, I observed that FUS is able bind directly and specifically to both pri-

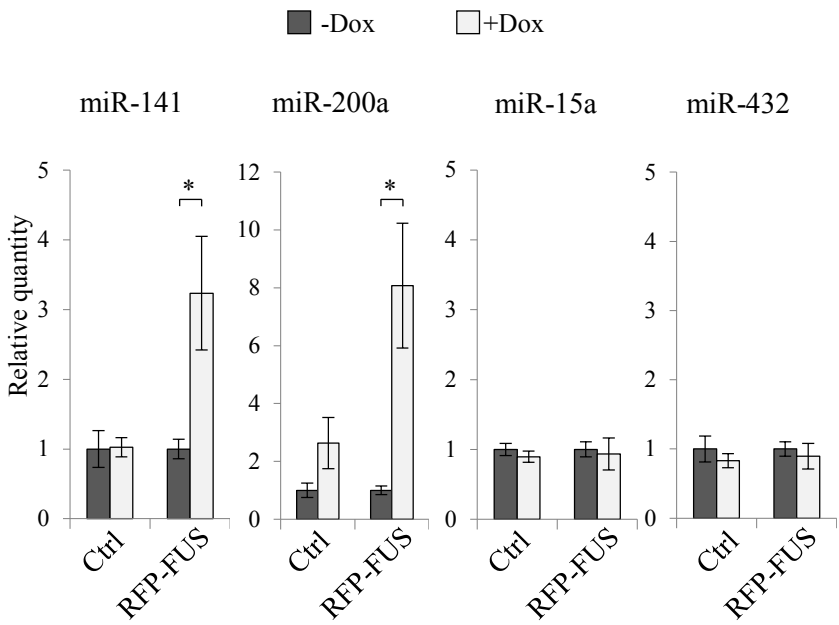
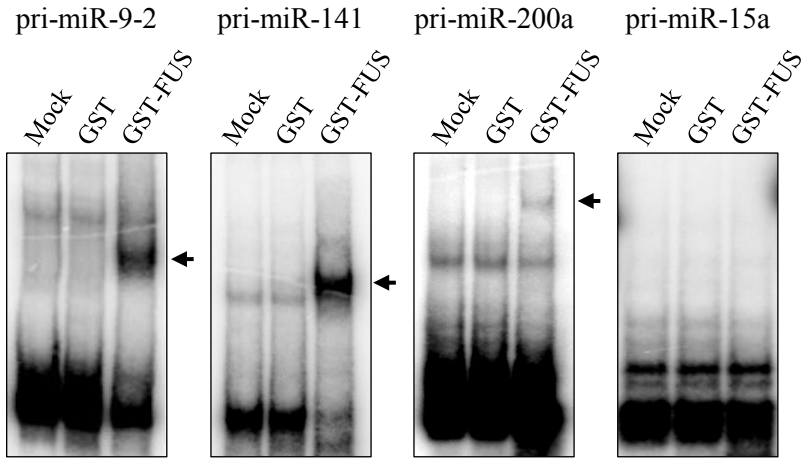


Figure 20. **FUS overexpression induces miR-141 and miR-200a upregulation**. Levels of miR-141 and miR-200a measured by qRT-PCR in SK-N-BE cells, carrying a RFP control construct (Ctrl) or the RFP-FUS expression cassette, grown for 48 hours in absence (-) or presence (+) of doxycycline (Dox). miR-15a and miR-432 were used as controls. Data were derived from at least three independent experiments; error bars represents s.e.m., significance was assessed by Unpaired Student's t-test (*P<0.05).

A



B

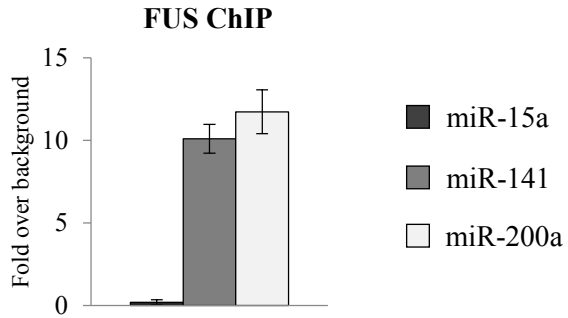


Figure 21. (**A**) Gel mobility shift assay using the indicated *in vitro* ^{32}P-labelled pri-miRNA transcripts with either GST or GST-FUS recombinant proteins. The arrow points to the specific RNA-protein complexes. (**B**) ChIP analysis with FUS-antibodies on chromatin from SK-N-BE cells expressing Dox-inducible flag-FUS cDNA. Genomic regions coding for miR-15a, miR-141 and miR-200a were analyzed. Co-amplifications were carried out with primers specific for miRNA and chromosome IV intergenic region. The histograms show the IP values on miRNA loci normalized for the intergenic region and expressed as enrichment over background signals (IgG). Error bars represent standard error from 3 independent experiments.

miR-141 and pri-miR-200a (Figure 21A). No binding was instead observed when control pri-miR-15a was used. According to our previous data indicating the chromatin localization of FUS, ChIP experiments with anti-FUS antibodies revealed a specific localization of FUS on the chromosomal loci encoding for miR-141 and miR-200a, while no localization was detected on the negative control, miR-15a (Figure 21B).
In conclusion, these data provide evidence for the existence of a feed forward regulatory loop in which FUS controls the expression levels of two miRNAs, which in turn regulate FUS accumulation (Figure 22). Interestingly, the disruption of this circuitry correlates with a mutation that establishes an ALS phenotype: whether the pathogenesis of the disease is due to the increased FUS levels or to the subsequent up-regulation of specific classes of miRNAs remains an interesting question to be addressed.

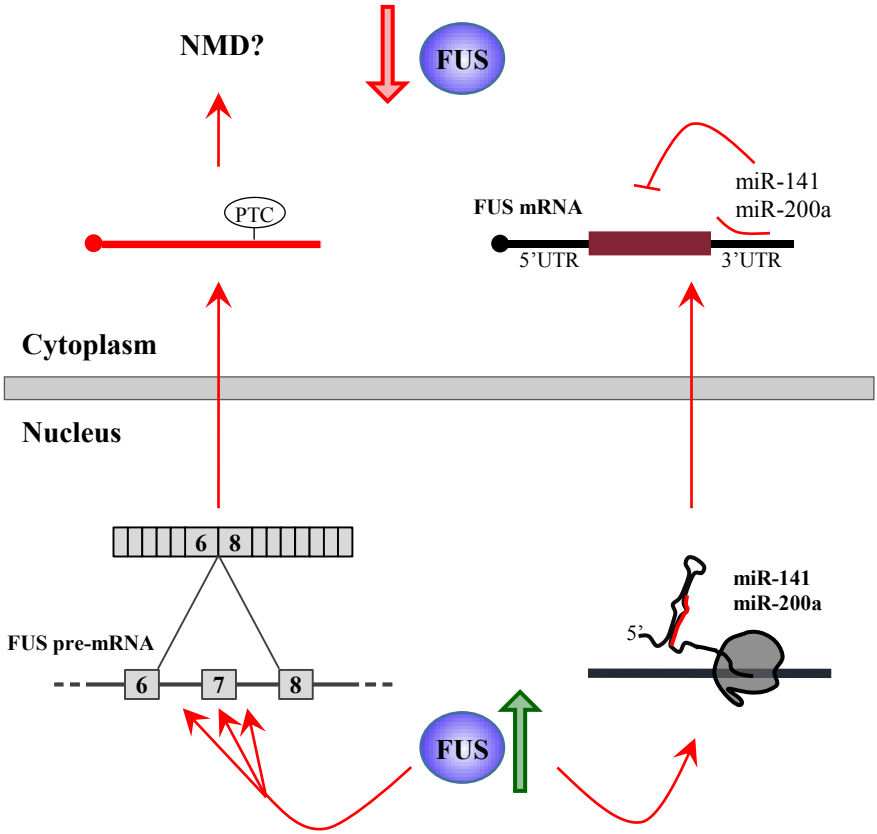


Figure 22. Model of FUS autoregolatory mechanisms. Left panel: increased levels of FUS are able to induce the skipping of exon 7 of its own pre-mRNA. This event leads to the formation of an isoform that is predicted to be degraded by non-sense mediated decay for the formation of a premature stop codon. Right panel: increased levels of FUS are able to induce the expression of miR-141 and miR-200a, that are able to target and repress FUS mRNA. These two mechanisms are able to restore the correct levels of FUS when they are increased. The alteration of part of these regulatory mechanisms can lead to ALS onset.

4 - DISCUSSION

FUS is a multifunctional nuclear protein, involved in RNA metabolism, which has been recently linked to familial forms of ALS, a severe age-dependent disorder causing degeneration of motoneurons in the brain and spinal cord. Since mutations seem to mainly affect the nucleus/cytoplasmic distribution of the protein, it has been suggested that these mutations may have a dual effect: i) loss of function in the nucleus and ii) toxic gain of function in the cytoplasm. Therefore, dosage alteration of the protein in the two compartments can provide a hint for understanding ALS pathology. FUS has been attributed a large number of functions in the nucleus mainly related to transcription and RNA processing, whereas cytoplasmic aggregated forms have been suggested to cause alteration in neuronal plasticity, or in nuclear RNA maturation and transport (Belly *et al*., 2005; Polymenidou *et al*., 2012).

Among the large repertoire of nuclear functions, I focused on the observation that FUS was described as a Drosha interactor (Gregory *et al.*, 2004). Here I demonstrated that the FUS protein has a dual function of interacting with specific pri-miRNA sequences and with Drosha. Moreover, I show that FUS binds to nascent pri-miRNA molecules and helps Drosha recruitment on the chromatin allowing efficient miRNA processing.

I also show that, among the others, FUS affects the biogenesis of miRNAs with a relevant role in neuronal function, differentiation and synaptogenesis such as miR-9, miR-125b and miR-132 (Laneve *et al*., 2007 and 2010; Packer *et al*., 2008; Edbauer *et al*., 2010; Pathania *et al*., 2012).

Notably, I observed that the accumulation levels of these miRNAs were lowered even when the residual amount of FUS was only half with respect to control. These data could explain why mutations affecting FUS nuclear dosage could have a remarkable negative effect on miRNA homeostasis, thus providing a possible correlation with the ALS pathogenesis. Due to the fact that ubiquitous miRNAs

are affected by FUS downregulation, one should envisage a more general toxic effect not restricted to the nervous system. However, several considerations could explain a higher susceptibility of neuronal cells: i) the miRNA downregulation is limited and only neuronal cells could be affected by such tiny changes; ii) the neuronal miRNA species identified play non-redundant essential functions; iii) protein delocalization and aggregate formation could be partially compensated in proliferating cells, while in post-mitotic neuronal cells these processes would have additive effects. The progressive accumulation and aggregation is indeed a phenomenon common to other neurodegenerative diseases due to proteins having the ability of forming amyloid-like fibers (Yamamoto *et al.*, 2011; Han *et al*., 2012).

It is important to underline that FUS plays multiple roles in the nucleus and in particular during transcription. ChIP and promoter microarrays have identified a large number of target genes regulated by this factor (Tan *et al*., 2012), thus indicating that miRNA biogenesis may represent only part of FUS activity.

It is well documented that FUS levels are crucial for the cell homeostasis. On one side, decreased levels of FUS protein in the nucleus has been described in ALS patients, and in FUS-null mice have been observed chromosomal instability and neurons with abnormal spine morphology (Hicks *et al*., 2000; Fuji *et al*., 2005). On the other side also increased amount of FUS protein has been described in ALS patients, and mice overexpressing wild-type human FUS develop an aggressive phenotype with an early onset of several pathological features observed in human ALS patients (Mitchell *et al*., 2013). Notably, have been identified mutations in the 3'UTR of FUS in ALS patients, in which a strong increase of FUS protein was described (Sabatelli *et al.*, 2013). Even though in these patients the protein is wild type, ALS still occurs most probably for the failure of a regulatory mechanism. Here I demonstrated the existence of two feed forward regulatory loops in which FUS controls its own levels (Figure 22). On one side I show a regulatory mechanism in which FUS binds directly its own pre-mRNA and induces skipping of exon 7. This event leads to the formation of an isoform with a premature stop codon that is

predicted to be degraded by nonsense-mediated decay. Therefore, in this case, in order to re-establish the correct amount of FUS, the surplus of the protein leads to the formation of an unfunctional RNA isoform. On the other side, I demonstrated that FUS induces the expression of two miRNAs (miR-141 and miR-200a), which in turn are able to regulate FUS accumulation. Interestingly, a 3'UTR mutation in the binding site for miR-141/200a (G48A) was found in two ALS patients. Therefore, the disruption of this circuitry might correlate with a mutation that establishes an ALS phenotype; however, whether the pathogenesis of the disease is due to the increased FUS levels or to the subsequent up-regulation of specific classes of miRNAs remains an interesting question to be addressed. Notably, the up-regulation of miR-200a was described in Alzheimer (Cogswell *et al*., 2008) and Huntington (Jin *et al*., 2012) neurodegenerative diseases; moreover, miR-200a predicted targets were implicated in regulating synaptic function, neurodevelopment, and neuronal survival, suggesting that deregulation of this miRNA, as a consequence of FUS mutation, might have a consistent impact on ALS pathogenesis. Finally, I show that the G48A mutation can be suppressed by *ad hoc* modified miRNAs suggesting the possibility of rescuing the correct FUS control and opening interesting perspectives in the treatment of this type of mutations.
Due to the fact that the pathological effects of FUS mutations are mainly restricted to neuronal cells, it is possible that FUS thresholds become critical only in these cells, and that miRNAs are part of the molecular mechanisms whose deregulation may have a relevant role in ALS pathogenesis.

5 - MATERIALS AND METHODS

Cell Cultures and Treatments. SK-N-BE cells were cultured in RPMI 1640 medium (Gibco), supplemented with 10% fetal bovine serum, 1-L-glutamine, and penicillin/streptomycin, and induced to differentiate by 10μM all-trans-Retinoic acid (RA, Sigma). SK-N-BE plasmid transfection was carried out using Lipofectamine and Plus Reagent (Invitrogen) according to the manufacturer's instructions while siRNAs targeting FUS coding region (Hs_FUS_4 FlexiTube siRNA, SI00070518, Qiagen) or 3'UTR (for sequence, see below) or control siRNA (AllStars Negative Control siRNA, Qiagen) were transfected using HiPerfect Transfection Reagent (Qiagen) according to the manufacturer's instructions.
For the generation of stable SK-N-BE cells expressing FUS protein, upon plasmid transfection (epB-Puro-TT derived plasmids and epiggyBac transposase vector) the cells were selected by Puromycin (1μg/ml) treatment and the expression of the different forms of FUS protein was induced by adding Doxycycline (0,2μg/ml) to the culture medium.
For the rescue experiments stable SK-N-BE cells expressing FLAG-FUS^{wt}, FLAG- FUS^{R521C} and FLAG-FUS^{P525L} were treated with siRNA against the 3'UTR of FUS (siFUS-3') for 6 days in RA. The last 2 days the cells were treated or not with Doxycycline (0,02μg/ml final concentration).
HeLa cells were cultured in D-MEM medium (Gibco), supplemented with 10% fetal bovine serum, 1-L-glutamine, and penicillin/streptomycin. HeLa plasmid and siRNA transfections were carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Plasmid construction. To generate the constructs overexpressing miRNAs, the genomic fragments containing pri-miR-9-2, pri-miR-124-2, pri-miR-212, pri-miR-132, pri-miR-141 were PCR amplified

(oligonucleotides sequences are listed below) and cloned using BglII and XhoI restriction sites of U1snRNA expression cassette (Denti *et al.,* 2004). Plasmid overexpressing pri-mir-125b-2 is described in Laneve *et al.,* 2007. Plasmid overexpressing pri-mir-200a (mouse) was cloned by Francesca De Vito using BglII and XhoI restriction sites of U1snRNA expression cassette. The vectors were transfected in combination with a plasmid carrying a modified snRNA U1 gene (U1#23; Denti *et al.,* 2006) to measure the efficiency of transfection.

The mutant plasmids overexpressing miR-141*mut* and miR-200a*mut* were obtained by double inverse PCR amplification on the previous cloned plasmids, in order to obtain the single point mutation of the mature miRNAs and the complementary substitution for rescuing a correct structure for the efficient processing.

For generating GST fused FUS protein, FUS cDNA was PCR amplified from vector pCMV6-AC (SC320263, OriGene Technologies) with the oligolucleotides FUS FW and FUS REV and inserted in BamHI and XhoI restriction sites of pGEX-4T-1 (Amersham Biosciences) raising FUS^{WT} vectors. The mutant form FUS^{R521C} was obtained by inverse PCR amplification on FUS^{WT} vectors using the oligonucleotides FUS R521C FW and FUS R521C RV.

For the generation of the transposable element vectors for inducible expression of FUS, cDNA from vector pCMV6-AC was amplified using the Flag-FUS FW, FUS WT RV, FUS R521C RV and FUS P525L RV and inserted into the epB-Puro-TT vector generating the Flag-FUS^{WT}, Flag-FUS^{R521C} and Flag-FUS^{P525L} plasmids. The transposable element vectors for inducible expression of RFP-FUS^{wt} and EGFP-FUS^{R521C} and EGFP-FUS^{P525L} were derived from the enhanced piggyBac (ePiggyBac) vector epB-Bsd-TRE described in Rosa *et al.* (2011). Briefly, a cassette encoding for the rtTA-Advanced protein (Clontech) was fused to the Puromycin or Blasticidin resistance coding sequences through a T2A self-cleavage peptide element, and put under the control of the ubiquitous pUbc promoter in the epB-Bsd-TRE vector. The resulting plasmids (epB-Puro-TT and epB-Bsd-TT) hold on the opposite direction the tetracycline-responsive promoter element (TRE), followed by a

short multicloning site. Therefore both elements of the TET-ON system are present in the same vector. The RFP and EGFP coding sequences, devoid of the stop codon, were then inserted in the epB-Puro-TT and epB-Bsd-TT plasmids, respectively, generating the epB-Puro-TT-RFP and epB-Bsd-TT-EGFP. Finally, the coding sequences of FUS, wild type or mutated, were cloned in frame with the fluorescent proteins, generating the epB-Puro-TT-RFP-FUS^{wt}, epB-Bsd-TT-EGFP- FUS^{R521C} and epB-Bsd-TT-EGFP- FUS^{P525L}.
The FUS-*G48A* mutant construct was obtained by inverse PCR amplification on FUS-*WT* plasmid (SC320263, OriGene Technologies) using the oligonucleotides FUS-G48A FW and RV while the FUS-*Δ3'UTR* mutant construct was generated by PCR amplification on FUS-*WT* plasmid using the oligonuclotides FUS-Δ3'UTR FW and RV.
To generate the constructs for the luciferase assay Luc-FUS-*WT*, the genomic fragment containing the 3'UTR was PCR amplified using the oligonucleotides FUS-3UTR NotI FW and FUS-3UTR NotI RV and cloned downstream the Renilla Luciferase open reading frame in psiCHECK2 vector (Promega) using NotI restriction sites. The mutant derivatives Luc-FUS-*Δseed* and Luc-FUS-*G48A* were obtained by inverse PCR amplification using the oligonucleotides FUS 3'UTR Δseed FW and RV, and FUS-G48A FW and RV respectively. For the rescue experiment a 164 nt long 3'UTR carrying the G48A substitution was used. This was generated by inverse PCR amplification on Luc-FUS-G48A construct using the FUS-G48Ashort FW and RV oligonucleotides.

Protein extraction and Western blot. Whole-cell protein extracts were prepared from SK-N-BE and HeLa cells lysed in RIPA buffer. Extracts were separated by electrophoresis on 4–12% poly-acrylamide gel (Invitrogen) and electroblotted onto nitrocellulose membrane (Protran, S&S, Drammen, Norway). The immunoblots were incubated with the following antibodies: anti-FUS/TLS (sc-47711, Santa Cruz), anti-DGCR8 (ab90579, Abcam), anti-Drosha (ab12286, Abcam), anti-N-Myc (sc-56729, Santa Cruz), anti-FlagM2 (Sigma), anti-GAPDH (sc-32233, Santa Cruz) as a loading

control. The densitometric analysis was performed using Image Lab software (Bio-Rad).

Luciferase assay. Luc-FUS-WT, Luc-FUS-Δseed and Luc-FUS-G48A plasmids were co-transfected with the plasmids expressing miR-141/200a and their mutant derivatives in SK-N-BE cells. After 48 hours of incubation cells were assayed with the Dual-Luciferase Assay (Promega).

RNA preparation and analysis. Total RNA was isolated using miRNeasy Mini Kit according to the manufacturer's instructions (Qiagen).
For the Northern blot assay 5μg of total RNA were fractionated on 10% poly-acrylamide gel in MOPS–NaOH (pH 7), 7 M Urea and transferred onto Amersham Hybond-NX nylon membrane (GE Healthcare). RNA cross-linking was performed in 0.16 M N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride and 0.13 M 1-methylimidazole (Sigma-Aldrich) at pH 8, for 2 hours at 60°C. DNA oligonucleotides complementary to the sequence of mature miR-9, miR-124, miR-125b, miR-132, U1#23 and to 5.8S-rRNA were ^{32}P-labeled and used as probes. Densitometric analysis was performed using Typhoon Imager (GE Healthcare) and ImageQuant software (Molecular Dynamics).

Quantitative RT- PCR analysis. cDNA generation was carried out using the miScript Reverse Transcription Kit (Qiagen). The Real-time PCR detection of miRNAs was performed using miScript SYBR-Green PCR Kit and DNA oligonucleotides by Qiagen, on a 7500 Fast Real-Time PCR (Applied Biosystem). The values obtained were normalized for snoRNA-U25. The qPCR detection of mRNAs was performed using the oligonucleotides listed below. For the detection of the skipping of exon 7 were used oligonucleotides specific for the isoform lacking the exon 7 (FUS exon 6 FW and FUS exon 6-8 REV). GAPDH was used as a loading control. The values were analyzed by the unpaired Student's t-test. P-values were calculated for samples from 3 independent experiments unless otherwise indicated.

miRNAs high-throughput analysis. 700ng of total RNA extracted from SK-N-BE cells were retrotranscribed using the TaqMan MicroRNA RT Kit (Applied Biosystems). The Real-time detection of the miRNA levels was performed using the TaqMan® Human MicroRNA Array A (Applied Biosystems) according to the manufacturer's instructions. The values obtained were normalized for snoRNA-U44.

Band-shift. Band-shift assays were carried out as previously described (Song *et al*., 2012) with minor modifications. Purified in vitro labelled transcripts were incubated with 6 nmoles of recombinant GST or GST-FUS proteins in the presence of increasing amount of cold tRNA competitor, from 50 to 250 molar excess. The complexes were separated by a 4% acrylamide non-denaturing gel. Densitometric analysis was performed using Typhoon Imager (GE Healthcare) and ImageQuant software.

Chromatin immunoprecipitation assay. ChIP analyses were performed on chromatin extracts from SK-N-BE cells according to manufacturer's specifications of MAGnify Chromatin Immunoprecipitation System kit (Invitrogen). Sheared Chromatin was immunoprecipitated with the following antibodies: anti-FUS/TLS (sc-47711, Santa Cruz), anti-Drosha (ab12286, Abcam), anti-Pol II (sc-889, Santa Cruz). The occupancy of the immunoprecipitated factor on miRNA loci was estimated by normalizing for the occupancy on tRNA coding region or chromosome IV intergenic region and expressed as enrichment over background (IgG). Densitometric analysis was performed using Typhoon Imager (GE Healthcare) and ImageQuant software (Molecular Dynamics). RNase treatment of the chromatin and the occupancy of the immunoprecipitated factor on miRNA loci were carried out as described in Morlando *et al.,* 2008. Oligonucleotide used for PCR amplifications are listed below.

Biotin pull-down. Binding of biotinylated transcripts to paramagnetic streptavidin Dynabeads (Dynal) and incubation with

nuclear lysate was carried out as described in Figueroa *et al.,* 2003. Biotilylated transcripts were obtained from PCR generated templates (oligonucleotides are listed below) using 0.35mM Biotin-16-UTP (Roche) as described previously (Dye & Proudfoot*,* 1999).

GST-FUS Purification FUS^{WT} and FUS^{R521C} were transfected in BL21 cells and induced with 0,5mM IPTG for 4h at 28°C. Cell pellets were resuspended in 5 ml of NET-N buffer (Tris-HCl pH 8 20mM, NaCl 100mM, NP-40 0.5%, EDTA 0,5mM) supplemented with a cocktail of protease inhibitor (Roche). After sonication the supernatant fractions were loaded on to Glutathione-Agarose resin (G4510, Sigma) and incubated for 1 hour at 4°C and then washed once with NET-N buffer and twice with NET (Tris-HCl pH 8 20mM, NaCl 100mM, EDTA 0,5mM). The recombinant GST-proteins were eluted with the elution buffer containing 20mM L-Gluthatione reduced and 100mM Tris-HCl pH 8.

Co-immunoprecpitation and GST-Pull down. Co-immunoprecipitation was perfomed using Immunoprecipitation kit - Dynabeads Protein G (Invitrogen) according to the manufacturer's instructions. To obtain the nuclear extracts, the cell pellets were resuspended with Buffer A (Tris-HCl pH 8 20mM, NaCl 10mM, $MgCl_2$ 3mM, Igepal 0,1%, glycerol 10%, EDTA 0,2mM) supplemented with protease inhibitor (Roche) and after centrifugation the nuclei were resuspended in Buffer C (Tris-HCl pH 8 20mM, NaCl 400mM, glycerol 20%, DTT 1mM) supplemented with protease inhibitor (Roche). After three cycles of incubation in liquid nitrogen followed by incubation at 37°C the nuclear extract was recovered by centrifugation.
The GST-Pull down experiments were carried out as described in Morlando *et al.,* 2004 with minor modification. 50µg of SK-N-BE nuclear extract were used instead of in vitro translated Drosha protein and the RNase treatment was carried out with RNase A (Sigma) at 20mg/ml final concentration.

Oligonucleotides used in this study:

Oligonucleotides used for cloning:

miR-9-2 -400 FW BglII	5′-GCCAGATCTAGGCTAAAGAGTCTT-3′
miR-9-2 +400 RV XhoI	5′-GCCCTCGAGGGTGCCTCCCAAAGG-3′
miR-124 -50 BglII FW	5′-CCGAGATCTGGTAATCGCAGTGGGTCTTA TAC-3′
miR-124 +50 XhoI RV	5′-CCGCTCGAGCTGACCCTGAGATGCTTTG GTG-3′
miR-212-132 BglII FW	5′-GGCAGATCTCTCTGCGAGCGGAGCTGTC CTC-3′
miR-212-132 XhoI RV	5′-GCGCTCGAGCCTCGGTGGACTCAGCCG-3′
miR-141 BglII FW	5'-ACTAGATCTCCCACCCAGTGCGATTTGTC-3'
miR-141 XhoI RV	5'-TCACTCGAGAACCAGTGTTTCCACATCT TGC-3'
miR-141 mut FW	5'-TTGTCTGGTAAAGATGGCTCCC-3'
miR-141 mut RV	5'-TGTTAGGAGCTTCACAATTAGACC-3'
miR-141 star mut FW	5'-ATGTTGGATGGTCTAATTGTGAAG-3'
miR-141 star mut RV	5'-TGTACTGGAAGATGGACCCAGG-3'
miR-200a BglII FW	5'-GGAAGATCTTATTGCGATGCATATACGGT CTC-3'
miR-200a XhoI RV	5'-ATTCTCGAGCTGTAGAGCTGAGACAGGC CCT-3'
miR-200a mut FW	5'-TTGTCTGGTAACGATGTTCAAAG-3'
miR-200a mut RV	5'-TGTTAGAGTCAAGCCAAGAAATC-3'
miR-200a star mut FW	5'-ATGCTGGATTTCTTGGCTTGAC-3'
miR-200a star mut RV	5'-TGTCCGGTAAGATGCCCAC-3'
FUS 3'UTR NotI FW	5'-ATTGCGGCCGCTTAGCCTGGCTCCCCAGG TTC-3'
FUS 3'UTR NotI RV	5'-ATTGCGGCCGCGTTTAATCTCTGCTCTCA AGG-3'
FUS 3'UTR Δseed FW	5'-CCCTCGTTATTTTGTAACCTTC-3'
FUS 3'UTR Δseed RV	5'-GGTACAGGACAAAAAGCTGTTC-3'
FUS G48A FW	5'-ATGTTACCCTCGTTATTTTGTAAC-3'
FUS G48A RV	5'-TGGGTACAGGACAAAAAGCT-3'
FUS-Δ3'UTR FW	5'-GCCGCATCCATGGACTACAAGGACGACGAT GACAAGATGGCCTCAAACGATTATACC-3'
FUS-Δ3'UTR RV	5'-GCCGCGGCCGCTTAATACGGCCTCTCCCTGC GATCC-3'
FUS-G48Ashort FW	5'-AACTAAAATGGTCACTTTTAATGG-3'
FUS-G48Ashort RV	5'-GAGCGGCCGCTGGCCGCAATA-3'

Oligonucleotides used for GST-FUS cloning:

FUS BamHI FW	5′-GCCGGATCCATGGCCTCAAACGATTATACC-3′
FUS XhoI RV	5′-GCCCTCGAGTTAATACGGCCTCTCCCTGCG-3′

FUS R521C FW	5′-TGCAGGGAGAGGCCGTATTAACTC-3′
FUS R521C RV	5′-ATCCTGTCTGTGCTCACCCCTG-3′

Oligonucleotides used for Flag-FUS cloning:

Flag FUS FW	5′-GCCGGATCCATGGACTACAAGGACGACG ATGACAAGATGGCCTCAAACGATTATACC-3′
FUS WT RV	5′-GCCAAGCTTTTAATACGGCCTCTCCCTGCGATCC-3′
FUS R521C RV	5′-GCCAAGCTTTTAATACGGCCTCTCCCTGCAATCC-3′
FUS P525L RV	5′-GCCAAGCTTTTAATACAGCCTCTCCCTGCGATCC-3′

Oligonucleotides for qPCR and q-RT-PCR

Pri-miR-9-2 FW	5′-GCCTGTGTGGGAAGCGAGTTG-3′
Pri-miR-9-2 RV	5′-GTCTTTCATTCTCACACGCTCCC-3′
Pri-miR-15a FW	5′-AACCTTGGAGTAAAGTAGCAGCAC-3′
Pri-miR-15a RV	5′-CCTTGTATTTTTGAGGCAGCAC-3′
Pri-miR-125b-2 FW	5′-AAGTCAGGCTCTTGGGACCT-3′
Pri-miR-125b-2 RV	5′-GGATGGGTCATGGTGAAAAC-3′
Pri-miR-132 FW	5′-TCTCCAGGGCAACCGTGGCTTTC-3′
Pri-miR-132 RV	5′-GCGTGGGCGTGCTGCGGGG-3′
Interg. Chr.4 FW	5′-TTCTGATTCTTAAAGGAGTGAC-3′
Interg. Chr.4 RV	5′-AATCATGCAGATAATGAC-3′
miR-141 FW	5'-TCCCCTGTAGCAACTGGTGAG-3'
miR-141 RV	5'-GGAGCCATCTTTACCAGACAGTG-3'
miR-200a FW	5'-CCCCTGTGAGCATCTTACCG-3'
miR-200a RV	5'-CCCATCCCTGGAGTAGGAGC-3'
FUS total FW	5'-TCAGCTAAAGCAGCTATTGACTGG-3'
FUS total RV	5'-GCCACCACCCCGATTAAAGTCTGC-3'
FUS endogenous FW	5'-CAGGGGTGAGCACAGACAGG-3'
FUS endogenous RV	5'-AATAACGAGGGTAACACTGGG-3'
FUS exon 6 FW	5'-CAGAGTGGTGGAGGTGGCAGCG-3'
FUS exon 8 RV	5'-ACGTGATCCTTGGTCCCGAG-3'
GAPDH FW	5'-GGAAGGTGAAGGTCGGAGTC-3'
GAPDH RV	5'-TTACCAGAGTTAAAAGCAGCCC-3'
U1#23 FW	5′-CAGGGGAAAGCGCGAACG-3′
U1#23 RV	5′-CGGCTTACCTGAAATTTTCG-3′

Oligonucleotides for Northern Blot analysis

alfa-miR-9	5′-TCATACAGCTAGATAACCAAAGA-3′
alfa-miR-124	5′-GGCATTCACCGCGTGCCTTA-3′
alfa-miR-125b	5′-TCACAAGTTAGGGTCTCAGGGA-3′
alfa-miR-132	5′-UAACAGUCUACAGCCAUGGUCG-3′
alfa-5.8S-rRNA	5′-GTCGATGATCAATGTGTCCTG-3′
alfa-U1#23	5′-TGAGGCTCTGCAAAGTTCCGAA-3′

Oligonucleotides used for in vitro transcription:

Pri-miR-9-2 T7 Prom FW	5′-TAATACGACTCACTATAGGGCA AGTATCCTGGACGACCACTC-3′
Pri-miR-9-2 RV	5′-GCCCTCGAGAGTATTCCTGACCT TTCTGGT-3′
Pri-miR-15a T7 prom FW	5′-TAATACGACTCACTATAGGGATT CTTTAGGCGCGAATGTGTG-3′
Pri-miR-15a RV	5′-GCTATCATAAGAGCTATGAAT-3′
Pri-miR-125b-2 T7 prom FW	5′-TAATACGACTCACTATAGGGAG GTAAAAGTCTAAGTGAACC-3′
Pri-miR-125b-2 RV	5′-CTCCTAGGCAGAATCTATGTATG-3′
Pri-miR-132 T7 Prom FW	5′-TAATACGACTCACTATAGGGTG ACGTCAGCCCGCCCCGCGC-3′
Pri-miR-132 RV	5′-GTCCCCAGCCCGCGGCTCGGGG-3′
Pri-miR-141 T7 Prom FW	5'-TAATACGACTCACTATAGGGTA GCAACTGGTGAGCGCGCA-3'
Pri-miR-141 RV	5'-TGGTCTTCAGGGCTCCCTGAAGGT-3'
Pri-miR-143 T7 Prom FW	5′-TAATACGACTCACTATAGGGCC ACAGGCCACCAGAGCGGAGC-3′
Pri-miR-143 RV	5′-AGCACTTACCACTTCCAGGCTG-3′
Pri-miR-192 T7 Prom FW	5′-TAATACGACTCACTATAGGGCT ACCGTGGCGACGCTCCCAGGC-3′
Pri-miR-192 RV	5′-GGATCTCTGCTGACTGCTGGAC-3′
Pri-miR-199a-2 T7 Prom FW	5′-TAATACGACTCACTATAGGGG AGGCTTTTCCTGAGGACCGGG-3′
Pri-miR-199a-2 RV	5′-CAAATGTCTTCTCCTTGGAAAC-3′
Pri-miR-200a T7 Prom FW	5'-TAATACGACTCACTATAGGGAGC CCCTGCCTGCCTGGCG-3'
pri-miR-200a RV	5'-CTCCGGATGTGCCTCGGTGG-3'
Pri-miR-212 T7 Prom FW	5′-TAATACGACTCACTATAGGGCGG AGCAGCAGAGCCCCCAGC-3′
Pri-miR-212 RV	5′-CCTGAGGGACGGGGACTGGG-3′
Pri-miR-370 T7 Prom FW	5′-TAATACGACTCACTATAGGGCTCA TTCTACAAACCGTACAAGTC-3′
Pri-miR-370 RV	5′-CTGCAGCAGCGCCCGAGCTCT-3′
Pri-miR-513a-1 T7 Prom FW	5′-TAATACGACTCACTATAGGGGA GCATTTGGTCTGGGATGCCAC-3′
Pri-miR-513a-1 RV	5′-CTACACCCCCATCCTCAGGGAC-3′
Pri-miR-628 T7 Prom FW	5′-TAATACGACTCACTATAGGGCA TAAAGGAGCAGCACCAGAATAG-3′
Pri-miR-628 RV	5′-GATCAAGGTTCAAAGCACTG-3′
tRNA Leucine T7 PromFW	5′-TAATACGACTCACTATAGGGAG GACAACGGGGACAGTAA-3′
tRNA Leucine RV	5′-TCCACCAGAAAAACTCCAGC-3′

siRNA 3'UTR of FUS/TLS (Qiagen):
5′-AAUAACGAGGGUAACACUGGG-3′

6 - REFERENCES

- Adam-Vizi V and Starkov AA (2010) Calcium and mitochondrial reactive oxygen species generation: how to read the facts. J Alzheimers Dis, 20:S413-S426.
- Andersson MK, Ståhlberg A, Arvidsson Y, Olofsson A, Semb H, Stenman G, Nilsson O, Aman P (2008) The multifunctional FUS, EWS and TAF15 proto-oncoproteins show cell type-specific expression patterns and involvement in cell spreading and stress response. BMC Cell Biol, 9:37.
- Appel SH, Zhao W, Beers DR, Henkel JS (2011) The microglial-motoneuron dialogue in ALS. Acta Myol. 30(1):4-8.
- Bagga S, Bracht J, Hunter S, Massirer K, Holtz J, Eachus R, Pasquinelli AE (2005) Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation. Cell, 122(4): 553-63.
- Barbato C, Ruberti F, Cogoni C (2009) Searching for MIND: microRNAs in neurodegenerative diseases. J Biomed Biotechnol, 2009:871313.
- Barber SC, Shaw PJ (2010) Oxidative stress in ALS: key role in motor neuron injury and therapeutic target. Free Radic Biol Med, 48(5):629-41.
- Bartel DP, Chen CZ (2004) Micromanagers of gene expression: the potentially widespread influence of metazoan microRNAs. Nat Rev Genet, (5):396-400.
- Beers DR, Henkel JS, Zhao W, Wang J, Huang A, Wen S, Liao B, Appel SH (2011) Endogenous regulatory T lymphocytes ameliorate amyotrophic lateral sclerosis in mice and correlate with disease progression in patients with amyotrophic lateral sclerosis. Brain, 134(Pt 5):1293-314.

- Belly A, Moreau-Gachelin F, Sadoul R, Goldberg Y (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, 379:152-157
- Belzil VV, Valdmanis PN, Dion PA, Daoud H, Kabashi E, Noreau A, Gauthier J, Hince P, Desjarlais A, Bouchard JP, Lacomblez L, Salachas F, Pradat PF, Camu W, Meininger V, Dupre N, Rouleau GA (2009) Mutations in FUS cause FALS and SALS in French and French Canadian populations. Neurology 73: 1176–1179
- Bendotti C, Marino M, Cheroni C, Fontana E, Crippa V, Poletti A, De Biasi S (2012) Dysfunction of constitutive and inducible ubiquitin-proteasome system in amyotrophic lateral sclerosis: implication for protein aggregation and immune response. Prog Neurobiol, 97(2):101-26.
- Bendotti C, Tortarolo M, Suchak SK, Calvaresi N, Carvelli L, Bastone A, Rizzi M, Rattray M, Mennini T (2001) Transgenic SOD1 G93A mice develop reduced GLT-1 in spinal cord without alterations in cerebrospinal fluid glutamate levels. J Neurochem, 79(4):737-46.
- Bernstein E, Caudy AA, Hammond SM, Hannon GJ (2001) Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature, 409(6818): 363-6.
- Bilsland LG, Sahai E, Kelly G, Golding M, Greensmith L, Schiavo G (2010) Deficits in axonal transport precede ALS symptoms in vivo. Proc Natl Acad Sci U S A, 107(47):20523-8.
- Bogdanov M, Brown RH, Matson W, Smart R, Hayden D, O'Donnell H, Flint Beal M, Cudkowicz M (2000) Increased oxidative damage to DNA in ALS patients. Free Radic Biol Med, 29(7):652-8.
- Brengues M, Teixeira D, Parker R (2005) Movement of eukaryotic mRNAs between polysomes and cytoplasmic processing bodies. Science, 310(5747): 486-9.

- Brennecke J, Stark A, Russell RB, Cohen SM (2005) Principles of miRNA-target recognition. PLoS Biol, 3(3):e85.
- Bueno MJ, Pérez de Castro I, Gómez de Cedrón M, Santos J, Calin GA, Cigudosa JC, Croce CM, Fernández-Piqueras J, Malumbres M (2008) Genetic and epigenetic silencing of microRNA-203 enhances ABL1 and BCR-ABL1 oncogene expression. Cancer Cell, 13(6):496-506.
- Carrí MT, Ferri A, Cozzolino M, Calabrese L, Rotilio G (2003) Neurodegeneration in amyotrophic lateral sclerosis: the role of oxidative stress and altered homeostasis of metals. Brain Res Bull, 61(4):365-74.
- Charcot JM (1874) De la sclérose latérale amyotrophique. Prog Med, 2:325-327, 341-342, 453-455.
- Charcot JM, Joffroy A (1869) Deux cas d'atrophie musculaire progressive avec lesions de la substance grise et des faisceaux antero-lateraux de la moelle epiniere. Arch Physiol Neurol Pathol, 2:744-54.
- Chekulaeva M, Mathys H, Zipprich JT, Attig J, Colic M, Parker R, Filipowicz W (2011) miRNA repression involves GW182-mediated recruitment of CCR4-NOT through conserved W-containing motifs. Nat Struct Mol Biol, 18:1218–1226.
- Chendrimada TP, Finn KJ, Ji X, Baillat D, Gregory RI, Liebhaber SA, Pasquinelli AE, Shiekhattar R (2007) MicroRNA silencing through RISC recruitment of eIF6. Nature, 447(7146):823-8.
- Chiò A, Restagno G, Brunetti M, Ossola I, Calvo A, Mora G, Sabatelli M, Monsurro MR, Battistini S, Mandrioli J, Salvi F, Spataro R, Schymick J, Traynor BJ, La Bella V (2009) Two Italian kindreds with familial amyotrophic lateral sclerosis due to FUS mutation. Neurobiol Aging 30: 1272–1275
- Cogswell JP, Ward J, Taylor IA, Waters M, Shi Y, Cannon B, Kelnar K, Kemppainen J, Brown D, Chen C, Prinjha RK, Richardson JC, Saunders AM, Roses AD, Richards CA (2008) Identification of miRNA changes in Alzheimer's disease brain

and CSF yields putative biomarkers and insights into disease pathways. J Alzheimers Dis, 14(1):27-41.

- Cozzolino M, Pesaresi MG, Gerbino V, Grosskreutz J, Carrì MT (2012) Amyotrophic lateral sclerosis: new insights into underlying molecular mechanisms and opportunities for therapeutic intervention. Antioxid Redox Signal. 17(9):1277-330.
- Deng HX, Hentati A, Tainer JA, Iqbal, Z., Cayabyab A, Hung WY, Getzoff ED, Hu P, Herzfeldt B, Roos RP, Warner C, Deng G, Soriano E, Smyth C, Parge HE, Ahmed A, Roses AD, Hallewell RA, Pericak-Vance MA, Siddique T (1993). Amyotrophic lateral sclerosis and structural defects in Cu,Zn superoxide dismutase. Science 261, 1047-1051.
- Doench JG, Sharp PA (2004) Specificity of miRNA target selection in translational repression. Genes Dev, 18(5):504-11.
- Doi H, Koyano S, Suzuki Y, Nukina N, Kuroiwa Y (2010) The RNA-binding protein FUS/TLS is a common aggregate-interacting protein in polyglutamine diseases. Neurosci Res, 66(1):131-3.
- Doi H, Okamura K, Bauer PO, Furukawa Y, Shimizu H, Kurosawa M, Machida Y, Miyazaki H, Mitsui K, Kuroiwa Y, Nukina N (2008) RNA-binding protein TLS is a major nuclear aggregate-interacting protein in huntingtin exon 1 with expanded polyglutamine-expressing cells. J Biol Chem, 283(10):6489-500.
- Dormann D, Rodde R, Edbauer D, Bentmann E, Fischer I, Hruscha A, Than ME, Mackenzie IR, Capell A, Schmid B, Neumann M, Haass C (2010) ALS-associated fused in sarcoma (FUS) mutations disrupt Transportin-mediated nuclear import. EMBO J, 29(16):2841-57.
- Dye MJ, Proudfoot NJ (1999) Terminal exon definition occurs cotranscriptionally and promotes termination of RNA polymerase II. Mol Cell, 3:371-378
- Edbauer D, Neilson JR, Foster KA, Wang CF, Seeburg DP, Batterton MN, Tada T, Dolan BM, Sharp PA, Sheng M (2010)

Regulation of synaptic structure and function by FMRP-associated microRNAs miR-125b and miR-132. Neuron, 65: 373-384

- Ferraiuolo L, Kirby J, Grierson AJ, Sendtner M, Shaw PJ (2011) Molecular pathways of motor neuron injury in amyotrophic lateral sclerosis. Nat Rev Neurol, 7(11):616-30.
- Figueroa A, Cuadrado A, Fan J, Atasoy U, Muscat EG, Muñoz-Canoves P, Gorospe M, Muñoz A (2003) Role of HuR in Skeletal Myogenesis through Coordinate Regulation of Muscle Differentiation Genes. Mol Cell Biol, 23:4991–5004
- Filipowicz W, Jaskiewicz L, Kolb FA, Pillai RS (2005) Post-transcriptional gene silencing by siRNAs and miRNAs. Curr Opin Struct Biol, 15(3):331-41.
- Friedman RC, Farh KK, Burge CB, Bartel DP (2009) Most mammalian mRNAs are conserved targets of microRNAs. Genome Res, 19(1):92-105.
- Fujii R, Okabe S, Urushido T, Inoue K, Yoshimura A, Tachibana T, Nishikawa T, Hicks GG, Takumi T (2005) The RNA binding protein TLS is translocated to dendritic spines by mGluR5 activation and regulates spine morphology. Curr Biol, 15(6):587-93.
- Fujii R, Takumi T (2005) TLS facilitates transport of mRNA encoding an actin-stabilizing protein to dendritic spines. J Cell Sci, 118(Pt 24):5755-65.
- Giraldez AJ, Mishima Y, Rihel J, Grocock RJ, Van Dongen S, Inoue K, Enright AJ, Schier AF (2006) Zebrafish MiR-430 promotes deadenylation and clearance of maternal mRNAs. Science, 312(5770):75-9.
- Girotti AW (1998) Lipid hydroperoxide generation, turnover, and effector action in biological systems, Journal of Lipid Research, vol. 39, no. 8, 1529–1542.
- Gregory RI, Yan KP, Amuthan G, Chendrimada T, Doratotaj B, Cooch N, Shiekhattar R (2004) The Microprocessor complex mediates the genesis of microRNAs. Nature, 432(7014):235-40.

- Grimson A, Farh KK, Johnston WK, Garrett-Engele P, Lim LP, Bartel DP (2007) MiRNA targeting specificity in mammals: determinants beyond seed pairing. Mol Cell, 27(1):91-105.
- Hallier M, Lerga A, Barnache S, Tavitian A, Moreau-Gachelin F (1998) The transcription factor Spi-1/PU.1 interacts with the potential splicing factor TLS. J Biol Chem, 273(9):4838-42.
- Han J, Lee Y, Yeom KH, Nam JW, Heo I, Rhee JK, Sohn SY, Cho Y, Zhang BT, Kim VN (2006) Molecular basis for the recognition of primary miRNAs by the Drosha-DGCR8 complex. Cell, 125(5):887-901.
- Han TW, Kato M, Xie S, Wu LC, Mirzaei H, Pei J, Chen M, Xie Y, Allen J, Xiao G, McKnight SL (2012) Cell-free formation of RNA granules: bound RNAs identify features and components of cellular assemblies. Cell, 149:768-779
- Hata A, Davis BN (2009) Control of microRNA biogenesis by TGFbeta signaling pathway-A novel role of Smads in the nucleus. Cytokine Growth Factor Rev, 20(5-6):517-21.
- Heo I, Joo C, Kim YK, Ha M, Yoon MJ, Cho J, Yeom KH, Han J, Kim VN (2009) TUT4 in concert with Lin28 suppresses microRNA biogenesis through pre-microRNA uridylation. Cell, 138(4):696-708.
- Hicks GG, Singh N, Nashabi A, Mai S, Bozek G, Klewes L, Arapovic D, White EK, Koury MJ, Oltz EM, Van Kaer L, Ruley HE (2000) Fus deficiency in mice results in defective B-lymphocyte development and activation, high levels of chromosomal instability and perinatal death. Nat Genet, 24(2):175-9.
- Hoell JI, Larsson E, Runge S, Nusbaum JD, Duggimpudi S, Farazi TA, Hafner M, Borkhardt A, Sander C, Tuschl T (2011) RNA targets of wild-type and mutant FET family proteins. Nat Struct Mol Biol 18: 1428–1431
- Iko Y, Kodama TS, Kasai N, Oyama T, Morita EH, Muto T, Okumura M, Fujii R, Takumi T, Tate S, Morikawa K (2004)

Domain architectures and characterization of an RNA-binding protein, TLS. J Biol Chem, 279(43):44834-40.

- Ilieva H, Polymenidou M, Cleveland DW (2009) Non-cell autonomous toxicity in neurodegenerative disorders: ALS and beyond. J Cell Biol, 187(6):761-72.
- Ince PG, Tomkins J, Slade JY, Thatcher NM, Shaw PJ (1998) Amyotrophic lateral sclerosis associated with genetic abnormalities in the gene encoding Cu/Zn superoxide dismutase: molecular pathology of five new cases, and comparison with previous reports and 73 sporadic cases of ALS. J Neuropathol Exp Neurol, 57(10):895-904.
- Izquierdo JM, Valcárcel J (2006) A simple principle to explain the evolution of pre-mRNA splicing. Genes Dev, 20(13):1679-84.
- Jeong SY, Rathore KI, Schulz K, Ponka P, Arosio P, David S (2009) Dysregulation of iron homeostasis in the CNS contributes to disease progression in a mouse model of amyotrophic lateral sclerosis. J Neurosci, 29(3):610-9.
- Jin J, Cheng Y, Zhang Y, Wood W, Peng Q, Hutchison E, Mattson MP, Becker KG, Duan W (2012) Interrogation of brain miRNA and mRNA expression profiles reveals a molecular regulatory network that is perturbed by mutant huntingtin. J Neurochem, 123(4):477-90.
- Johnson BS, Snead D, Lee JJ, McCaffery JM, Shorter J, Gitler AD (2009) TDP-43 is intrinsically aggregation-prone, and amyotrophic lateral sclerosis-linked mutations accelerate aggregation and increase toxicity. J Biol Chem, 284(30):20329-39.
- Kandel ER, Schwartz JH and Jessel TM (1991). Diseases of the Motor Unit. In Principles of Neural Sciences (Norwalk, Appleton & Lange), pp. 248-250.
- Katsuno M, Tanaka F, Sobue G. Perspectives on molecular targeted therapies and clinical trials for neurodegenerative

diseases. J Neurol Neurosurg Psychiatry. 2012 Mar;83(3):329-35. Review.

- Kawahara Y, Zinshteyn B, Chendrimada TP, Shiekhattar R, Nishikura K (2007) RNA editing of the microRNA-151 precursor blocks cleavage by the Dicer-TRBP complex. EMBO Rep, 8(8):763-9.
- Kornblihtt AR, de la Mata M, Fededa JP, Munoz MJ, Nogues G (2004) Multiple links between transcription and splicing. RNA, 10(10):1489-98.
- Kosik KS (2006) The neuronal microRNA system. Nat Rev Neurosci, 7(12):911-20.
- Kozomara A, Griffiths-Jones S (2013) miRBase: annotating high confidence microRNAs using deep sequencing data. Nucleic Acids Res, 2013 Nov 25 [Epub ahead of print].
- Kuroda M, Sok J, Webb L, Baechtold H, Urano F, Yin Y, Chung P, de Rooij DG, Akhmedov A, Ashley T, Ron D (2000) Male sterility and enhanced radiation sensitivity in TLS(-/-) mice. EMBO J, 19(3):453-62.
- Kwiatkowski TJ Jr, Bosco DA, Leclerc AL, Tamrazian E, Vanderburg CR, Russ C, Davis A, Gilchrist J, Kasarskis EJ, Munsat T, Valdmanis P, Rouleau GA, Hosler BA, Cortelli P, de Jong PJ, Yoshinaga Y, Haines JL, Pericak-Vance MA, Yan J, Ticozzi N, Siddique T, McKenna-Yasek D, Sapp PC, Horvitz HR, Landers JE, Brown RH Jr (2009) Mutations in the FUS/TLS gene on chromosome 16 cause familial amyotrophic lateral sclerosis. Science, 323(5918):1205-8.
- Lagier-Tourenne C, Cleveland DW (2009) Rethinking ALS: the FUS about TDP-43. Cell, 136: 1001-1004.
- Lagier-Tourenne C, Polymenidou M, Cleveland DW (2010) TDP-43 and FUS/TLS: emerging roles in RNA processing and neurodegeneration. Hum Mol Genet, 19(R1):R46-64.
- Lagier-Tourenne C, Polymenidou M, Hutt KR, Vu AQ, Baughn M, Huelga SC, Clutario KM, Ling SC, Liang TY, Mazur C, Wancewicz E, Kim AS, Watt A, Freier S, Hicks GG, Donohue

JP, Shiue L, Bennett CF, Ravits J, Cleveland DW, Yeo GW (2012) Divergent roles of ALS-linked proteins FUS/TLS and TDP-43 intersect in processing long pre-mRNAs. Nat Neurosci, 15(11):1488-97.

- Landgraf P, Rusu M, Sheridan R, Sewer A, Iovino N, Aravin A, Pfeffer S, Rice A, Kamphorst AO,Landthaler M, Lin C, Socci ND, Hermida L, Fulci V, Chiaretti S, Foa R, Schliwka J, Fuchs U, NovoselA, Muller RU, Schermer B, Bissels U, Inman J, Phan Q, Chien M, Weir DB, Choksi R, De Vita G,Frezzetti D, Trompeter HI, Hornung V, Teng G, Hartmann G, Palkovits M, Di Lauro R, Wernet P,Macino G, Rogler CE, Nagle JW, Ju J, Papavasiliou FN, Benzing T, Lichter P, Tam W, BrownsteinMJ, Bosio A, Borkhardt A, Russo JJ, Sander C, Zavolan M, Tuschl T (2007) A mammalian microRNAexpression atlas based on small RNA library sequencing. Cell, 129:1401–1414.
- Laneve P, Di Marcotullio L, Gioia U, Fiori ME, Ferretti E, Gulino A, Bozzoni I, Caffarelli E (2007) The interplay between microRNAs and the neurotrophin receptor tropomyosin-related kinase C controls proliferation of human neuroblastoma cells. Proc Natl Acad Sci USA, 104: 7957–7962
- Laneve P, Gioia U, Andriotto A, Moretti F, Bozzoni I, Caffarelli E (2010) A minicircuitry involving REST and CREB controls miR-9-2 expression during human neuronal differentiation. Nucleic Acids Res 38: 6895–6905
- Lee RC, Feinbaum RL, Ambros V (1993) The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell, 75(5):843-54.
- Lee Y, Ahn C, Han J, Choi H, Kim J, Yim J, Lee J, Provost P, Rådmark O, Kim S, Kim VN (2003) The nuclear RNase III Drosha initiates miRNA processing. Nature, 425(6956):415-9.
- Lee Y, Morrison BM, Li Y, Lengacher S, Farah MH, Hoffman PN, Liu Y, Tsingalia A, Jin L, Zhang PW, Pellerin L, Magistretti PJ, Rothstein JD (2012) Oligodendroglia metabolically support axons and contribute to neurodegeneration. Nature, 487(7408):443-8.

- Lin MT, Beal MF (2006) Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. Nature, 443(7113):787-95.
- Lin S, Fu XD (2007) SR proteins and related factors in alternative splicing. Adv Exp Med Biol, 623:107-22.
- Liu J, Valencia-Sanchez MA, Hannon GJ, Parker R (2005) MiRNA-dependent localization of targeted mRNAs to mammalian P-bodies. Nat Cell Biol, 7(7):719-23.
- Liu N, Williams AH, Kim Y, McAnally J, Bezprozvannaya S, Sutherland LB, Richardson JA, Bassel-Duby R, Olson EN (2007) An intragenic MEF2-dependent enhancer directs muscle-specific expression of microRNAs 1 and 133. Proc Natl Acad Sci USA, 104, 20844–20849.
- Liu X, Fortin K, Mourelatos Z (2007) MicroRNAs: Biogenesis and Molecular Functions. Brain Pathology, 18(1):113-121
- Logroscino G, Traynor BJ, Hardiman O, Chio' A, Couratier P, Mitchell JD, Swingler RJ, Beghi E (2008) Descriptive epidemiology of amyotrophic lateral sclerosis: new evidence and unsolved issues. J Neurol Neurosurg Psychiatry, 79:6-11.
- Logroscino G, Traynor BJ, Hardiman O, Chiò A, Mitchell D, Swingler RJ, Millul A, Benn E, Beghi E; EURALS (2010) Incidence of amyotrophic lateral sclerosis in Europe. J Neurol Neurosurg Psychiatry, 81(4):385-90.
- López-Bigas N, Audit B, Ouzounis C, Parra G, Guigó R (2005) Are splicing mutations the most frequent cause of hereditary disease? FEBS Lett, 579(9):1900-3.
- Mackenzie IR, Rademakers R, Neumann M (2010) TDP-43 and FUS in amyotrophic lateral sclerosis and frontotemporal dementia. Lancet Neurol, 9(10):995-1007
- Maniatis T, Reed R (2002) An extensive network of coupling among gene expression machines. Nature, 416(6880):499-506.
- Maniatis T, Tasic B (2002) Alternative pre-mRNA splicing and proteome expansion in metazoans. Nature, 418(6894):236-43.

- Mathonnet G, Fabian MR, Svitkin YV, Parsyan A, Huck L, Murata T, Biffo S, Merrick WC, Darzynkiewicz E, Pillai RS, Filipowicz W, Duchaine TF, Sonenberg (2007) MicroRNA inhibition of translation initiation in vitro by targeting the cap-binding complex eIF4F. N Science, 317(5845):1764-7
- Michlewski G, Guil S, Caceres JF (2010) Stimulation of pri-miR-18a processing by hnRNP A1. Adv Exp Med Biol, 700: 28–35
- Michlewski G, Guil S, Semple CA, Cáceres JF (2008) Posttranscriptional regulation of miRNAs harboring conserved terminal loops. Mol Cell, 32(3):383-93.
- Miller RG, Mitchell JD, Moore DH (2012) Riluzole for amyotrophic lateral sclerosis (ALS)/motor neuron disease (MND). Cochrane Database Syst Rev, 3:CD001447.
- Mitchell JC, McGoldrick P, Vance C, Hortobagyi T, Sreedharan J, Rogelj B, Tudor EL, Smith BN, Klasen C, Miller CC, Cooper JD, Greensmith L, Shaw CE (2013) Overexpression of human wild-type FUS causes progressive motor neuron degeneration in an age- and dose-dependent fashion. Acta Neuropathol, 125(2):273-88.
- Montes M, Becerra S, Sánchez-Álvarez M, Suñé C (2012) Functional coupling of transcription and splicing. Gene, 501(2):104-17.
- Morlando M, Ballarino M, Greco P, Caffarelli E, Dichtl B, Bozzoni I (2004) Coupling between snoRNP assembly and 3' processing controls box C/D snoRNA biosynthesis in yeast. EMBO J, 23:2392-2401
- Morlando M, Ballarino M, Gromak N, Pagano F, Bozzoni I, Proudfoot NJ (2008) Primary microRNA transcripts are processed co-transcriptionally. Nat Struct Mol Biol, 15(9):902-9.
- Mourelatos Z, Dostie J, Paushkin S, Sharma A, Charroux B, Abel L, Rappsilber J, Mann M, Dreyfuss G (2002) miRNPs: a novel class of ribonucleoproteins containing numerous miRNAs. Genes Dev, 16(6):720-8.

- Nakaya T, Alexiou P, Maragkakis M, Chang A, Mourelatos Z (2013) FUS regulates genes coding for RNA-binding proteins in neurons by binding to their highly conserved introns. RNA, 19(4):498-509.
- Nelson LM, McGuire V, Longstreth WT, Jr., Matkin C (2000). Population-based case-control study of amyotrophic lateral sclerosis in western Washington State. I. Cigarette smoking and alcohol consumption. Am J Epidemiol, 151:156-163.
- Newman MA, Thomson JM, Hammond SM (2008) Lin-28 interaction with the let-7 precursor loop mediates regulated microRNA processing. RNA, 14, 1539-1549.
- Osler W (1880) On heredity in progressive muscular atrophy as illustrated in the Farr family of Vermont. Archives of Medicine, 4: 316–20.
- Packer AN, Xing Y, Harper SQ, Jones L, Davidson BL (2008) The bifunctional microRNA miR-9/miR-9* regulates REST and CoREST and is downregulated in Huntington's disease. J Neurosci, 28(53):14341-6.
- Pagani F, Raponi M, Baralle FE (2005) Synonymous mutations in CFTR exon 12 affect splicing and are not neutral in evolution. Proc Natl Acad Sci USA, 102(18):6368-72.
- Papadeas ST, Kraig SE, O'Banion C, Lepore AC, Maragakis NJ (2011) Astrocytes carrying the superoxide dismutase 1 (SOD1G93A) mutation induce wild-type motor neuron degeneration in vivo. Proc Natl Acad Sci USA, 108(43):17803-8
- Pasinelli P, Brown RH (2006) Molecular biology of amyotrophic lateral sclerosis: insights from genetics. Nat Rev Neurosci, 7: 710-723.
- Pasquinelli AE, Reinhart BJ, Slack F, Martindale MQ, Kuroda MI, Maller B, Hayward DC, Ball EE, Degnan B, Müller P, Spring J, Srinivasan A, Fishman M, Finnerty J, Corbo J, Levine M, Leahy P, Davidson E, Ruvkun G (2000) Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. Nature, 408(6808):86-9.

- Pathania M, Torres-Reveron J, Yan L, Kimura T, Lin TV, Gordon V, Teng ZQ, Zhao X, Fulga TA, Van Vactor D, Bordey A (2012) miR- 132 enhances dendritic morphogenesis, spine density, synaptic integration, and survival of newborn olfactory bulb neurons. PLoS ONE, 7: e38174.
- Polymenidou M, Lagier-Tourenne C, Hutt KR, Bennett CF, Cleveland DW, Yeo GW (2012) Misregulated RNA processing in amyotrophic lateral sclerosis. Brain Res, 1462: 3-15
- Prasad DD, Ouchida M, Lee L, Rao VN, Reddy ES (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):3717-29.
- Rademakers R, Stewart H, Dejesus-Hernandez M, Krieger C, Graff-Radford N, Fabros M, Briemberg H, Cashman N, Eisen A, Mackenzie IR (2010) Fus gene mutations in familial and sporadic amyotrophic lateral sclerosis. Muscle Nerve, 42(2):170-6.
- Reenan RA (2001) The RNA world meets behavior: A-I pre-mRNA editing in animals. Trends Genet, 17, 53–56.
- Régal L, Vanopdenbosch L, Tilkin P, Van den Bosch L, Thijs V, Sciot R, Robberecht W (2006) The G93C mutation in superoxide dismutase 1: clinicopathologic phenotype and prognosis. Arch Neurol, 63(2):262-7
- Richter JD, Sonenberg N (2005) Regulation of cap-dependent translation by eIF4E inhibitory proteins. Nature, 433(7025):477-80
- Robberecht W, Philips T (2013) The changing scene of amyotrophic lateral sclerosis. Nat Rev Neurosci, 14(4):248-64.
- Rogelj B, Easton LE, Bogu GK, Stanton LW, Rot G, Curk T, Zupan B, Sugimoto Y, Modic M, Haberman N, Tollervey J, Fujii R, Takumi T, Shaw CE, Ule J (2012) Widespread binding of FUS along nascent RNA regulates alternative splicing in the brain. Sci Rep, 2:603

- Rosa A, Brivanlou AH (2011) A regulatory circuitry comprised of miR-302 and the transcription factors OCT4 and NR2F2 regulates human embryonic stem cell differentiation. EMBO J, 30:237-248
- Rosen DR, Siddique T, Patterson D, Figlewicz DA, Sapp P, Hentati A, Donaldson D, Goto J, O'Regan JP, Deng HX, Rahmani Z, Krizus A, McKenna-Yasek D, Cayabyab A, Gaston SM, Berger R, Tanzi RE, Halperin JJ, Herzfeldt B, Van den Bergh R, Hung WY, Bird T, Deng G, Mulder DW, Smyth C, Laing NG, Soriano E, Pericak–Vance MA, Haines J, Rouleau GA, Gusella JS, Horvitz HR and Brown RH Jr (1993) Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis. Nature, 362(6415):59-62.
- Rothstein JD, Martin LJ, Kuncl RW (1992) Decreased glutamate transport by the brain and spinal cord in amyotrophic lateral sclerosis. N Engl J Med, 326(22):1464-8.
- Rouleau G, Meijer I (2007) CSCI/RCPSC Henry Friesen lecture: the past and the future of neurogenetics. Clin Invest Med, 30: E269–73.
- Rowland LP, Mitsumoto H, Przedborski S (2010) Amyotrophic lateral sclerosis, progressive muscular atrophy, and primary lateral sclerosis. In: Rowland LP, Pedley TA, editors. Merritt's Neurology. 12 ed. Philadelphia: Lippincott, Williams & Wilkins, 802–808.
- Rybak A, Fuchs H, Smirnova L, Brandt C, Pohl EE, Nitsch R, Wulczyn FG (2008) A feedback loop comprising lin-28 and let-7 controls pre-let-7 maturation during neural stem-cell commitment. Nature Cell Biol, 10, 987–993.
- Sabatelli M, Conte A, Zollino M (2013) Clinical and genetic heterogeneity of amyotrophic lateral sclerosis. Clin Genet, 83(5):408-16.
- Sabatelli M, Moncada A, Conte A, Lattante S, Marangi G, Luigetti M, Lucchini M, Mirabella M, Romano A, Del Grande A, Bisogni G, Doronzio PN, Rossini PM, Zollino M (2013) Mutations in the 3' untranslated region of FUS causing FUS

overexpression are associated with amyotrophic lateral sclerosis. Hum Mol Genet, 22(23):4748-55.

- Shaw PJ (2005) Molecular and cellular pathways of neurodegeneration in motor neurone disease. J Neurol Neurosurg Psychiatry, 76(8):1046-57.
- Shaw PJ, Ince PG, Falkous G, Mantle D (1995) Oxidative damage to protein in sporadic motor neuron disease spinal cord. Annals of Neurology, vol. 38, no. 4, pp. 691–695.
- Siddique T, Figlewicz DA, Pericak-Vance MA, Haines JL, Rouleau G, Jeffers AJ, Sapp P, Hung WY, Bebout J, McKenna-Yasek D, Deng G, Horvitz HR, Gusella JF, Brown RH Jr and Roses AD (1991) Linkage of a gene causing familial amyotrophic lateral sclerosis to chromosome 21 and evidence of genetic-locus heterogeneity. N Engl J Med, 324(20):1381-4.
- Song X, Wang X, Arai S, Kurokawa R (2012) Promoter-associated noncoding RNA from the CCND1 promoter. Methods Mol Biol, 809:609-622
- Sun Z, Diaz Z, Fang X, Hart MP, Chesi A, Shorter J, Gitler AD (2011) Molecular determinants and genetic modifiers of aggregation and toxicity for the ALS disease protein FUS/TLS. PLoS Biol, 9(4):e1000614.
- Tan AY, Manley JL (2010) TLS inhibits RNA polymerase III transcription. Mol Cell Biol, 30(1):186-96.
- Tan AY, Riley TR, Coady T, Bussemaker HJ, Manley JL (2012) TLS/FUS (translocated in liposarcoma/fused in sarcoma) regulates target gene transcription via single-stranded DNA response elements. Proc Natl Acad Sci USA 109: 6030–6035
- Thermann R, Hentze MW (2007) Drosophila miR2 induces pseudo-polysomes and inhibits translation initiation. Nature, 447:875–878.
- Thomas M, Alegre-Abarrategui J, Wade-Martins R (2013) RNA dysfunction and aggrephagy at the centre of an amyotrophic lateral sclerosis/frontotemporal dementia disease continuum. Brain, 136(Pt 5):1345-60.

- Tohgi H, Abe T, Yamazaki K, Murata T, Ishizaki E, Isobe C (1999) Increase in oxidized NO products and reduction in oxidized glutathione in cerebrospinal fluid from patients with sporadic form of amyotrophic lateral sclerosis. Neurosci Lett, 260(3):204-6.
- Uranishi H, Tetsuka T, Yamashita M, Asamitsu K, Shimizu M, Itoh M, Okamoto T (2001) Involvement of the pro-oncoprotein TLS (translocated in liposarcoma) in nuclear factor-kappa B p65-mediated transcription as a coactivator. J Biol Chem, 276(16):13395-401.
- Vance C, Rogelj B, Hortobágyi T, De Vos KJ, Nishimura AL, Sreedharan J, Hu X, Smith B, Ruddy D, Wright P, Ganesalingam J, Williams KL, Tripathi V, Al-Saraj S, Al-Chalabi A, Leigh PN, Blair IP, Nicholson G, de Belleroche J, Gallo JM, Miller CC, Shaw CE (2009) Mutations in FUS, an RNA processing protein, cause familial amyotrophic lateral sclerosis type 6. Science, 323(5918):1208-11.
- Verma A, Tandan R (2013) RNA quality control and protein aggregates in amyotrophic lateral sclerosis: a review. Muscle Nerve, 47(3):330-8.
- Viswanathan SR, Daley GQ, Gregory RI (2008) Selective blockade of microRNA processing by Lin28. Science, 320(5872):97-100.
- Wang ET, Sandberg R, Luo S, Khrebtukova I, Zhang L, Mayr C, Kingsmore SF, Schroth GP, Burge CB (2008) Alternative isoform regulation in human tissue transcriptomes. Nature, 456(7221):470-6.
- Wang WY, Pan L, Su SC, Quinn EJ, Sasaki M, Jimenez JC, Mackenzie IR, Huang EJ, Tsai LH (2013) Interaction of FUS and HDAC1 regulates DNA damage response and repair in neurons. Nat Neurosci, 16(10):1383-91.
- Wang X, Arai S, Song X, Reichart D, Du K, Pascual G, Tempst P, Rosenfeld MG, Glass CK, Kurokawa R (2008) Induced ncRNAs allosterically modify RNA-binding proteins in cis to inhibit transcription. Nature, 454(7200):126-30.

- Wightman B, Ha I, Ruvkun G (1993) Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. Cell, 75(5):855-62.
- Wijesekera LC, Leigh PN (2009) Amyotrophic lateral sclerosis. Orphanet J Rare Dis, 4:3.
- Wu JY, Maniatis T (1993) Specific interactions between proteins implicated in splice site selection and regulated alternative splicing. Cell, 75(6):1061-70.
- Yamamoto A, Simonsen A (2011) The elimination of accumulated and aggregated proteins: A role for aggrephagy in neurodegeneration. Neurobiology of Disease, 43:17–28
- Yeo G, Holste D, Kreiman G, Burge CB (2004) Variation in alternative splicing across human tissues. Genome Biol, 5(10):R74.
- Yi R, Qin Y, Macara IG, Cullen BR (2003) Exportin-5 mediates the nuclear export of pre-miRNAs and short hairpin RNAs. Genes Dev, 17(24):3011-6.
- Zhang H, Kolb FA, Jaskiewicz L, Westhof E, Filipowicz W (2004) Single processing center models for human Dicer and bacterial RNase III. Cell, 118(1): 57-68.
- Zhou Y, Liu S, Liu G, Oztürk A, Hicks GG (2013) ALS-Associated FUS Mutations Result in Compromised FUS Alternative Splicing and Autoregulation. PLoS Genet, 9(10):e1003895.
- Zinman L, Cudkowicz M (2011) Emerging targets and treatments in amyotrophic lateral sclerosis. Lancet Neurol, 10(5):481-90.
- Zinszner H, Sok J, Immanuel D, Yin Y, Ron D (1997) TLS (FUS) binds RNA in vivo and engages in nucleo-cytoplasmic shuttling. J Cell Sci, 110 (Pt 15):1741-50.

List of publications

- Di Carlo V, Grossi E, Laneve P, Morlando M, **Dini Modigliani S**, Ballarino M, Bozzoni I, Caffarelli E (2013) TDP-43 Regulates the Microprocessor Complex Activity During In Vitro Neuronal Differentiation. Mol Neurobiol, 48(3):952-63

- Morlando M[1], **Dini Modigliani S**[1], Torrelli G, Rosa A, Di Carlo V, Caffarelli E, Bozzoni I (2012) FUS stimulates microRNA biogenesis by facilitating co-transcriptional Drosha recruitment. EMBO J, 31(24):4502-10.
  [1]*These authors contributed equally to this work*