ALS-RELATED FUS PROTEIN IS MISLOCALIZED TO CYTOPLASM AND RECRUITED INTO STRESS GRANULES IN FIBROBLASTS OF ASYMPTOMATIC FUS P525L MUTATION CARRIERS

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ABSTRACT

Symptoms onset in Amyotrophic Lateral Sclerosis (ALS) occur when over 70% of motor neurons is already lost, suggesting a relatively long pre-symptomatic phase. The description of several genes linked to ALS (e.g., SOD1, FUS, TARDP, C9orf72) has now allowed identification of pre-symptomatic carriers.

These pre-symptomatic (or even preclinical) carriers can be followed up with the aim to identify the very early clinical disease-related changes or a valuable biomarker. These efforts seem at present the best approach for the implementation of an early symptomatic therapy or for the disease prevention.

In this work, we studied the expression of FUS protein in cultured skin fibroblasts from pre-symptomatic FUS P525L mutation carriers.

We cultured skin fibroblasts from two sisters belonging to an ALS family with FUS P525L mutation and carrying the same mutation, one healthy control and two patients with sporadic ALS (sALS), with no identified gene mutations. The two carriers were clinically asymptomatic, while the two patients fulfilled the El-Escorial and Awaji criteria for definite ALS.

Western blot and immunocytochemistry were performed with specific antibodies to study the expression and subcellular localization of FUS protein in the skin fibroblasts.

In sporadic ALS, FUS protein showed an almost exclusive nuclear localization. In the healthy control, FUS was also mostly nuclear, with some cells showing a faint cytoplasmic expression. In the FUS P525L mutation carriers, the protein was strongly expressed in both nucleus and
cytoplasm in most cells, with a relatively high proportion of cells showing an exclusive cytoplasmic FUS localization.

Furthermore, either heat-shock or dithiothreitol were applied to assess the effect of stress on FUS expression and cytoplasmic redistribution. Stress induced cytoplasmic granules formation in all subjects, and FUS was recruited into them. After a single stress exposure, we observed in all subjects a time-dependent decrease of cells containing granules. However, granules persisted longer in fibroblasts from the two FUS P525L carriers, where the number of granules per cell was found to be higher than HC and sALS, suggesting that these granules may accumulate during chronic stress and thus be the precursors of the pathological FUS inclusions.

These data might represent an early molecular change occurring before ALS onset, suggesting a transient pre-aggregative state.
INTRODUCTION
1. Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is the third most common neurodegenerative disease in the world. It is characterized by progressive neuronal loss and degeneration of upper motor neuron (UMN) and lower motor neuron (LMN) (figure 1). Patients affected with ALS typically suffer from progressive muscle weakness and atrophy and usually die from respiratory insufficiency within 2-3 years of symptoms onset. (Hong-Fu Li and Zhi-Ying Wu, 2016). ALS is an incurable disease and the only modifying therapy is limited to riluzole, with a modest effect on disease progression (Senda Ajround-Driss and Teepu Siddique, 2014).

Fig. 1 Representation of upper and lower motor neuron. (Rowland 2011)
The incidence of the disease is 2-3 per year per 100,000 population, with a risk of developing ALS of 1:350 for men and 1:400 for women (S. Morgan and R.W. Orrell, 2016).

The etiology of ALS is not fully understood, but many factors have been considered, including gene’s mutations, mitochondrial damage, oxidative stress, glutamate excitotoxicity, viral infection, protein aggregation, environmental factors (figure 2). In addition microglia and astrocytes seem to be involved (J JM LOan et al., 2012).

![Cellular and molecular processes mediating neurodegeneration in ALS.](image)

**Fig. 2** Cellular and molecular processes mediating neurodegeneration in ALS.

The recent advances in research on ALS have established that the disease has a robust genetic basis (Kinsley L and Siddique T., 2001; Renton AE1 et al., 2014) while the putative contribution of non-genetic risk factors is still under investigation (Ingre C et al., 2015; Al-Chalabi A et al., 2013; Bradley WG et al., 2013).
Since the identification of first causative gene in 1993, a growing number of ALS-causative genes associated with Mendelian inheritance have been identified. To date, at least 21 chromosomal regions containing 19 identified genes have been linked to ALS. These genes offered a unique opportunity to decipher the molecular mechanisms of neuronal degeneration (Senda Ajround-Driss and Teepu Siddique, 2014).

In 90% of cases ALS is sporadic (sALS) and it is only in 10% of the case genetically transmitted (fALS) (figure 3). Among those 20% of fALS cases which are caused by the mutation in SOD1 gene, 4-5% of fALS cases are the result of mutations in TARDBP and FUS genes, more than 30% of fALS cases are associated with C9ORF72 mutations and the rest are due to the mutations in alsin, senataxin, spatacsin, vesicle associated membrane protein associated protein B (VAPB), angiogenin (ANG), factor induced gene 4 (FIG4), optineurin (OPTN) and perhaps other unknown genes (table 1) (Chen S. et al., 2013).

Fig. 3 – ALS genetic: 90% of cases are sporadic (SALS); 10% are FALS, with C9ORF72, SOD1, TARDBP and FUS being the main altered genes.
Table 1. The genetic of fALS

Among the genes involved in ALS, Cu,ZnSOD1, FUS, TARDP and C9orf72 are those most frequently screened for mutations in familial and sporadic cases (Renton AE1 et al., 2014; Lattante S. et al., 2015). Furthermore, recent evidence suggests that the intermediate CAG expansions of the spinocerebellar ataxia-related genes, ATXN-1 and ATXN-2, are playing a role as genetic risk factors in the disease (Wang M-D. et al., 2014; Conforti FL et al., 2012).

Similarly to other common neurodegenerative disorders, when ALS-related symptoms and signs start, the majority of the disease-specific neurons (i.e., over 70%) are already lost (Arasaki K and Tamaki
M., 1998; Brooks BR et al., 2000). Given that the onset of ALS is generally subtle, and because of the lack of a reliable biological marker, the diagnosis is often reached after a relatively long delay (Cellura E. et al., 2012) when further motor neurons are degenerated and the disease is generally in a full-blown stage.

This has been one of the main reasons why nearly all clinical trials on ALS, carried out in the last twenty years, have repeatedly failed (Mitsumoto H. et al., 2014).

![Diagram of the clinical stages of ALS]

It becomes therefore very important to understand the pathophysiological and the clinical changes occurring in ALS at the very beginning or even at the pre-symptomatic stage, in order to implement early and potentially effective therapeutic interventions (Benatar M. and Wuu J., 2012).
2. **Fused in Sarcoma (Fus)**

Fused in sarcoma/translocated in liposarcoma (FUS/TLS) is an RNA/DNA binding protein involved in the pathogenesis of the neurodegenerative disorder amyotrophic lateral sclerosis. The FUS/TLS gene, localized on chromosome 16, consists of 15 exons and 14 introns (Morohoshi et al., 1998) and span about 12 Kb.

Chromosomal translocation of FUS/TLS was first found in human myxoid liposarcomas and acute myeloid leukaemia and results in the production of an oncogenic fusion protein formed by the N-terminus of FUS and the DNA-binding domain of an endogenous transcription factor such as CHOP and ERG (Ichikawa et al., 1994; Crozat et al., 1993; Rabbitts et al., 1993).

FUS belongs to the FET-protein family along with EWS (Ewing sarcoma) and TAF15 (TATA binding protein-associated factor 15 (Bertolotti et al., 1996; Law et al., 2006; Tan & Manley, 2009). This family of proteins is involved in all stages of RNA metabolism, and also interacts with DNA and proteins.

FUS gene is ubiquitously expressed in human tissues and in cultured cell lines but is absent from cardiac endothelium and muscle cells and melanocytes (Aman et al., 1996; Andersson et al., 2008; Morohoshi et al., 1998). The subcellular localization of the FUS/TLS protein is cell-type dependent. FUS/TLS is mainly a nuclear protein, but it is also present in the cytoplasm of many cells, except hepatocytes, where FUS/TLS is only present in the cytoplasm (Andersson et al., 2008).
The molecular weight of human FUS protein is 53,426 daltons although it migrates as a band at approximately 70 kDa in SDS-PAGE. (Gal et al., 2011).

FUS is a 526 amino acid multidomain protein composed of a Ser, Gly, Gln and Tyr-rich region containing degenerate repeats of S-Y-S-G/Q sequences at the N-terminal region, a glycine-rich domain, RNA-recognition motif (RRM), a zinc finger domain (ZFD), arginine-glycine-glycine rich domains (RGG1, RGG2) and C-terminal nuclear localization signal (NLS) (Dormann D et al., 2012).

FUS binds RNA in vitro and in vivo (Crozat, et al., 1993; Zinszner et al., 1997) and is involved in several mRNA-processing steps, such as transcription regulation (Uranishi et al., 2001), RNA splicing (Meissner et al., 2003), and RNA transport including nucleo-cytoplasmic shuttling (Zinszner et al., 1997).

FUS also binds single- and double-stranded DNA and plays a role in DNA repair through homologous DNA pairing and recombination (Baechtold et al., 1999), and acts as a transcriptional regulatory sensor of DNA damage signals to assure genomic integrity (Wang et al., 2008).
FUS deficient mice indeed show high levels of chromosomal instability and perinatal mortality (Hicks et al., 2000) and an increased sensitivity to ionizing radiation (Kuroda et al., 2000).

To date over 50 different FUS mutations have been identified in patients with ALS, and these mutations account for about 4% of fALS and 1% sALS cases in the Caucasian population (Liu X. et al., 2015).

Many ALS-associated FUS mutations are mostly clustered in the C-terminal proline-tyrosine nuclear localization signal (PY-NLS) and impair Transportin-mediated nuclear import of FUS (Bentmann E. et al., 2012). Generally, transportins bind to the nuclear localization signal (NLS) of FUS proteins in the cytoplasm. The transportin/FUS complexes cross the nuclear pore complex (NPC) through the interactions of the transportin with nucleoporins. In the nucleus, transportins are bound by
Ran-GTP, which releases FUS. Transportins are then recycled to the cytoplasm in association with Ran-GTP. On the cytoplasm face of the NPC, Ran hydrolyzes its bound GTP into GDP and dissociates, freeing the transportin for a new import cycle (Twyffels et al., 2014).

Interestingly, mutations that show a very severe nuclear import defect, such as P525L, cause an unusually early disease onset and rapid disease progression, suggesting that impaired nuclear import of FUS is causally linked to the disease.

So far, it is unclear whether reduced motor neuron (MN) survival in ALS patients is due to loss of nuclear function or gain of a still unidentified toxic function in the cytoplasm, or a combination of both (Lenzi et al, 2015).
3. **Fused in Sarcoma (Fus) and Stress Granules**

Cytoplasmic inclusions containing Fused in Sarcoma (FUS) are a hallmark of ALS. The origin and nature of pathological inclusions remains poorly understood. It has been proposed that the formation of these pathological inclusions may be due to misregulation of the stress granules (SG) response.

Stress granules are cytosolic structures that form transiently upon exposure of cells to stressful conditions, such as heat shock (42-44°C), osmotic shock, UV irradiation, or substances that elicit mitochondrial and/or oxidative stress (e.g. DTT) (Bentmann E. et al, 2012). SGs function to sequester, silence and/or degrade RNA transcript as part of a mechanism that adapts patterns of local RNA translation to facilitate the stress response (Wolozin et al., 2012). They contain polyadenylated RNA, small ribosomal sub-units, translation initiation factors (eIF3, eIF4E, eIF4G), and RNA binding proteins (RBPs) (Aulas A. et al., 2015). Among the latter group, TIA-1 (T-cell restricted intracellular antigen-1) and G3BP1 (Ras GAP SH3 domain-binding protein 1) are the two most commonly studied and utilized SG markers (Kedersha et al., 1999; Tourriere et al., 2003; Gilks et al., 2004). Finally, SGs dissolved 1-3 hrs after the stress is removed, and were inhibited by cycloheximide treatment (Kedersha et al., 1999).

Many RNA binding proteins (FUS, TDP43, SMN) linked to neurodegenerative diseases associate with SGs in cell culture, and co-localize with SG markers in cells undergoing stress. Mutations in RNA binding proteins appear to increase their propensity to aggregate and to form SGs (Wolozin 2012).
Recently, a two-hit model has been proposed to account for cytoplasmic FUS toxicity in ALS (Dormann et al., 2010). Cytoplasmic mislocalization of FUS, either through genetic mutations or other unidentified factors, represents the first hit. The first hit alone may not be sufficient to cause disease.

However, a second hit (oxidation, heat-shock, viral infection or hypoxia) stemming from cellular stress directs cytoplasmic FUS into stress granules.

Localization of FUS to SGs is independent of its glycine-rich prion-like domain but does require its capacity to bind mRNA (Anderson et al., 2008; Bentmann et al., 2012; Daigle et al., 2013). Intriguingly, mutant FUS localized to SGs will further recruit wild type FUS protein (Vance et al., 2013).

So, cytoplasmic mislocalization of FUS protein, followed by cellular stress, contributes to the formation of cytoplasmic aggregates that sequester FUS, disrupt RNA processing and initiate motor neuron degeneration (Vance et al., 2013).
RESEARCH OBJECTIVES
The aim of this study was to point out the presence of biochemical alterations among the members of a Sicilian family with FUS P525L mutation carriers, which might cause an aggressive form and an early onset of the disease.

More specifically, we carried out a biochemical and immunocytochemical characterization of the FUS protein expression using primary cultures fibroblasts obtained through skin biopsy from FUS P525L mutation asymptomatic carriers in order to investigate possible alteration of the normal protein intracellular localization without signs or symptoms of the disease. We demonstrate that mutant P525L FUS is mislocalized to the cytoplasm of fibroblasts of the asymptomatic mutation carriers. Both wild-type and mutant FUS are sequestered into cytoplasmic foci after a stressful insult; however, the cytoplasmic granules of FUS P525L fibroblasts persist longer and appear more numerous, than those of healthy controls and sALS, suggesting a pre-aggregative state.
SUBJECTS, MATERIALS AND METHODS
1. Subjects

All subjects and patients involved in this study underwent a genetic counselling and gave their informed consent for the genetic testing and skin biopsy. All experiments were carried out according to the World Medical Association Declaration of Helsinki.

A Sicilian ALS family carrying a FUS P525L was recently described in a collaborative study (Chiò et al., 2009). This FUS mutation is located at the C-terminus of the FUS protein, in the Nuclear Localization Signal (NLS) domain.

As shown in Fig 1, in this family there were several members affected, and we could follow up the proband (IV-1), a girl with a bulbar-onset form of the disease who died at the age of 22 years because respiratory failure.

Three healthy siblings of the proband (two sisters (IV-2 and IV-5), turquoise circles in Fig. 1, and one brother (IV-3), green square in Fig. 1, after a careful genetic counselling, took the decision to undergo a genetic testing for FUS P525L mutation. Sequencing of the FUS exon fifteen gene revealed that the two sisters, but not the brother, were carrying the P525L mutation. The two sisters agreed to be submitted to a thorough clinical, biochemical and neurophysiological (i.e., EMG/ENG in the four limbs, including sternocleidomastoid and trapezius muscles, following the Awaji criteria (De Carvaho et al., 2008), and motor evoked potentials) workup in order to unveil signs and/or symptoms of ALS.

The neurological examination did not disclose abnormalities; the biochemical and neurophysiological evaluations were all within normal range. A neurological, biochemical and neurophysiological follow-up was then planned to be performed every six months. At the time of this
study, we are at the third follow-up: the two IV-2 (mutation carrier 1, MC1) and IV-5 (mutation carrier 2, MC2) sisters are healthy, without signs or symptoms of motor neuron degeneration.

The brother without mutation (IV-3) accepted to be a healthy control (HC) in this study (HC1). Another HC is a 58-year-old man (HC2), unrelated to the FUS P525L ALS family.

We finally included two sporadic clinically-definite ALS patients, who had been diagnosed according to the current clinical and neurophysiological diagnostic criteria (De Carvaho et al., 2008; Brooks et al., 2000).

![Pedigree of the family with P525L mutation](image)

Fig. 1 Pedigree of the family with P525L mutation
2. Skin biopsy and Human Fibroblasts Culture

A punch biopsy was obtained from the volar region of the left arm of each subject enrolled. After having disinfected and anesthetized the interested area, the skin will be cut with the help of a biopsy punch, with a rotary and compressive movement. The obtained sample is put into a tube containing cold PBS, a phosphate buffer whose function is to slow down cellular metabolism and avoid oxidative stress. Each skin biopsy was then cut into four pieces, each of which was cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% calf serum, 2 mM L-glutamine, 5 mM pyruvate, 100 U/mL penicillin and 100 µg/mL streptomycin. The medium was changed every three-four days until the fibroblasts were grown to confluence. Fibroblasts obtained from the skin biopsy were maintained in culture through passages on flask. The culture medium was changed every three days. Each biopsy was coded to ensure subject’s anonymity.

Fibroblast cultures, despite being peripheral cells, offer many advantages for the genetic and biochemical studies of neurological diseases as they are easy to cultivate, maintain and preserve. The cellular system of the in vitro cultured dermic fibroblasts allows us to:

- study patients with similar cellular and molecular characteristics, which will be compared with those of cells obtained from non-affected subjects (healthy control);
- analyze the in vivo mutated proteins: an in vitro cellular system is a good model to study the in vivo organism, as some of the characteristics of the skin cells are maintained by the cultivated cells;
• study the effects of a genetic mutation causing the disease on the biological mechanisms activated in the cell, like for instance cellular survival, growth and migration.

3. Stress induction

Human fibroblasts were placed either on glass coverslips (for immunocytochemistry, cells and stress granules counting) and incubated twenty-four hours at 37°C in a 5% CO2 atmosphere. Two stress paradigms were used to acutely induce stress granule (SG) formation: i) a heat-shock, made by floating the cell plate in a 44°C pan of water in a CO2 incubator for one hour (Kedersha et al., 1999; Dorman et al., 2010); ii) a Dithiothreitol (DTT) treatment according to Maharjan et al. (2016). Shortly, cells were incubated with medium containing 3 mM DTT in a CO2 incubator for three hours to induce stress. DTT was then washed off by changing medium. Control cells were kept in the incubator.
4. Subcellular Fractionation

Subcellular fractions (nuclei and cytoplasm) were prepared from human fibroblasts at ~ 80% confluence, as previously described (La Bella et al., 2000) (figure 2). Cells were resuspended in RSB 100 Buffer (10mM Tris-HCl, pH 7.4; 100mM NaCl; 2.5mM MgCl2), containing protease inhibitors (Sigma, St. Louis, MO) and digitonin (40 µg/ml) and incubated for 10 minutes at 4°C.

The digitonin stock solution was dissolved in ethanol because the alcoholic solution of digitonin forms insoluble complexes with cholesterol (plasma membrane) but it does not form these with cholesterol esters and then it preserves the nuclei.

Cells were then disrupted by passage through a 25 G needle, and centrifuged at 900xg for 10 min at 4°C. The pellet obtained corresponded to the nuclei and the supernatant corresponded to the cytoplasm, see schema in Fig 2. Later the nuclei were resuspended in RSB 100 buffer with protease inhibitors. All fractions were finally equalized by volume and stored at -80°C until further use.

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**Fig. 2** Flow chart for the subcellular fractionation.
5. **SDS-page and Western blotting**

Subcellular fractions were thaw on ice, mixed with SDS-PAGE sample buffer, and boiled at 95°C for 5 min. All samples were spun down for 2 min, loaded on to a 12% polyacrylamide gel and run at 35mA for about 1 h. Proteins were then electrobotted on to PVDF filters (0,45µm pore size, Millipore). Blots were blocked with 10% milk in PBS with 0,2% Tween-20 (PBS-T) for 2-3 h at room temperature, and then incubated with 1:1200 dilution polyclonal FUS antibody (Proteintech Group, Inc., Chicago, USA) overnight at 4 °C. This antibody was raised against a peptide between amino acids 52 and 400 (GeneBank accession number: BC026062;). Secondary (HRP)-conjugated donkey antirabbit antibody (Chemicon, Damstadt, Germany) was used at a dilution of 1 : 7000 in 1% milk/PBS-T.

After washing the filters with PBS-T, protein bands were visualized using SuperSignal® West Pico Chemiluminescent Substrate (Pierce Biotecnology, Rockford, IL, USA). The detection of chemiluminescence was performed by ChemiDoc-It Imaging Systems (UVP, Cambridge, UK).

In some experiments, filters were washed and stripped out of the bound antibodies (La Bella et al., 2000). After re-blocking, a membrane was reused for detection of α-tubulin, a protein stained with a specific monoclonal antibody, at 1:1250 dilution (Sigma, St. Louis, MO), and the other for detection of SP1 ( 1:1000; Santa Cruz Biotechnology), a nuclear fraction marker. Densitometry of the immunoblots was performed with the Image J software.
6. Immunofluorescence

Skin fibroblasts were cultured on glass coverslips until ~70% confluency. Fibroblasts from each subject were plated in duplicate.

Cells were rinsed twice with ice-cold PBS and then fixed with 4% paraformaldehyde. After washing with PBS, cells were permeabilized with 0.5% Triton X-100 containing 50mM L-lysine in PBS and incubated with blocking buffer (4% BSA/2% horse serum in PBS). For immunofluorescence staining, cells were incubated overnight with polyclonal FUS antibody (1:500; Proteintech Group, Inc., Chicago, USA).

For double staining experiments and stress granules detection after stress induction, we used both a polyclonal FUS antibody and a monoclonal anti-TIA-R antibody (BD Transduction LaboratoriesTM, San José, CA, USA), a specific marker of stress granules, at 1:500 dilution (Kawakami et al., 1992). Cells were incubated with the primary antibodies overnight at 4°C.

Cells were then washed with PBS containing 0.1% Triton X-100 and then incubated with Cy3- conjugated donkey anti-rabbit antibody (1:1000; Chemicon Intl. Inc., Temecula, CA, USA) for 90 minutes at room temperature. The secondary antibody used for TIA-R detection was a FITC-conjugated donkey anti-mouse antibody at 1:500 dilution (Chemicon Intl. Inc., Temecula, CA, USA).

After four rinses with PBS, coverslips were finally mounted on glass slides and stored at 4°C until analysis.

Laser confocal microscopy was performed with a Zeiss LSM 5 EXCITER confocal microscope with argon ion laser set at 548 nm for
Cy3 excitation or 495 nm for FITC excitation. Digitized series of optical sections were recorded and images for each staining reconstructed.

7. **Cell counting and subcellular FUS expression.**

For cell counting, coverslips with the coded fibroblasts from the two sporadic ALS (sALS), the two asymptomatic P525L mutation carriers and the healthy controls were placed under an Olympus Microscope with the fluorescence lamp set at 548 nm for Cy3 excitation and 495 nm for FITC excitation.

The pattern of FUS staining in the plated cells was categorized as follows: i) cells with exclusive nuclear FUS staining (the cytoplasmic staining being faint or absent); ii) cells with both a nuclear and a cytoplasmic staining; iii) cells with only a cytoplasmic staining.

For each experiment, ~3000 cells from each subject were plated in duplicate. Two of us (MLB and FDF) blindly and independently recorded each pattern of staining present in 10 adjacent microscopic frames, i.e. 5 upward and 5 downward from the center of the rounded coverslip. A microscopic frame is an area delimited by a rectangle projected on the dish, and it is a tool of the inverted IX70 Olympus Microscope. Therefore, a relatively large microscopic field in each coverslip was analyzed twice. The agreement between the two counting researchers on the total number of cells per well was above 85%. Agreement for each pattern of staining was always above 90%.

Each experiment was carried out in duplicate.
8. **Evaluation of the number cells containing stress granules**

After stress induction with either heat-shock or DTT, the presence and morphology of the stress granules in immunofluorescence, using both FUS and TIA-R antibodies, was observed and recorded. Each glass coverslip was coded and the proportion of TIA-R-positive cells containing stress granules, over the total number of cells present in five consecutive microscopic frames, was independently counted by two of us (LBM and AN). The analysis was made at different times after the end of the stressful insult (i.e., T0, T1.5, T3.0, T4.5, T6.0, T9.0, T12 and T24 hours). We took pictures of the five consecutive microscopic frames with an Olympus C-5060 Camedia digital camera. Pictures were then transferred to a computer for analysis. Inter-rater agreement was above 88%; hence, data from the two independent counts were pooled together.

9. **Evaluation of the number of stress granules per cell**

The number of granules per cell was also counted. Shortly, each coverslip was coded, and after a single stress insult, cells were double stained for FUS and TIA-R at different time points (i.e., T0, T1.5, T3.0, T4.5, T6.0 hours). Pictures of 20 individual FUS- and TIA-R-stained cells, present in two-three adjacent microscopic frames of the IX70 Olympus microscope, were taken.

Pictures were transferred to a computer and two of us (MLB and AN), blind for the experiment, independently counted the number of TIA-R-positive granules per cell at each time point after stress.
10. Data analysis and statistics

Data obtained by counting the cells showing the different patterns of FUS staining in each subject were calculated as the percent of the total number of counted cells and expressed as mean + SD of three separate experiments. Data from either the two HCs or the two sALS were pooled. Given the high level of agreement between the two counting researchers, the data from each duplicate count were pooled.

The proportion of cells containing granules at different times after stress insults, in each subject group, was represented as mean + SD of two different experiments. Data from each FUS P525L mutation carrier were analyzed individually. Finally, the number of granules per cell at different times after stress insults were expressed as mean + SD of 20 cells per subject in two independent experiments. Data from the healthy subjects and sALS were pooled.

For the statistical analysis, we used the SIGMASTAT 3.5 software package (Systat Software Inc., San Jose, CA, USA). Differences in the proportion of the different patterns of FUS staining, the granule-containing cells and the number of granules per cell for each subject group were analyzed with one-way ANOVA, followed by post-hoc Dunnett’s analysis. p values <0.05 were considered significant.
RESULTS
The fibroblasts cultured from the skin biopsies of the healthy subject, the two FUS P525L mutation carriers and the two sporadic ALS showed a similar gross morphology. Cells were apparently healthy in culture.

Fig. 1 Fibroblasts from the IV-2 FUS P525L mutation carrier.
1. **FUS is mislocalized to cytoplasm in cells of asymptomatic FUS P525L mutation carriers**

To study the subcellular localization of FUS protein in the skin fibroblasts, we used the digitonin method to isolate the nuclei from the whole cytoplasm (Fig. 2). The two nuclear and cytoplasmic fractions were equalized by volume.

![Fig. 2 Nuclei](image)

We then performed immunoblot studies with a polyclonal anti-FUS antibody and found a relatively strong FUS protein expression in the nuclear fraction of all samples examined (Fig 3A e 3B). FUS expression in the cytosol extracts was instead very different between samples.
Fig. 3 Differential expression of FUS protein in the nuclei (N) and cytoplasm (C) of fibroblasts from the two FUS P525L mutation carriers (MC1 and MC2), a healthy control (HC) and two sALS patients. Fractions were incubated with a polyclonal anti-FUS antibody. α-Tubulin and Sp1 antibodies marked the cytoplasm and nuclear fractions, respectively. (C) Densitometric analysis of the relative FUS-related band intensity of the nuclear and cytoplasmic fractions from HC, MC1, MC2 and sALS. Data are expressed as mean ± SD of three independent blots. * p < 0.05 one-way ANOVA with post-hoc Dunnett’s analysis.
Cytosolic FUS protein expression was strong in the two FUS P525L mutation carriers, faint in the healthy subject and almost absent in the two sporadic ALS. Therefore, FUS protein appears specifically mislocalized in the cytoplasm of preclinical FUS P525L mutation carriers. α-tubulin and Sp1 antibodies allowed identification of the nuclear and cytoplasmic fractions (Fig 3A).

The relative densitometry of the FUS-related bands in the nuclear and cytoplasmic fractions confirmed that the protein level was significantly higher in the cytoplasm of the two FUS P525L mutation carriers compared to the HCs and sALS (p < 0.05 one-way ANOVA with post-hoc Dunnett’s analysis; Fig. 3C).
To further confirm the subcellular mislocalization of FUS protein in the two preclinical FUS P525L mutation carriers, we performed immunocytochemical studies on the cultured skin fibroblasts.

**Fig. 4** FUS mislocalization to the cytoplasm of the fibroblasts of FUS P525L mutation carriers. (A) Representative confocal images of immunofluorescence experiments performed with the polyclonal anti-FUS antibody on fibroblasts from the healthy control # 2, FUS P525L mutation carrier 1 and sporadic ALS 1. (B) Comparison of the number of cells expressing FUS in the nucleus (N) only, in the cytoplasm (C) only or in both nucleus and cytoplasm (N + C). hatched bars: healthy control (pedigree # IV-3; black bars: FUS P525L carrier 1 (pedigree # IV-2); white bars: FUS P525L carrier 2 (pedigree # IV-5); grey bars: sporadic ALS (combined # 1 and # 2). Data are expressed as % of the total analyzed cells (mean + SD of three separate experiments done in duplicate wells). * p < 0.05 one-way ANOVA with post-hoc Dunnett’s analysis.
Fig. 4A shows the pattern of FUS staining in representative cells of the different subjects studied. In the healthy subject (HC2), FUS was expressed in the nucleus in almost all cells. A weak cytoplasmic staining was seen in about twenty percent of the cultured cells.

In the FUS P525L mutation carriers, most cells (i.e., over 70 percent) showed a diffuse nuclear and cytoplasmic staining, with about ten percent of the cultured cells with an exclusive cytoplasmic staining. Cells with an exclusive nuclear staining were a minority in these subjects. Finally, in fibroblasts from sALS, FUS was mostly expressed in the nucleus, with a few cells showing a concomitant nuclear and a cytoplasmic staining. Nuclear or cytoplasmic aggregates were not seen in any of the cultured cells.

In the majority of cells from the two preclinical FUS P525L mutation carriers, FUS was expressed in both nucleus and cytoplasm at a percent significantly higher than the healthy subject and the two sporadic ALS patients (p < 0.05 one-way ANOVA with post-hoc Holm-Sidak analysis) (figure 4B).

These experiments further demonstrate that FUS is mislocalized in the cytoplasm of presymptomatic FUS P525L mutation carriers.
2. Cellular stress induces cytoplasmic FUS recruitment into stress granules

The evidence that C-terminal FUS mutations can lead to protein recruitment into stress granules (Dormann et al., 2010; Vance et al., 2013), prompted us to study the effect of stress on wild-type and the mutant P525L FUS proteins redistribution in the cells. Heat-shock and DTT were the two stressors used to transiently insult the fibroblasts. TIA-R antibody was used as a marker of the stress granules. In control conditions, FUS was mostly nuclear in sALS and HCs, while, as expected, the mutated protein was diffusely expressed in the cytoplasm of the MC1 and MC2 fibroblasts (Fig 5A, D, G, J and Fig 6A, D, G, J). TIA-R showed in all control cells a nuclear and cytoplasmic staining (Fig 5B, E, H, K and Fig 6B, E, H, K). Merging the pictures highlighted a co-localization of both proteins in the nucleus and, for the FUS P525L fibroblasts, in the cytoplasm (Fig 5C, F, I, L and Fig 6C, F, I, L).
Fig. 5 e 6 Cytosolic FUS is recruited to SG upon treatment with various stressors. Fibroblasts were subjected to dithiothreitol (DTT) 3mM for 3 hrs (A) and to heat shock (44°C) for 1 h (B). Cells were fixed and stained with antibody anti-FUS (red) and anti-TIAR (green). Stress induced cytoplasmic granules formation in all subjects, and FUS was recruited into them.

Heat-shock (44°C, one hour) induced the appearance of TIA-R-positive stress granules, but with a heterogeneous morphology: a number of cells showed a diffuse cytoplasmic micro-granulation (e.g., Fig. 5B1, K1), while in other cells stress granules appeared well rounded (e.g., Fig. 5E1, H1). Note that this heterogeneous pattern of stress granules was detectable in the fibroblasts of all subjects under study (i.e., healthy controls, sALS patients, asymptomatic FUS P525L mutation carriers), the proportion of cells showing the micro-granulation being slightly predominant (data not shown).

In fibroblasts of sALS, stress induced FUS redistribution to the cytoplasm (Fig. 5A1), where it fully co-localized with TIA-R into stress granules (Fig. 5C1). In cells of the HCs, FUS remained mostly nuclear.
(Fig. 5D1) where it overlapped with TIA-R (Fig. 5F1). In the fibroblasts of MC1 and MC2, mutated P525L FUS co-localized with TIA-R into stress granules, both in the micro-granules or in bigger, punctuate, granules (Fig. 5G1, H1, I1 and Fig 5J1, K1, L1).

We conclude that heat-shock induces in fibroblasts the appearance of either diffuse micro-granules or dotted stress granules, and that it causes FUS redistribution into the cytoplasm of the cells from sALS patients. When localized to the cytoplasm, FUS is fully recruited into stress granules, irrespective of their specific morphology.

When 3mM DTT was used as stressor, all fibroblasts, irrespective of the subject they were derived from, showed TIA-R-positive dotted stress granules in the cytoplasm (Fig. 6B1, E1, H1, K1). FUS remained mostly nuclear in HCs, with a very few FUS-positive granules detectable (Fig. 6D1); in sALS, the protein relocated to the cytoplasm and in stress granules (Fig. 6A1). Merging the pictures revealed FUS-TIA-R co-localization only in the nucleus for HCs (Fig. 6F1) and in the stress granules for sALS (Fig 6C1). Similarly, DTT stress induced a full recruitment of cytoplasmic mutated P525L FUS into stress granules (Fig. 6G1 and J1) where it co-localized with TIA-R (Fig. 6H1, K1) in all cells: see merged FUS-TIA-R pictures in Fig.6 I1 and L1. Therefore, the two stress paradigms confirm that cytoplasmic FUS, either wild-type or with P525L mutation, can be recruited into stress granules.
3. Cell viability and proliferation after stress induction

After the end of the stressful insult we counted cells at different time (i.e., T0, T1.5, T3.0, T4.5, T6.0, T9.0, T12 and T24 hours). We observed that fibroblasts from all subjects appeared to be able to eventually recover after 6 hours from the heat stress treatment, but they underwent a growth arrest following treatment with DTT (figure 7). So, with regards to cell viability and proliferation, fibroblasts from our subjects with FUS P525L mutation were not more susceptible to damage from heat shock and DTT than heathy control and sALS.

**Fig 7.** Effect of HS (44°C, 30 min) and DTT exposure (3 mM/3 hrs) on cell viability and proliferation.
4. Long persistence of the stress granules in the cytoplasm of FUS P525L fibroblasts

Recently, it has been suggested that FUS mutated at the C-terminal may induce neurodegeneration through a two-hit mechanism, in which FUS can be permanently sequestered into stress granules (Buratti & Baralle, 2010; Dorman et al., 2010). This hypothesis prompted us to verify whether cytoplasmic FUS, either wild type or mutated, when recruited into stress granules after acute stress, would lead to a pathological persistence of these structures in the fibroblasts.

As DTT-induced stress always led to a homogeneous appearance of discrete foci in the cells, to avoid any miscounting we chose this stress paradigm to evaluate the proportion of fibroblasts containing TIA-R-positive granules at different times after the stressful insult.

![Graph showing percent of cells with stress granules after DTT treatment.](Image)

**Fig. 8** Percent of cells with stress granules after DTT treatment.
As shown in Fig 8, immediately after stress (T0) almost all cells contained granules, irrespective of the subject the fibroblasts came from. However, while the granule-containing cells of the HCs rapidly decreased, becoming negligible 9 – 12 hours after stress, the proportion of granule-containing cells in the FUS P525L fibroblast population from the two mutation carriers (MC1 and MC2) remained significantly higher than the HCs up to 4.5-6 hours after the insult (p < 0.05 at 1.5, 3.0 and 4.5 hours after stress for FUS P525L fibroblasts from carrier 1 and carrier 2 vs pooled healthy controls, one-way ANOVA with post-hoc Dunnett’s analysis).

Note that at 6.0 hours after stress, the FUS P525L fibroblast population from MC1 showed a proportion of cells with granules significantly higher than all other groups.

The fibroblasts from the two sALS patients (data were pooled) showed an intermediate behavior. Cells containing FUS-positive stress granules decreased over time, but at a rate slightly slower than the HCs (Fig. 8).

A 9.0 hours after stress or later (cells were counted up to 24 hours) no differences between groups could be seen, the proportion of granule-containing cells being very low in all.

To further evaluate the time course of the DTT-induced stress granules in the fibroblasts, we counted for each subject the number of TIA-R-positive granules per cell.
As shown in TAB. I, immediately after the stress insult, the number of cytoplasmic stress granules per cell was not different among groups. However, while the number of granules regularly decreased in fibroblasts of HCs and sALS patients, it remained relatively high in the FUS P525L fibroblasts even at six hours after stress (p < 0.05 at 1.5, 3.0, 4.5 and 6.0 hours after stress for FUS P525L fibroblasts from MC1 and p < 0.05 at 1.5 and 6.0 hours after stress for FUS P525L fibroblasts from MC2, both vs pooled healthy controls; one-way ANOVA with post-hoc Dunnett’s analysis).

<table>
<thead>
<tr>
<th>Subject</th>
<th>Time after DTT exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T 0</td>
</tr>
<tr>
<td>Healthy Controls</td>
<td>68 ± 19</td>
</tr>
<tr>
<td>(two subjects)</td>
<td></td>
</tr>
<tr>
<td>Mutation Carrier 1</td>
<td>75 ± 23</td>
</tr>
<tr>
<td>Mutation Carrier 2</td>
<td>73 ± 20</td>
</tr>
<tr>
<td>sALS (two patients)</td>
<td>71 ± 34</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD. * p < 0.05 vs healthy controls, ANOVA with post-hoc Dunnett’s analysis.
We conclude that stress granules tend to persist in the FUS P525L fibroblasts and, to lesser extent, in sALS fibroblasts. Therefore, when the mutated FUS P525L protein is recruited into stress granules, it might induce in the cells a transient pre-aggregative state.
DISCUSSION
This study presents, at our knowledge, the first evidence of a cytoplasmic mislocalization of FUS in skin fibroblasts of presymptomatic FUS P525L mutation carriers. Acute stress induced cytoplasmic FUS recruitment into stress granules. The granules containing the mutant P525L FUS persist in the cells longer than granules with wild-type FUS, suggesting a transient pre-aggregative state. This condition is likely to be a necessary step, though not sufficient, to induce motor neuron degeneration. These results support the two-hit hypothesis of mutant FUS-mediated motor neuron degeneration, in which FUS-containing stress granules may transform into permanent aggregates when either a long-lasting stress or another type of cellular insult is applied (Buratti & Baralle, 2010; Dorman et al., 2010; Aulas & Vande Velde, 2015).

FUS is a 526 aminoacid protein that was originally described as a component of a fusion oncoprotein associated with liposarcoma (Crozat et al., 1993), and it is involved in RNA transcription, processing and transport (Crozat et al., 1993; Renton et al., 2014). Furthermore, FUS is also involved in mRNA export and mRNA transport to neuronal dendrites (Fujii & Takumi, 2005).

In human neurons, FUS is physiologically present in the nucleus, with a low level of expression in the cytoplasm. A very similar profile has been also detected in the skin fibroblasts (Andersson et al., 2008).

Recently, it has been shown that mutations in the FUS gene are responsible for some 5% of familial and 1% of sporadic ALS (Renton et al., 2014; Lattante et al., 2015). Mutant FUS protein has been observed in cytosolic inclusions in motor neurons, with a concomitant reduction of its nuclear staining (Kwiatkowski et al., 2009; Vance et al., 2009;
Bäumer et al., 2010). Furthermore, FUS appears to be misplaced to the cytoplasm of cells transfected with the mutant protein (i.e., R521C, R521H, R521C) (Kwiatkowski et al., 2009; Vance et al., 2009), in neurons of ALS patients with P525L mutation (Bäumer et al., 2010; Ito et al., 2011) and skin fibroblasts from patients carrying the R521C mutation and mutations in the 3’ untranslated region of the gene (Sabatelli et al., 2013). In fibroblasts of subjects with sALS, FUS maintains an nearly exclusive nuclear expression (Sabatelli et al., 2013; Kariya et al., 2014).

Our study shows that in the fibroblasts of sporadic ALS patients, with no known mutations, FUS is expressed in the nucleus, whereas in the healthy subject the protein shows also a weak, though detectable, cytoplasmic localization, a data fully consistent with other published works (Ito et al., 2011; Andersson et al., 2008; Kariya et al., 2014). In the two presymptomatic FUS P525L mutation carriers, FUS protein appears heavily misplaced to the cytoplasm, a biochemical abnormality similar to that reported with patients with symptomatic ALS and carrying FUS mutations (Kwiatkowski et al., 2009; Vance et al., 2009; Bäumer et al., 2010; Ito et al., 2011; Sabatelli et al., 2013).

However, while P525L FUS cytoplasmic mislocalization represents a specific biochemical signature of the mutation in the asymptomatic carriers, it is not an indicator of an ongoing neurodegenerative process. Mislocalized P525L FUS in these cells is, in fact, the result of the altered NLS domain that prevents the protein translocation to the nucleus (Dorman et al., 2010). It basically denotes the presence of the mutation.
The molecular pathway involving mutated FUS and leading to the motor neuron degeneration is still imperfectly known. However, it has been suggested that a putative early event might be the abnormal assembling of the cytoplasmic misplaced protein into stress granules (Dorman et al., 2010; Vance et al., 2013; Aulas & Vande Velde, 2015; Lenzi et al., 2015).

Stress granules are transient cytoplasmic foci containing RNA-binding proteins, mRNA, non-translating messenger ribonucleoproteins and several translation initiation factors, that assemble in response to a variety of cellular stresses (Anderson & Kedersha, 2009). Granule disassembling occurs when the stress insult is terminated.

We challenged the fibroblasts from the different subjects with two acute stress paradigms, i.e., heat-shock and DTT, and found that all cells reacted by forming TIA-R positive cytoplasmic stress granules. TIA-R is a specific marker of the stress granules (Kedersha et al., 1999; Anderson & Kedersha, 2009). Noteworthy, TIA-R/mutant P525L FUS-positive granules persisted in the cells longer, and they were more abundant than the cells from healthy controls and sALS patients with TIA-R/wild-type FUS-positive granules, suggesting a dose-effect. That is, the more FUS present in the cytoplasm the higher amount recruited into the stress granules. This explains why we observed more DTT-induced granules in the mutant FUS P525L fibroblasts than in the healthy controls.

In addition, we found no evidence of toxicity associated with treatment with stressors, as measured by cell counting. In particular fibroblasts from all subjects appeared to be able to eventually recover after 6 hours from the heat stress treatment, but they underwent a growth arrest following treatment with DTT (Pani et al., 2000).
Interestingly, in fibroblasts of sALS patients, acute stress (either heat-shock or DTT exposure) induced a cytoplasmic redistribution also of the wild-type FUS which then assembled into stress granules. However, this occurred to a lesser extent than asymptomatic FUS P525L mutation carriers. An indirect support to this result comes from the demonstration that wild-type FUS can be found in cytoplasmic inclusions, containing other proteins (i.e., TDP-43, ubiquitin, p62) and in skein-like inclusions in motor neurons of sporadic ALS (Deng et al., 2010; Keller et al, 2012). Most data on the effect of stress on the FUS cellular expression have been obtained with transfections of the wild-type FUS gene on cell lines or using cells from healthy controls (Dorman et al., 2010; Ito et al., 2011; Vance et al., 2013), showing a pattern of staining similar to what we have seen on cells from our healthy controls. This might not be true for the sALS cells, were other putative disease-related variables may cooperate in facilitating FUS mislocalization to the cytoplasm and recruitment to stress granules under stress conditions.

We suggest that the FUS-positive stress granules, either from the sALS or the mutation carriers, might actually represent an early step towards the development of the cellular inclusions observed in both sALS and mutant FUS motor neurons (Kwiatkowski et al., 2009; Vance et al., 2009; Bäumer et al., 2010; Deng et al., 2010; Keller et al, 2012).

The stress granules in the fibroblasts of asymptomatic FUS P525L mutation carriers might, therefore, represent a pre-aggregative state of the FUS protein. This condition is reversible, and it is certainly not sufficient to explain the whole neurodegenerative process leading to motor neuron cell death in FUS P525L ALS. Also, fibroblasts may not be suitable for the study of all neurodegenerative changes leading to
motor neuron death. Yet, they remain at present the best model for the evaluation of some ALS-related mechanistic processes in living ALS patient and in an asymptomatic ALS gene mutation carriers. An alternate possibility could be the use motor neurons derived from stem cells from fibroblasts of either ALS patients or asymptomatic mutation carriers reprogrammed to a pluripotent state (Dimos et al., 2008; Favarelli et al., 2014; Lenzi et al., 2015). This approach, although not a laboratory routine, is worth a focused effort in the near future, as it may help the disclosure of specific neurodegenerative pathways in asymptomatic mutation carriers.

The two sisters with FUS P525L mutation are now in their third year of follow up, and they are still asymptomatic; the repeated diagnostic workups have been negative. As the FUS P525L mutation is known to be particularly aggressive (Conte et al., 2012), a careful surveillance is performed to detect very early clinical, neurophysiological and biochemical changes indicating the onset of the disease. Unfortunately, we could not obtain fibroblasts from the proband (IV-1 in the pedigree of the FUS P525L family,), which could have given useful information about the biochemical changes in a subject with the actual disease.

All biochemical changes we have seen up to now in the fibroblasts of the asymptomatic FUS P525L mutation carriers might represent early steps of the neurodegenerative process, being, however, insufficient to explain it fully. Moreover, they cannot at present be adopted as preclinical biomarkers of the disease.
FINAL CONCLUSIONS AND FUTURE PROSPECTS
During my PhD period at the Laboratory of Neurochemistry Laboratory, Department of Experimental Biomedicine and Clinical Neurosciences, University Hospital "Paolo Giaccone" in Palermo, I worked on the study of the expression and characterization of FUS protein in fibroblasts of ALS patients and FUS P525L mutation carriers. I have come to the following conclusions:

- Molecular abnormalities occur early in fibroblasts of preclinical asymptomatic FUS P525L mutation carriers (aggressive mutation in the FUS gene);
- Mislocalization of FUS protein takes place in absence of signs and/or symptoms of motor neuron degeneration;
- Cytoplasmic granules form in all subjects following acute stress, and FUS recruitment into them.
- Granules persist longer in fibroblasts from the two FUS P525L carriers, and number of granules per cell is higher than HC and sALS.

These represent important biochemical changes suggesting a role of the protein as a preclinical molecular signature before the disease onset.

This may provide new biological and pathological insights into the understanding of the pathogenesis of FUS-related ALS and prevent, through targeted therapeutic strategies, motor neurons degeneration.
**Future Prospects:**

As this study showed that mutated FUS protein is recruited into stress granules, suggesting a transient pre-aggregative state, we will more investigate in order to understand mechanisms involved in the switching in pathological FUS inclusions.

To reach these aims we will proceed as follow:

- To expose fibroblasts to repeated and chronic stresses;
- To inhibit the formation of stress granules with cycloheximide or emetine;
- To modulate, with activators or inhibitors, autophagic process: failure to clear stress granules by autophagic processes may promote ALS pathogenesis.

Simultaneously we will study the eventual role of the cellular senescence in subcellular distribution of FUS protein.
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