Role of FET proteins in neurodegenerative disorders

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Abstract

Neurodegenerative disorders such as Alzheimer disease (AD), frontotemporal dementia (FTD), amyotrophic lateral sclerosis (ALS), Parkinson disease (PD), Huntington’s disease (HD), and multiple sclerosis (MS) affect different neuronal cells, and have a variable age of onset, clinical symptoms, and pathological features. Despite the great progress in understanding the etiology of these disorders, the underlying mechanisms remain largely unclear. Among the processes affected in neurodegenerative diseases, alteration in RNA metabolism is emerging as a crucial player. RNA-binding proteins (RBPs) are involved at all stages of RNA metabolism and display a broad range of functions, including modulation of mRNA transcription, splicing, editing, export, stability, translation and localization and miRNA biogenesis, thus enormously impacting regulation of gene expression. On the other hand, aberrant regulation of RBP expression or activity can contribute to disease onset and progression. Recent reports identified mutations causative of neurological disorders in the genes encoding a family of RBPs named FET (FUS/TLS, EWS and TAF15). This review summarizes recent works documenting the involvement of FET proteins in the pathology of ALS, FTLD, essential tremor (ET) and other neurodegenerative diseases. Moreover, clinical implications of recent advances in FET research are critically discussed.

Introduction

FUS (Fused in liposarcoma), EWS (Ewing Sarcoma) and TAF15 (TATA binding associated factor 15) belong to the FET family of DNA and RNA binding proteins, together with the Drosophila ortholog Cabeza. FET proteins are highly conserved and ubiquitously expressed. They contain several conserved domains: a serine-tyrosine-glycine-glutamine (SYGQ) domain embedded in the DNA activation domain (AD), 3 glycine-arginine (RGG) rich regions that affect RNA binding, one conserved RNA-binding domain (RBD, formed by a RNA-recognition motif, RRM), and a zinc finger domain that is also involved in nucleic acid binding (Fig. 1).1,2

FET proteins display several physiological roles within the cell. They are involved in multiple steps of DNA/RNA metabolism and in the maintenance of genomic stability.3,4,5 Moreover, all members of the FET family interact with the transcription pre-initiation complex, formed by the RNA Polymerase II (RNAPII) and the TFIID complex, and with several transcription factors.1,2,6,7 In addition to transcription, FET proteins affect also alternative splicing by recruiting the spliceosome machinery and splicing factors, such as the heterogeneous nuclear ribonucleoproteins (hnRNPs) and the SR (Serine-Arginine) proteins, to nascent pre-mRNAs.8-13 Importantly, FET proteins are physiologically subjected to different environmental signals that induce post-translational modifications in their RBD and in the RGG domains, thus modulating their activity.14 In this way, FET proteins can couple signal transduction network with modulation of transcription and RNA metabolism, thus globally impacting on gene expression programs.

FET proteins are involved in sarcoma translocations that give rise to in frame fusion proteins comprising the N-terminal part of a FET protein and a transcription factor belonging to the ETS family at the carboxy-terminus.15,16 These aberrant chimeras are potent transcription factors that guide oncogenic transformation.

FET proteins are expressed in most human tissues, where they mainly localize into the cell nucleus.17 Interestingly, FUS protein is also localized in dendritic granules and spines in neurons where it plays a role in mRNA transport into dendrites, which represents an essential process for local protein synthesis and synaptic plasticity.18,19 Recently, it has been suggested the involvement of FET proteins in neurological diseases, such as frontotemporal lobar degeneration (FTLD) and amyotrophic lateral sclerosis (ALS), where they have been found in cytoplasmic aggregates.20-24 For instance, it was shown that TAF15 and EWS co-accumulate with FUS in neuronal and glial cytoplasmic inclusions in FTLD patients.20 Remarkably, the cells bearing cytoplasmic inclusions frequently show reduced nuclear staining of all FET proteins.20 Although it has been recognized as a pathological hallmark of these diseases, the mechanism leading to cytoplasmic mislocalization of FET proteins in neurodegenerative disease is not well understood yet. In particular, it is still unresolved whether neurodegeneration is caused by the loss of an essential function displayed by FET proteins, and/or other RNA binding proteins (“loss-of-function”), or by a gain of toxic properties of these protein aggregates (“gain-of-function”), or by a combination of both. In this regard, recent data appear
to support a disease model in which motor neuron degeneration occurs through a toxic gain of function mechanism that does not involve the normal activity of these RBPs. In this manuscript we will review and discuss recent papers highlighting the critical role played by FET proteins in neurological disorders.

**Genetic models of FET ablation in mice**

The physiological functions of FUS and EWS have been recently unveiled by the analyses of mouse knockout models of the *Fus/Tls* and *Ewsr1* (encoding EWS) genes, whereas *Taf15* mouse knockout models have not been developed yet. FET mutant

![Diagram of FET proteins](image)

**Figure 1.** Schematic representation of the 3 members of the FET family (FUS, EWS and TAF15). FET proteins share the same domain structure. SYGQ = serine, tyrosine, glycine and glutamine; RGG = represents a region enriched in arginine-glycine-glycine motifs; RRM = RNA-binding domain; ZF = Cys2/Cys2-type zinc finger motif; PY = proline-tyrosine nuclear localization signal (NLS). In the figure, a schematic overview of FUS, EWS and TAF15 mutations identified in ALS patients is represented. del = deletion; ins = insertion; fs = frameshift; X = stop.
mice display very similar phenotypes, such as perinatal lethality, sterility, enhanced radiation sensitivity and defects in B cell development.\textsuperscript{4,27,28}

**Mouse models of Ewsr1 gene ablation**

Ewsr1 knockout mice are sterile, due to meiotic arrest and apoptosis of pachytene spermatocytes.\textsuperscript{4} Notably, EWS protein is critically important for the completion of meiosis in both males and females. After the formation of bivalents, synapsed chromosomes initiate homologous recombination, which is essential for proper segregation of chromosomes. Ewsr1-null spermatocytes display a reduction in the number of MLH1 foci, which mark the sites of recombination. As a consequence, Ewsr1-null spermatocytes undergo massive apoptosis and gamete maturation is completely arrested.\textsuperscript{4} Ewsr1\textsuperscript{−/−} mice display also aging-like characteristics, such as kyphosis, reduced bone density and loss of subcutaneous fat.\textsuperscript{4} EWS protein is also required for the completion of B cell development and Ewsr1-null mice display a severe lymphopenia, whereas erythropoiesis is normal.\textsuperscript{4} Furthermore, Ewsr1-null mice display smaller thymus and spleen compared with their littermates, reflected in a markedly reduced cellularity of these organs, with a slight decrease in the proportion of T cells and a marked reduction in both immature and mature B cell populations.\textsuperscript{1} Further studies indicated that Ewsr1\textsuperscript{−/−} mice exhibit a progressive and severe postnatal atrophy of haematopoietic organs due to pronounced reduction in the number of lymphoid progenitor stem cells compared with wild type mice, while the myeloid progenitors are not affected, suggesting that the drop in the lymphoid population might result from the skewing of stem progenitor cells toward the myeloid lineage.\textsuperscript{29} Notably, mice with deficiency in DNA repair genes display a similar pattern of haematopoietic lineage skewing as that described for Ewsr1\textsuperscript{−/−} mice.\textsuperscript{30} Related to this similar phenotype, Ewsr1\textsuperscript{−/−} mice are highly susceptible to ionizing radiation, and 2 genetic screens identified EWSR1 gene as required for resistance to ionizing radiations and to the treatment with the topoisomerase I inhibitor camptothecin.\textsuperscript{4,31,32} Since in human cells EWS protein was shown to regulate the alternative splicing of genes involved in the DNA damage response (DDR), like ABL1 and CHEK2, it is possible that EWS plays a direct role in the DDR by modulating the expression of genes involved in this biological response.\textsuperscript{5}

Lastly, Ewsr1 deficient mice display drastically reduced intercapular brown adipose tissue (BAT) compared to their wild type littermates.\textsuperscript{33} Ewsr1 mutant brown preadipocytes fail to differentiate due to the loss of Bmp7 (bone morphogenic protein), expression, which is a critical early factor for brown adipogenesis.\textsuperscript{33} Moreover, mouse embryonic fibroblasts (MEFs) lacking Ewsr1 fail to undergo adipogenesis, due to a significant reduction in the expression of early adipogenic regulators such as Bmp2, Bmp4 Cebp\textbeta{}, and Cebp\textgreek{o} (CCAA[T/enhancer binding protein \textbeta{} and \textgreek{o}).\textsuperscript{34}

**Mouse models of Fus/Tls gene ablation**

Similar to deletion of the Ewsr1 gene, inactivation of Fus/Tls in mice leads to defects in B cell development and fertility defects, but the molecular mechanisms affected by EWS and FUS deficiencies are different.\textsuperscript{27,28} Matings of Fus/Tls\textsuperscript{−/−} animals with wild-type counterparts revealed complete male sterility and reduced fertility of females.\textsuperscript{27} Interestingly, FUS protein is completely excluded from the synapsed X-Y chromosomes, a chromatin region transcriptionally inactive also called sex body.\textsuperscript{27,35} In Fus/Tls\textsuperscript{−/−} testes, most homologous chromosomes do synapse; however, the few that fail to execute this essential step properly are sufficient to interfere with meiosis accomplishment, thus causing male sterility.\textsuperscript{27} Fus/Tls\textsuperscript{−/−} haematopoietic stem cells (HSCs) are highly susceptible to radiation both in vitro and in vivo and show delayed repair of radiation-induced DNA damage.\textsuperscript{36} The proliferation and differentiation of Fus/Tls\textsuperscript{−/−} haematopoietic progenitors appear normal in vitro. However, the number of colony-forming cells present in long-term cultures is significantly reduced. Fus/Tls\textsuperscript{−/−} HSCs have impaired long-term repopulating capacity and fail to repopulate in recipient mice.\textsuperscript{36} Furthermore, FUS protein displays an intrinsic role in the proliferative response of B cells to specific mitogenic stimuli and it is required for the maintenance of genomic stability.\textsuperscript{36} These observations demonstrate that EWS and FUS display similar but non-redundant functions in vivo.

**Neuronal functions of FET proteins**

No major neuronal defects have been reported in Fus/Tls or Ewsr1 knockout mice, highlighting the possibility that EWS and FUS functions are not essential for normal neuronal development and/or that they play redundant roles in neurons, carried out by the other member of the family on demand. Although Fus/Tls\textsuperscript{−/−} mice do not manifest ALS- or ET-like phenotypes until nearly 2 years, they show distinct histological and behavioral alterations upon aging, including vacuolation in hippocampus, hyperactivity, and reduction in anxiety-like behavior.\textsuperscript{37} Knockout mice show changes in the expression of genes related to neurological diseases, including upregulation of Taf15 and Hnmpa1, while they have normal morphology of RNA-related granules.\textsuperscript{37} Moreover, FUS has been shown to transport the Nd1-L mRNA, encoding an actin-stabilizing protein, to neuronal dendrites.\textsuperscript{38} Since Fus/Tls\textsuperscript{−/−} hippocampal neurons display abnormal spine morphology, this defect could be attributed to actin destabilization by the improper supply of Nd1-L mRNA to the dendrites. Nevertheless, exogenous expression of FUS in Fus/Tls\textsuperscript{−/−} neurons did not rescue the abnormal spine phenotypes. Thus, FUS nucleo-cytoplasmic shuttling during neuronal maturation may be critical for the recruitment of Nd1-L mRNA into the pool of ribonucleoproteins near the spines. This function might be relevant for the acquisition of neuronal activity at early developmental stages and not recoverable by exogenous expression of the protein in mature neurons.\textsuperscript{38} Thus, although FUS depletion causes phenotypes possibly related to neuropsychiatric and neurodegenerative conditions, they appear distinct from ALS and ET.

Notably, postnatal elimination of FUS in mice has no effect on motor neuron survival, providing unequivocal evidence that FUS loss-of-function alone does not underlie motor neuron degeneration.\textsuperscript{25} Furthermore, postnatal elimination of the endogenous FUS in a hFUSP525L mutant mouse model has no effect on the initiation and early progression of motor neuron loss, indicating that mutant FUS toxicity does not involve an
excess of FUS activity. Recently a novel mouse model lacking the last exon of Fus/Tls gene has been generated, resulting in the production of a truncated FUS protein without the nuclear localization signal (NLS) that localizes almost exclusively to the cytoplasm. Remarkably, this Fus/Tls ΔNLS knockin mouse model displays expression and splicing alterations consistent with loss of FUS nuclear function. However, unlike Fus/Tls−/− mice, they exhibit perinatal motor neuron loss, rescued by motor neuron expression of wild-type FUS. These findings indicate that cytoplasmic FUS mislocalization exerts not only a nuclear loss of function effect, but also a toxic gain of function within the cytoplasm of motor neurons required to trigger neuronal death.

The presence of only one member of the FET family in fruitflies renders the ablation phenotype more severe in locomotion, longevity, and neuromuscular junction (NMJ) expansion. In fact, ablation of the Drosophila FET homologue cabeza led to decreased adult viability, locomotor disabilities at both larval and adult stage, and shorter life span. All these phenotypes were rescued by transgenic wild-type Drosophila Caz (cabeza) or human FUS, revealing a remarkable conservation of protein function during evolution. Remarkably, selective inactivation of cabeza in adult neurons did not affect motor performance and life span, documenting the requirement of neuronal Caz during development, but not for the maintenance of adult neuronal function. Thus, loss of neuronal FUS/Caz function is not sufficient to cause adult motor neuron degeneration in fruitflies.

Genetic ablation of FUS in zebrafish led to abnormal motor behavior measured as a deficient touch-evoked escape response and reduced outgrowth of hyperbranched axons from motor neurons. Moreover, Fus depleted zebrafish displayed aberrant structure of the neuromuscular junctions (NMJs) with reduced success of synaptic transmission. Ewsr1 knockdown in zebrafish leads to defects in the brain and spinal cord. In particular, upon depletion of Ewsr1 gene cells of the central nervous system (CNS) were more prone to apoptosis. This was probably due to the fact that EWS protein interacts with a neuronal specific factor that regulates neuronal outgrowth and prevents apoptotic cell death by binding the promoter of target genes.

Collectively from these reports we can conclude that in lower organisms the phenotype associated with genetic ablation models becomes more severe. Thus, a partial redundancy in FET function could be responsible of the milder neuronal phenotype associated with FET deficiency in mammals.

**FET proteins in neurodegenerative diseases**

**Amyotrophic lateral sclerosis**

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease that primarily targets motor neurons. The average age at onset is 60 years, and annual incidence is 1 to 2 per 100,000 individuals. ALS leads to a progressive muscular weakness caused by the degeneration of motor neurons in the brain and spinal cord, thus resulting in paralysis and death due to respiratory failure within a few years from onset. Approximately 10% of ALS is dominantly inherited, while the remaining 90% of patients (referred to as sporadic) do not have familial history. Autosomal dominant familial ALS (FALS) is clinically and pathologically indistinguishable from the sporadic disease (SALS). Few genes have been associated with familial ALS, including SOD1, the gene encoding superoxide dismutase 1; ANG, the gene encoding angiogenin; expanded repeats in the C9ORF72, the chromosome 9 open reading frame 72 and TARDBP, encoding TAR DNA binding protein TDP-43. The discovery of mutations in another RNA/DNA binding protein, FUS, rapidly followed the identification of TDP-43 mutations in ALS. FUS/TLS mutations account for about 5% of cases of FALS. Notably, mutant FUS proteins share common features with mutant TDP-43. In fact, in ALS, mutated TDP-43 is partially excluded from the nuclei of neurons, while it accumulates in cytoplasmic aggregates, and most patients with FUS/TLS-associated ALS disease display FUS-immunoreactive cytoplasmic inclusions.

Since 2009, when the first FUS/TLS mutation was associated to ALS, more then 50 mutations have been identified in this locus (Fig. 1). Most of them are missense mutations, with few exceptions, and they mainly cluster in 2 regions. Approximately two-thirds of mutations are located in exons 12–15. These exons encode the zinc-finger motif domain, the second and third RGG domain, and the nuclear localization signal. The remaining mutations are located in exons 3–6, which encode the QGSY-rich and the first RGG domains. Importantly, none of the ALS-FUS cases investigated showed alteration in the subcellular distribution of TAF15 or EWS and there is not evidence of co-accumulation of these proteins in the FUS-positive pathological inclusions. Thus, cytoplasmic accumulation of FUS per se does not trigger alterations in the subcellular distribution of its homologues and does not lead to sequestration of TAF15 and EWS into FUS inclusions.

Several ALS-associated FUS mutations occur within the nuclear localization signal (NLS) and impair nuclear import to a degree that correlates with the age of disease onset. Nuclear import of FUS depends on transportin, and interference with this transport pathway is sufficient to cause cytoplasmic redistribution and recruitment of FUS into stress granules. Other FUS/TLS mutations affect the splice-acceptor site of intron 13 and induce skipping of exon 14, leading to the C-terminal truncation of FUS protein (p.G466VfsX14, Fig. 1), with a dramatic increase in the cytoplasmic localization of truncated FUS protein compared to wild-type FUS. These observations suggest that forced accumulation of FUS in the cytoplasm leads to aggregates that might exert toxic effects and trigger neuron degeneration.

Development of animal models of the disease greatly helped the understanding of the molecular mechanisms underlying the involvement of FET proteins in ALS. Overexpression of mutant (R521C substitution), but not normal, human FUS in rats induced progressive paralysis and displayed a substantial loss of neurons in the cortex and hippocampus, accompanied by ubiquitin aggregation. Interestingly, although FUS transgenic rats that overexpressed the wild-type human FUS were asymptomatic at young ages, at advanced ages they showed a significant loss of cortical and hippocampal neurons, demonstrating that increased expression of normal FUS is sufficient to induce neuron death accompanied by ubiquitin aggregation, thus recapitulating some features of ALS and FTLD.
Similarly, FUS overexpression in mice showed significant loss of motor neurons, coupled with pathological phenotype recapitulating many aspects of FUS-ALS. The phenotype strongly depends on the expression level of the protein, and is associated with the translocation of FUS protein to the cytoplasm.58

Overexpression of the ALS-linked mutant of FUS R521H in zebrafish caused motor deficiency.42 Moreover, the generation of transgenic C. elegans worms expressing mutant or wild-type (WT) human FUS, showed that only the mutant proteins mislocalize in the cytoplasm and cause progressive motor dysfunction, with phenotype severity that was directly correlated with the severity of the illness caused by the same mutation in humans.58 Fly models of FUS also recapitulate several features of the human disease.59,60 Targeted expression of FUS in the fly brain and motor neurons led to the pupal lethality and larval locomotor defects.60 Moreover, conditional expression of mutant FUS in neurons drastically reduce the life span and climbing abilities compared to FUS wild type flies.61,62 From these reports we can argue that these models closely replicate key features of the human illness.

Recently, genetic variants in TAF15 and EWSR1 genes have been reported in a small number of ALS patients, supporting a more general role for FET proteins in neurodegeneration.63–65 TAF15 and EWS were identified as candidate RBPs in a yeast functional screen with the aim of identifying human proteins containing RNA recognition motifs (RRM) with properties similar to FUS and TDP-43.64 Genomic sequencing of exons 13–16 of TAF15 in 735 individuals diagnosed with ALS, allowed the identification of 3 patient-specific nonsynonymous missense variants (G391E, R408C, and G473E; Fig. 1). These variants were all located in highly conserved regions of TAF15 and correspond to the analogous regions where many FUS mutations are located, comprising the RGG domain and the PY-motif in the C-terminal domain, which plays a role in nuclear localization.65,66 Importantly, these ALS-related variants of TAF15 showed an accumulation in cytoplasmic foci of dendrites and axons.64 Analogously, the exons (15–18) of EWSR1 gene encoding the same conserved region of the protein were sequenced in 817 individuals diagnosed with ALS and in 1082 healthy individuals.65 This approach identified 2 missense mutations in EWSR1 gene in 2 unrelated ALS patients with sporadic disease.65 These mutations are located in the first (G511A) and second (P552L) RGG of EWS (Fig. 1) and neither one was present in 1082 sequenced controls, strongly supporting their clinical significance.65 Remarkably, transfection of wild type and mutated EWSR1 into primary motor neurons cultured from rat embryos revealed that wild type EWS primarily localized to the nucleus, whereas both ALS-specific variants showed increased cytoplasmic and neuritic accumulation. Even more importantly, mutated TAF15 and EWS cause neurodegeneration when expressed in Drosophila.64,65

All mutant forms of FET protein have increased aggregation tendency and altered subcellular localization and they have been proposed to disrupt RNA and protein homeostasis. Nevertheless, the pathogenic mechanism by which mutated FET proteins causes neurodegeneration associated with ALS is still unknown. Several factors could be involved in FET-mediated toxicity, including stress granule formation, the presence of prion-like domain, arginine methylation, and/or myosin regulation of their RNA targets (Fig. 2).67,71 These issues will be critically discussed below.

Frontotemporal dementia

Frontotemporal dementia (FTD) is a clinical syndrome characterized by progressive deterioration in behavior, personality and/or language skills, with relative preservation of memory.72 FTD is the second most common form of dementia after Alzheimer disease and results from degeneration of the cortex of the frontal and temporal lobes, often in conjunction with the degeneration of subcortical regions.73 This pattern of degeneration gives rise to a spectrum of behavioral, language, and movement disorders. The molecular bases of FTD are heterogeneous, leading to phenotype variability. As mentioned, a common feature of FTD is the selective degeneration of the frontal and temporal lobes, and the term “frontotemporal lobar degeneration” (FTLD) is often used.72

Although most cases are considered sporadic, FTLD displays a strong genetic component, with a family history occurrence in approximately 40% of cases, and an autosomal dominant pattern of inheritance observed in 25–50% of these patients.73,74 Mutations in the microtubule associated protein tau gene (MAPT) and in progranulin gene (PGRN), both located on chromosome 17, were identified in families with FTLD.75,76 Recently, the presence of GGGGCC hexanucleotide repeat expansions in the first intron of the c9orf72 gene were identified as the most common genetic cause of familial FTLD, accounting for approximately 20% of all familial cases.22,57,77 Moreover, mutations in 2 other genes, VCP, encoding the vasolin-containing protein, and CHMP2B, encoding the chromatin-modifying protein 2B, are responsible for a minority of familial FTLD cases.78

Most cases of FTLD presented abnormal intracellular accumulation of disease-specific proteins. Initially, only the FTLD subgroup characterized by the aggregation of hyper-phosphorylated tau protein in neurons and glia, classified as FTLD-tau, was described.79 However, most cases of FTLD are not associated with tau pathology, but are instead characterized by ubiquitin positive neuronal inclusions, then termed FTLD-U.80 TDP-43 was also reported as a ubiquitinated pathological protein in several cases of FTLD-U (subsequently renamed FTLD-TDP).81 Recently, aberrant localization of FUS protein has been described as a component of the inclusions in several subtypes of FTLD, now included in the FTLD-FUS group. FTLD-FUS includes cases with ubiquitin-positive inclusions (FTLD-U), neuronal intermediate filament inclusion disease (NIFID) and basophilic inclusion body disease (BIBD).21,80 Notably, only few FUS mutations have been reported in patients with an FTLD or FTLD-ALS phenotype.81,82 However, further studies in large FTLD cohorts observed no mutations, suggesting that FUS has only a limited role in the genetic etiology of FTLD.21,82,83 Moreover, abnormal co-accumulation of all 3 FET proteins into pathological inclusions has been described in all subtypes of FTLD-FUS.84,85 Biochemical analysis of proteins extracted from FTLD-FUS brains revealed increased insolubility of all FET proteins, without other obvious disease-associated changes, such as truncation or abnormal protein phosphorylation.21
Since only rarely mutations in the \textit{FUS} gene are causal for FTLD, it is still debated what causes \textit{FET} protein translocation. In the case of \textit{FUS}, it is known that, under normal physiological conditions, this protein shuttles between the nucleus and the cytoplasm through the nuclear pore and its nuclear import is assisted by transportin1 (TRN1), which is responsible of most of the nucleocytoplasmic transport traffic within the cell.\cite{86} Binding to TRN1 is dependent on the PY motif located at the carboxy-terminus of \textit{FUS}.\cite{87} Interference with this transport pathway leads to cytoplasmic redistribution and recruitment of \textit{FUS} into stress granules. Thus, \textit{FUS} protein mislocalization in cases of FTLD-FUS might be due to a general defect of transportin-mediated nuclear import.\cite{55} Indeed, inhibition of transportin-mediated nuclear import resulted in recruitment and co-localization of all \textit{FET} proteins into stress granules, while alterations in the subcellular distribution of other transportin cargos such as hnRNP A1 and SAM68 was not affected.\cite{356,87} This observation suggests that \textit{FET} proteins are particularly sensitive to alteration of this pathway. Another possibility is that the normal nuclear import of \textit{FET} proteins is affected by abnormal post-translational modifications occurring in the FTLD condition. In this regard, it has been shown that arginine methylation and phosphorylation modulate \textit{FET} proteins nucleo-cytoplasmic transport, protein–protein interactions and stability. Arginine methylation of a site adjacent to the C-terminal PY motif impairs TRN1-mediated nuclear import of \textit{FUS}.\cite{88} In addition, the N terminus of \textit{FUS} contains a low-complexity domain that can aggregate into fibrils mimicking features of RNA granules; aggregation of the \textit{FUS} low-complexity domain can be disrupted by DNA-PK-mediated phosphorylation.\cite{88,89} On the other hand, \textit{FUS} phosphorylation on tyrosine 526 by Src kinases reduces its interaction with TRN1 and might consequentially affect the transport of \textit{FUS} into the nucleus.\cite{90} Thus, abnormal localization of \textit{FUS} protein could also result from post-translational modifications triggered at the onset of FTLD.

TAF15 co-localization with aberrant \textit{FUS} was reported in all cases analyzed, while subtle disease-specific differences were observed for EWS. Interestingly, TAF15 localization was differentially regulated in HeLa and neuronal HT22 cell lines. Although predominantly nuclear in both cell lines, in HT22 cells TAF15 co-localized in the cytoplasm with a subset of RNA granules, thus supporting the hypothesis of a role for TAF15 in RNA transport and/or local RNA translation in neurons.\cite{91}

Hence, co-accumulation of all \textit{FET} proteins in the characteristic pathologic inclusions seems a specific feature of FTLD-FUS, but not of ALS-FUS. Moreover, in all ALS cases presenting \textit{FUS} inclusions, mutations in the \textit{FUS}/\textit{TLS} gene caused the

**Figure 2.** Impairment of nucleo-cytoplasmic shuttling of \textit{FET} proteins contributes to neurodegenerative disease and muscular atrophy. In normal conditions, \textit{FET} proteins display several physiological functions, including transcription, pre-mRNA splicing, mRNA biogenesis, stress granules formation and mRNA stability, by binding target RNAs both in the nucleus and in the cytoplasm and are engaged in nucleo-cytoplasmic shuttling (a). Mutations within the NLS or NES of \textit{FET} proteins and factors arising during aging, like reduced expression of nuclear transport factors or oxidative damage, as well as post-translational modifications, such as phosphorylation, arginine methylation and proteolytic cleavage events that remove the NLS, can cause cytoplasmic accumulation of \textit{FET} proteins within stress granules (b). Such stressors may also include environmental toxins or mutations in neuro-protective/stress protective genes, such as \textit{GRN} or \textit{VCP}. Since the formation of stress granules is a reversible process, this step can potentially be reversed upon release of stress or by upregulation of protective factors, like chaperones or neuronal growth factors, such as Progranulin. However, persistent cellular stress or genetic risk factors leads to the conversion of \textit{FET}-containing stress granules into large pathologic inclusions. This in turn can cause degeneration of motoneurons and muscular atrophy (c).
disease, whereas only a few FTLD cases with FUS inclusions harbor FUS/TLS mutations.

**Essential tremor disease**

Essential tremor (ET) is a neurodegenerative disorder characterized by postural and/or kinetic tremor. It is one of the most common adult-onset movement disorders. The main feature is postural tremor of the arms, but head, legs, trunk, voice, jaw, and facial muscles may also be involved. Notably, in most families affected by the disease, autosomal dominant inheritance can be demonstrated.92

Recent exome sequencing of a large pedigree of individuals within a French-Canadian family affected by an autosomal dominant form of familial ET revealed a nonsense mutation in FUS/TLS gene (c.868C>T) as pathogenic.93 This ET variant in exon 9 of FUS/TLS corresponds to a stop mutation (p.Q290X). The nonsense mutation is located in the predicted nuclear exporting signal (NES) of FUS protein (amino acids 289–298), which is a short amino acid sequence that targets the protein for export from the nucleus to the cytoplasm through the nuclear pore complex. The corresponding transcribed mRNA is mainly degraded by nonsense mediated decay (NMD), suggesting a loss-of-function disease mechanism.93 Moreover, a novel risk variant c.1176G>A (p.M392I) was identified as potential ET susceptibility factor.94 This variant resided in exon 12 of the FUS/TLS gene, causing the substitution of Met392 with Ile.94 Exome sequencing analysis has also recently identified a non-sense mutation in FUS, p.R377W, in one patient with family history of disease, with one brother diagnosed with parkinsonism and one sister and one uncle with possible ET. This mutation was located in the second RGG domain of FUS protein, in which mutations for ALS and FTD have not been described.95 Furthermore, sequencing of the EWSR1 gene in ET patients identified a rare p.R471C substitution in a single subject with familial ET. The pathogenicity of this substitution remains equivocal, as DNA samples from relatives were not available and the genotyping of 404 additional ET subjects did not reveal any further carriers.96 Collectively, these studies suggest that mutations in FET proteins may underlie the pathology of ET and further studies are needed to elucidate the mechanisms of such pathological effects.

**Parkinson disease**

Parkinson disease (PD) is a progressive disorder of the nervous system that affects several regions of the brain devoted to the control of balance and movement. In addition, PD can affect emotions and thinking ability (cognition).97 Generally, PD onset occurs after age 50 (late-onset disease), although early-onset symptoms before this age and even before age 20 have been described (referred to as juvenile-onset PD).98 Despite familial history had been recognized as a common feature in PD, only in the last 15 years the contribution of genetics has been deeply explored. These studies have conducted to the identification of few genes that are clearly responsible for mendelian forms of the disease, either with autosomal dominant (SNCA, LRRK2) or recessive (PARK2/Parkin, PINK1, DJ-1, ATP13A2) inheritance.98 Given the involvement of FUS mutations in ET etiopathology, the role of FUS variants was also investigated in PD but it did not identify any novel non-synonymous variant affecting the subjects’ susceptibility to PD.99

**Alzheimer disease**

Alzheimer disease (AD) is the most common form of dementia. Genetic factors are involved in 25–40% of AD patients and, in some cases, AD segregates as an autosomal dominant trait in families.100 To date, 3 genes have been identified that, when mutated, cause AD: the Aβ amyloid precursor protein gene (APP), the presenilin 1 (PSEN1) and the presenilin 2 (PSEN2) genes. Together, these mutations are responsible for 30–50% of autosomal dominant AD cases, and about 0.5% of all AD cases.100 No mutations in FET genes have been reported in AD patients.

**FET proteins and RNA processing: New potential targets in neurodegenerative disease**

As discussed above, dysregulation of RBPs is emerging as a prominent pathogenic mechanism underlying ALS. Most ALS-related FUS mutants display disrupted nucleo-cyttoplasmic shuttling, leading to increased cytoplasmic levels of the protein and formation of aggregates. Cytoplasmic accumulation and aggregation of FUS, TAF15, and EWS was also found in a subset of FTLD cases, further supporting the hypothesis of a dysregulation of FET-dependent RNA metabolism in the disease.54 Thus, identification of common RNA targets driving or contributing to pathogenesis is an intriguing open challenge.

FUS and EWS preferentially bind polyU and polyG sequences.101,102 RNA binding is mediated by the RBD, while the 3 RGG motifs Cooperate with the RBD to increase the affinity for RNA.9,13,101–104 The RGG motifs are sites of post-translational modifications that modulate RNA binding affinity and affect protein-protein interactions.9,14 The carboxy-terminus of TAF15 contains 20 copies of Gly-Gly-Tyr-Gly-Gly-Asp-Arg repeats; this region is likely to have a role in RNA binding.14,103 Importantly, FUS was initially identified in purified human spliceosomes assembled in vitro, while in vitro splicing assays demonstrated its implication in the regulation of alternative splicing.9,106,107 FUS protein crosslinks the pre-mRNA 3’ splice site during the second step of splicing.108 On the other hand, EWS was initially shown to interact with the branch-point recognizing protein BBP/SF1 and with U1C, one of the protein components of U1 snRNP, suggesting a role in modulating 3’ and/ or 5’ splice-site recognition.109,110

Identification of the RNA targets bound by FET proteins in vivo is an essential step toward elucidation of their functions. FET RNA targets were reported for FLAG-tagged proteins expressed in 293T cells, revealing prominent binding of FUS to introns, while EWS and TAF15 preferentially bind coding sequences and 3’UTRs in target mRNAs.111 Notably, FUS and TAF15 crosslink sequences nearby the 3’ splice site, while EWS preferentially binds near the 5’ splice sites of target pre-mRNAs.111,13 UV crosslinking and immunoprecipitation sequencing (CLIP-seq) experiments highlighted an EWS signature in exonic regions nearby the 5’ splice sites. Mechanistically,
EWS recruitment of U1snRNP and U2AF to the flanking splice sites of the target exons, favoring their definition and, consequently, their inclusion. RNA targets for endogenous FUS in mouse brains and human autopsy brains document that nascent RNAs constitute the major class of substrates for FUS. Interestingly, no significant overlap between the binding sites or splicing changes is seen between FUS and TDP-43, although both proteins are directly involved in ALS and they both bind and regulate genes with long introns and important for neuronal development, highlighting the possibility that binding of TDP-43 or FUS to long introns may facilitate transcription elongation and ensure normal and correct splicing of long introns, preventing unproductive splicing events. Nevertheless, a subset of common targets was identified, opening the possibility of RNA regulation as a key mechanism underlying pathophysiological features of ALS and FTD diseases. FUS regulates splicing of genes coding for other RBPs by binding to their highly conserved introns. These results have important implications for understanding the impact of FUS in neurodegeneration, as they suggest that perturbations of FUS may have widespread effects in the neuronal transcriptome via dysregulation of the processing of various RBP transcripts. Among them, FUS regulates its own transcript by repression of exon 7 splicing, thus leading to a transcript subjected to NMD. FUS protein harboring R521G, R522G or ΔExon15 ALS-related mutations show deficiencies in both exon 7 repression and autoregulation of FUS protein levels. These observations suggest that compromised autoregulation of FUS can directly exacerbate the pathogenic accumulation of the protein in the cytoplasm in neurons of ALS patients. Importantly, FUS, EWS and TAF15 target pre-mRNAs largely overlap in Hek293 cells, while mutated FUS proteins (FUS-R521G or FUS-R521H) display an elevated fraction of unique crosslinks, pointing toward an altered, rather than disrupted, binding profile. Gene categories related to unfolded protein response (UPR) and endoplasmic reticulum (ER), are overrepresented among transcripts uniquely bound by cytoplasmic FUS mutants, suggesting that their dysregulation may contribute to the formation of cytoplasmic aggregates. In mouse and human brain tissues, FUS affects the expression of genes important for neuronal function, including synaptic genes. FUS mRNA targets of relevance for neurodegenerative disorders include SOD1, the genes encoding medium and heavy chains of neurofilament (NEFL, NEFM, NEFH) and the glutamate transporter (EAAT2), ubiquilin 1 and 2 (UBQLN1 and UBQLN2) and MAPT. Comparison of FUS mRNA targets in mouse versus human brain revealed a relatively high degree (69%) of overlap, indicating that FUS-RNA interactome is evolutionarily conserved.

More recently, UV crosslinking and immunoprecipitation sequencing (CLIP-seq) experiments highlighted an EWS signature in exonic regions nearby the 5’ splice sites. Mechanistically, EWS recruitment of U1snRNP and U2AF to the flanking splice sites of the target exons, favoring their definition and, consequently, their inclusion. FET proteins bind to both coding and noncoding RNAs. FUS, EWS and TAF15 associate with Drosha and with the microprocessor complex. Moreover, EWS crosslinks different classes of noncoding RNAs while FUS binds directly and contributes to the biogenesis of a subset of miRNAs with roles in neuronal function, differentiation and synaptogenesis. Notably, global loss of miRNAs through Dicer knockdown results in neurodegeneration, suggesting that misregulation of miRNA biogenesis may contribute to neurodegeneration through chronic changes in synaptic functions. Thus, it is possible that transcriptome alterations detected in FTD and ALS patients derive, at least in part, from disruption of miRNA networks, potentially linked to improper FET proteins function. Indeed, recent studies using miRNA microarrays or RNA sequencing to assess alterations in the miRNA landscape in FTD-ALS revealed extensive changes in the expression of these small noncoding RNAs.

As mentioned above, FET proteins display multiple roles in the RNA metabolism, from transcription to pre-mRNA splicing, miRNA processing, lncRNAs, miRNA stability, transport and control of mRNA translation, and they are even involved in the formation of stress granules (for a review see Ref ). Although their regulatory activities are similar, they regulate distinct RNA targets and show different and not-redundant functions both in normal and in pathogenetic conditions.

Now that genome-wide analyses have provided thousands of coding and noncoding RNAs either bound or processed by FET proteins, the next goal will be to determine whether these genes are dysregulated in neurodegenerative diseases bearing mutations in FET genes. Moreover, if a causative link is established, it will be crucial to assess whether disease-related phenotypes can be rescued by restoring the regulation of the disease-causing targets.

**Prion-like domain in FET proteins**

Increasing evidence supports a common mechanism driving neurodegeneration in clinically different conditions. Most neurodegenerative diseases, in fact, share the abnormal accumulation of misfolded insoluble proteins that aggregate in the neurons of affected individuals. In prion disease, the infectious agent is the prion, a misfolded pathogenic protein with the ability to self-propagate and to transmit its conformation to other proteins, thus driving encephalopathy. In neurodegenerative disease, instead, the misfolded aberrant proteins act as a seed for aggregation, thus sequestering their native isoforms and converting them into pathological aggregates, which gradually enlarge by recruitment and conversion of further native proteins, ultimately leading to the disease phenotype. Notably, bioinformatics analysis revealed that many human RBPs, including TDP-43 and FET proteins, contain predicted "prion-like" domains, enriched in uncharged polar amino acids (such as asparagine, glutamine and tyrosine) and in glycine residues. This finding raises the intriguing possibility that mutations in these aggregation-prone RBPs can trigger neurodegenerative diseases by acting as seeds in a prion-like fashion. Chronic cellular stress conditions or mutations in the amino acid sequence may convert the RBPs that are highly concentrated in the stress granules into irreversible protein aggregates. In turn, these protein aggregates can critically perturb protein homeostasis thus driving neurodegeneration.
The prion-like domain of TDP-43 is located at the carboxy-terminus of the protein (residues 277–414), while that of FUS is embedded in the N-terminal region (residues 1–239), but this RBP contains an additional potential prion-region in the first RGG domain (residues 391–405). Importantly, the prion-like domain of TDP-43 plays a critical role in protein aggregation, since its deletion prevents aberrant TDP-43 misfolding. Conversely, elevated expression of the C-terminal portion of TDP-43 containing the prion-like domain elicits toxicity and cytoplasmic TDP-43 aggregation in yeast and worm models. Unlike TDP-43, FUS fragments that harbor the prion-like domain (amino acids 1–238) do not aggregate, unless they also contain a C-terminal RGG domain (amino acids 374–422). Thus, communication between different domains of the protein is necessary to promote FUS aggregation. Importantly, both TDP-43 and FUS were found in stress granules of ALS patients. Association of TDP43 with stress granules strongly depends on the presence of the prion-like domain, as deletion of this region inhibited the process. In the case of FUS, the RGG-zinc finger domain is the most important domain for stress granules recruitment, although the glycine-rich domain and the RRM domain also contribute to FUS relocalization, whereas the prion-like glutamine-rich domain is dispensable. Notably, mutations within this domain resulted in enhanced kinetics of stress granule formation and increased size of the granules. Depending on the type of stress, FUS rapidly shuttles from the nuclear liquid compartments to the cytoplasm; then, a population of FUS converts from a liquid state to an aggregated state, which resembles the pathological state of ALS patients harboring mutations in the FUS protein. This conversion from liquid to solid is accelerated either by mutations in the prion-like domain that induce the early onset of ALS or by raising the protein concentration. Similarly to FUS, both TAF15 and EWS harbor a N-terminal prion-like domain (amino acids 1–149 and 1–280, respectively). Moreover, TAF15 and EWS proteins were shown to form cytoplasmic aggregates in yeast and to display toxic features.

As mentioned above, the accumulation of misfolded protein structures is a recurring and unifying facet of different neurodegenerative diseases, able to propagate disease from single or multiple sites of origin. Interestingly, many of the misfolded proteins found in these pathological inclusions are expressed in almost all cells, but they only seem to misfold and confer toxicity in specific neurons (i.e. motor neurons in ALS, dopaminergic neurons in PD). Agents able to revert protein misfolding and restore proteins to native form would likely have high therapeutic value. Indeed, a protein chaperone system able to rescue previously aggregated proteins has been described in yeast, but it has no metazoan orthologs. This system is formed by the protein disaggregate Hsp104 and the 2 chaperones Hsp70 and Hsp40. Remarkably, modifications of Hsp104 are able to disaggregate and rescue deleterious TDP-43, FUS, and TAF15 misfolding, whereas misfolded EWS was not buffered by Hsp104 variants. Thus, rescue of the disease-associated proteins to their non-pathogenic states through the development of an engineered chaperon system might provide a highly promising strategy for halting or even reversing the progression of devastating neurodegenerative diseases. Further understanding of the mechanisms regulating protein processing and underlying protein aggregation, as well as of the toxic effects of misfolded proteins in the pathogenesis of neurodegeneration, will help in the development of rationally designed therapies to treat or prevent these disorders.

Concluding remarks

FET proteins play key roles in the regulation of gene expression, thus displaying similar but not redundant functions within the cell. The recent discovery of the implication of FET proteins in neurodegenerative diseases renewed the interest in elucidating their physiological functions. To date, it is still not clear which, if any, endogenous function of FET proteins is involved in the pathogenesis of these diseases. Many ongoing studies suggest a prion-like mechanism underlying the pathogenesis of ALS, as well as of other neurodegenerative disorders. The prion-like seeding and spreading of the proteins implicated in the disorder could trigger, from a single protein-misfolding event, an autocatalytic protein-protein pathological cascade, thus causing neurodegeneration. In this scenario, impairment of FUS autoregulation of its own RNA might contribute to create a feed-forward positive loop of protein production and aggregation, resulting in sequestration of newly produced FUS, together with its RNA targets, into the cytoplasmic aggregates. Importantly, further understanding of the role of FET proteins in neurodegenerative diseases might lead to the development of new tools and techniques for early diagnosis and identification of therapeutic targets for the treatment and prevention of these disorders.

The discovery of the involvement of FUS/TLS, EWS and TAF15 in ALS and in other neurodegenerative diseases reinforces the role of altered RNA processing as a driving mechanism in neurodegeneration. Well known examples include errors in RNA metabolism from loss of survival of motor neurons (SMN) in spinal muscular atrophy (SMA) and FMRP in fragile-X mental retardation, and many others (for a review see Refs. ). Overall the emerging roles of FET proteins discussed in this review add considerable support to the proposal that defects in RNA processing play a central role in neurodegeneration.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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