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Understanding the role of TDP-43 and FUS/TLS in ALS and beyond

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Summary

Dominant mutation in two DNA/RNA binding proteins, TDP-43 and FUS/TLS, are causes of inherited Amyotrophic Lateral Sclerosis (ALS). TDP-43 and FUS/TLS have striking structural and functional similarities, implicating alterations in RNA processing as central in ALS. TDP-43 has binding sites within a third of all mouse and human mRNAs in brain and this binding influences the levels and splicing patterns of at least 20% of those mRNAs. Disease modeling in rodents of the first known cause of inherited ALS – mutation in the ubiquitously expressed superoxide dismutase (SOD1) – has yielded non-cell autonomous fatal motor neuron disease caused by one or more toxic properties acquired by the mutant proteins. In contrast, initial disease modeling for TDP-43 and FUS/TLS has produced highly varied phenotypes. It remains unsettled whether TDP-43 and FUS/TLS mutants provoke disease from a loss of function or gain of toxicity or both. TDP-43 or FUS/TLS misaccumulation seems central not just to ALS (where it is found in almost all instances of disease), but more broadly in neurodegenerative disease, including frontal temporal lobular dementia (FTLD-U) and many examples of Alzheimer's or Huntington's disease. (182 words)

Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive adult-onset neurodegenerative disorder that leads to paralysis and ultimately death within 2–5 years. The disease is characterized by the selective loss of motor neurons in the brain and spinal cord leading to fatal paralysis. Most cases of ALS are sporadic, but 10% are inherited in a dominant manner (familial ALS). Mutations in eight genes have now been identified to cause typical ALS (Table 1). The most common cause of inherited ALS, which accounts for twenty percent of the familial cases, has been shown to be associated with missense mutations in the gene encoding cytoplasmic Cu/Zn superoxide dismutase (SOD1), which is involved in intracellular detoxification of superoxide. Much work on transgenic mice constitutively expressing ALS-linked mutations in SOD1, which develop late onset motor neuron death and muscle atrophy like that seen in ALS, has led to two pivotal discoveries in mutant SOD1 mediated toxicity: (1) death of motor neurons is not due to a loss of SOD1 activity since mice expressing dismutase inactive mutant SOD1 develop ALS like symptoms [1] and (2) mutant SOD1 synthesis within the motor neurons contributes to disease onset whereas damage within the glial cells expressing mutant SOD1 accelerates disease progression [2–5]. With nine prominent hypotheses emerging for how SOD1 mutations lead to selective death of motor neurons (reviewed in [6]), it seems likely now that for SOD1 mutants, ALS pathogenesis may come from the convergence of several toxic pathways acting in different cell types.

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TDP-43 and FUS/TLS: a paradigm shift in ALS

In 2006 [7,8], the beginnings of a paradigm shift in ALS emerged with identification of the 43 kDa transactive response DNA-binding protein (TDP-43) as a major component of ubiquitinated protein aggregates found in the central nervous system (CNS) of patients with sporadic ALS or in the most common form of frontotemporal dementia called FTLD-U (Frontotemporal Lobar Degeneration with Ubiquitinated inclusions). This quickly prompted direct sequencing of the gene encoding TDP-43 (*TARDBP*) as a means to identify mutations in TDP-43 as causative of cases of sporadic and familial ALS, as well as rare cases of FTLD [9–11]. To date, at least 44 mutations in TDP-43 (Figure 1) now account for ~5% of ALS (combining familial and sporadic ALS cases) [12]. Rapidly thereafter, mutations in another RNA/DNA binding protein, known by two names (Fused in Sarcoma or Translocated in liposarcoma; referred to here as FUS/TLS), were identified as a primary contributor to familial ALS [13,14]. A total of 43 mutations (Figure 1) have now been described in patients with or without apparent familial history, corresponding to ~4% of familial (and rare sporadic) ALS cases [12].

TDP-43 and FUS/TLS proteinopathies

Although mutations in TDP-43 and FUS/TLS have been reported only in ALS and FTLD patients, TDP-43 and FUS/TLS immunoreactive inclusions have been observed in the nucleus and cytoplasm of both neurons and glial cells. This is true not just in ALS and FTLD, but also in a range of other neurodegenerative diseases, including Huntington's, Alzheimer's and Parkinson's disease [15]. The widespread presence of TDP-43 or FUS/TLS inclusions in so many disorders has led to a new nomenclature for the set of such diseases: TDP-43 or FUS/TLS proteinopathies [12,16].

A striking characteristic of TDP-43 pathology that is now well established in ALS, as well as in other neurodegenerative diseases (although the frequency and when it occurs during disease course remains unclear), is the nuclear clearance of TDP-43 concomitantly with its cytoplasmic mislocalization [12]. Pathogenesis is thus likely driven, at least in part, by loss of normal nuclear TDP-43 function(s). Although FUS/TLS is also a predominantly nuclear protein and is structurally close to TDP-43 (Figure 1), the redistribution of FUS/TLS from the nucleus to the cytoplasm is apparently less complete [12,17]. FUS/TLS and TDP-43 proteinopathies also differ by the absence in FUS/TLS of most of the post-translational modifications (such as hyperphosphorylation, ubiquitination and cleavage) that have been reported for TDP-43 [15,18]. Thus, if common mechanisms underlie toxicity mediated by TDP-43 and FUS/TLS, this divergence in the biochemical properties of the proteins may suggest that these alterations are not key contributors to pathogenesis.

Identifying TDP-43 RNA targets

Immunoprecipitation of RNAs interacting with TDP-43, followed by high-throughput sequencing has now been reported by three groups [19–21]. By crosslinking the RNAs to their bound proteins prior to immunoprecipitation (known as CLIP-seq or a modified version called iCLIP), two teams identified TDP-43 binding to more than third of all mouse [19] and probably an equivalent number of human [21] brain mRNAs. Immunoprecipitation of TDP-43 without crosslinking (known as RIP-seq, a technique that allows the identification of RNA binding partners but not the binding sites) also identified more than 4,000 RNAs bound by TDP-43 in primary cortical rat cells [20]. Finally, using conventional cloning and sequencing approaches [22], TDP-43 immunoprecipitation after UV-crosslinking in human neuroblastoma cell lines has led to the identification of >120 target RNAs. A comprehensive comparison of these newly reported data sets to identify conserved TDP-43 RNA targets is now eagerly anticipated.

A consensus has emerged from *in vitro* [20,22,23] and *in vivo* [19,21] approaches that the primary *in vivo* binding site for TDP-43 is a GU-rich motif, corroborating *in vitro* findings from a decade earlier [24]. Nevertheless, GU-rich motifs are neither necessary nor sufficient for TDP-43 binding [19]. Most of the TDP-43 binding sites lie deep within introns – far from splice junctions [19–22], many of which are downstream of silenced exons [21]. A broad influence of TDP-43 on mature mRNA levels and splicing was established following depletion of TDP-43 either by siRNA in cell culture [21] or from the adult mouse nervous system (by infusion of a TDP-43 complementary antisense oligonucleotide) [19]. The combination of high-throughput sequencing and splicing-sensitive microarrays has identified changes in abundance of >600 mRNAs and altered splicing patterns of 965 mRNAs in the adult mouse brain upon depletion of TDP-43 [19].

Among the mRNAs whose levels or processing is affected by TDP-43 in both mouse [19] and human [21] settings are those encoding proteins related to neuronal function and development and/or that have been implicated in neurological diseases. Indeed, RNAs whose levels are most depleted by reduction of TDP-43 are derived from genes with the longest introns (average size >100 kb) and that encode proteins involved in synaptic activity (including a N-methyl-D-aspartate receptor, an ionotropic glutamate receptor, and neurexins 1 and 3) [19]. Since genes with significantly longer introns are preferentially expressed in human and mouse nervous systems relative to other tissues, this requirement provides at least one component of selective vulnerability to neurons from the loss of nuclear TDP-43 that has been universally reported in ALS.

TDP-43 also binds and/or regulates expression and splicing patterns of several additional disease-related pre-mRNAs [19–21] including those encoding FUS/TLS, progranulin, and tau (mutations in which cause ALS and/or FTD), parkin and MEF2D (which are involved in Parkinson's disease), as well as huntingtin and the ataxins. The last of these RNA targets has become of high interest with report that an intermediate length CAG expansion in ataxin 2 may be a contributor to ALS [25]. Among the many now identified TDP-43 target mRNAs are two previously implicated: histone deacetylase 6 (HDAC6), [26,27]) and the NF-L neurofilament subunit [28], both of which are reduced in motor neurons of ALS patients. TDP-43 neither binds to nor does its reduction affect the level or splicing of the mRNA encoding superoxide dismutase (SOD1) in mice [19] or humans [29].

Overall, TDP-43 binds to and affects procession of a very long list of RNA targets with it probably acting in multiple steps of RNA processing.

TDP-43 RNA targets in FTLD and ALS

iCLIP and RNA sequencing analyses directly from brains of FTLD patients identified significantly increased expression of, and therefore increased TDP-43 binding to two non-coding RNAs: the nuclear paraspeckle assembly transcript 1 (NEAT1) and the metastasis associated lung carcinoma (MALAT1)), both of which are themselves thought to function in splicing and regulation of gene expression). In contrast, expression of transcripts involved in synaptic activity such as neurexin 3 (NRXN3) and the glial excitatory amino acid transporter -2 (EAAT2), accompanied by diminished TDP-43 binding, were significantly decreased in FTLD brains compared to those from healthy patients [21]. Comparison of TDP-43-RNA complexes in nuclear versus cytoplasmic fractions of healthy and FTLD brain tissues revealed that more than 90% of TDP-43 interactions were with nuclear pre-RNAs both in healthy and FTLD patients. Similarly, isolation of spinal cord motor neurons from sporadic ALS patients (by laser capture microdissection) coupled with genome exon splicing arrays has revealed alterations in expression and splicing of hundreds of transcripts, albeit no significant modifications in the mRNAs encoding TDP-43 or FUS/TLS [30].

Auto-regulation of TDP-43 synthesis

Multiple teams have identified an auto-regulatory mechanism that determines the level of TDP-43 synthesis: TDP-43 controls its own expression at least in part by direct binding to the 3′untranslated region (UTR) of its own RNA transcript [19,21,23]. Auto-regulation has been documented not only in cell culture [19,21,23] but also in mice, with expression of a TDP-43-encoding transgene without the regulatory 3′UTR driving significant reduction of endogenous TDP-43 mRNA and protein [19,31,32,48]. The detailed mechanism underlying auto-regulation is unsettled. One team has argued that TDP-43 auto-regulates its synthesis, at least in part by directly binding to and enhancing splicing of an intron in the 3′ UTR of its own transcript, thereby triggering nonsense-mediated RNA degradation of the spliced mRNA [19]. Another group has argued that TDP-43 binding to its own 3′UTR promotes RNA instability and degradation by the exosome machinery [23]. Regardless, self-regulation of TDP-43 expression seems all but certain to represent an important contributor to ALS pathogenesis following an initiating event that yields reduction in nuclear TDP-43 levels (by mutation and/or cytoplasmic aggregation), thereby driving elevated TDP-43 synthesis.

FUS/TLS RNA targets

To date only few FUS/TLS RNA binding partners have been reported and a sequence binding motif(s) has not been identified. RNAs apparently bound include actin-stabilizing protein (Nd1-L), thought to play a role in actin reorganization in spines [33] and HDAC6, which has also been shown to be bound by TDP-43 [27], thus suggesting that both FUS/TLS and TDP-43 proteins can function in common biological pathways. Use of high-throughput approaches (such as those described above for TDP-43) is now essential to identify the spectrum of FUS/TLS RNA targets and establish normal function of FUS/TLS.

Identifying TDP-43 and FUS/TLS protein partners

Various interacting proteins have been reported for either TDP-43 or FUS/TLS or both (as reviewed in [15]). Most of these have been found by coupling immunoprecipitation of normal or ALS-linked mutants of TDP-43 to mass spectrometry. Despite using different starting biological tissues, numerous hnRNPs, splicing factors and RNA binding proteins have been consistently identified as TDP-43 binding partners [18,20,27,34,35]. In addition to previous identification of Drosha as a TDP-43 binding partner [36], several other components involved in microRNA processing were established as TDP-43 interacting proteins using two-step tandem-affinity purification coupled to quantitative mass spectrometry analysis [35] (using stable isotope labeling amino acids in cell culture or SILAC, an approach that allows identification of proteins of low abundance while eliminating abundant contaminants [37]).

Interaction of TDP-43 and FUS/TLS

The most striking finding of the search for TDP-43 partners is association between FUS/TLS and TDP-43. This was detected in human cell lines expressing either transiently [27,34] or stably [35] a tagged form of wild-type TDP-43 and further validated for proteins expressed at endogenous levels [27,35]. TDP-43 and FUS/TLS were found to be present in two major high molecular weight complexes: a smaller complex (of 300–400 KDa) which was enriched in nuclei and a higher molecular weight (>1 MDa) complex in the cytoplasmic fraction [27]. Proteomic analysis of the higher molecular weight complex revealed the presence of multiple ribosome and translation factors [34]. No consensus has yet emerged about the association between FUS/TLS and ALS-linked TDP-43 mutants [27,35]. While FUS/TLS binding was found to be significantly enhanced by two TDP-43 mutants (Q331K and M337V) in isogenic HeLa cell lines expressing a GFP fused transgene at near endogenous levels [35], no clear evidence for increased interactions of TDP-43 mutants

(M337V, A315T, D169G and R361S) to FUS/TLS was observed in transiently transfected HeLa cells expressing a HA tagged transgene at high levels [27]. The proposed increased association of mutants of TDP-43 with FUS/TLS may be due to the surprising observation that the stability of the mutants is significantly enhanced compared to unmutated TDP-43, with mutant protein half-lives twice that of wild-type [35]. This striking finding may at least in part account for the accumulation of TDP-43 aggregation reported in ALS patients. Furthermore, if an increased interaction of FUS/TLS with TDP-43 ALS-linked mutants is confirmed in a more disease-relevant setting such as autopsied ALS brain tissues, it will suggest that this aberrant association may generate alterations of the normal function(s) of both TDP-43 and FUS/TLS, thus indicating a possible convergence of pathogenic pathways by both proteins in ALS. It will now be of high interest not only to identify the interacting partners of FUS/TLS, but also to quantitatively compare complexes containing normal TDP-43 or FUS/TLS versus ALS-linked mutants of both proteins.

Animal modeling of TDP-43 and/or FUS/TLS mediated toxicity

Multiple attempts to model ALS-like disease in fruit flies, worms, zebrafish, mice and rats from altered TDP-43 levels or mutation have now been reported [32,38–50] (Table 2). Two have been reported for FUS/TLS [51,52]. Despite the many modeling studies, no consensus has emerged on two key mechanistic questions concerning mutant TDP-43 mediated toxicity: 1) is toxicity from a gain of toxic property(ies), loss of function, or both and 2) in which cell types does the mutant act to drive toxicity (i.e., is the disease mechanism non-cell autonomous)?

Disease modeling in *Drosophila, C. elegans* and zebrafish

Drosophila, Caenorhabditis elegans (*C. elegans*) and zebrafish all have a homologue of TDP-43. Since these models have been proved to be very powerful genetic tools to study disease mechanisms, a number of studies have reported the effects of TDP-43 disruption or elevated expression of either wild-type or mutants of human TDP-43 in these organisms.

Loss of TDP-43—Complete loss of TDP-43 function produced by either partial or complete deletion of the *Drosophila* homologue TBPH and down-regulation of TBPH (using small interference RNAs or siRNA) is deleterious in flies, leading to semi-lethal [42,53,54] or lethal [26] phenotypes. The surviving null flies display defects in locomotive behavior, neuromuscular junctions, reduced lifespan [53], reduced dendritic branching [54] as well as axonal loss and neuronal death [42]. Similar knock-down of TDP-43 in zebrafish using antisense morpholino oligonucleotides causes a motor phenotype consisting of shorter motor axons, excessive disorganized branching as well as swimming deficits but without clear lethality [40]. Interestingly, the motor abnormalities observed in both flies and zebrafish can be partially rescued by wild-type human TDP-43 [40,53]. Altogether these findings highlight how TDP-43 is essential both in invertebrates and vertebrates.

Increased levels of wild-type or mutant TDP-43—A consensus from multiple reports of expression of wild-type human TDP-43 in neurons of worms [38,43], flies [39,42,44,54,55] and zebrafish embryos [40,41] reveals neurodegeneration accompanied by decreased locomotive activity [38,42,43], motor deficits [40–42], motor neuron loss [42], paralysis and reduced lifespan [39,55]. Not surprisingly, the severity of the phenotypes correlates with the levels at which the transgene was accumulated in the neurons [39]. Unsolved is how the toxicity mediated by wild-type TDP-43 in such organisms relates to the situation in human disease. To date copy number variation of the *TARDBP* gene has not been found in humans nor are brain mRNA levels changed in most patients with various TDP-43 proteinopathies [15].

Nevertheless, in zebrafish, worms and flies, expression of human TDP-43 carrying ALS-linked mutations uniformly produces greater toxicity than wild-type TDP-43. For example, compared with zebrafish embryos expressing wild-type TDP-43, mutant TDP-43 expressing ones develop shorter motor axons, more aberrant branching and more severe motor impairments [40,41]. Similarly, expression of mutant forms of TDP-43 in the neurons of worms or flies causes a more profound neurotoxicity than expression of the wild-type protein [43,44]. No conclusion can be drawn in the fly and worm models about selective neuronal vulnerability in these examples, as toxicity simply followed the transgene expression pattern. Widespread expression in zebrafish larvae has been reported. However, toxicity has been quantified only for motor axons, leaving it unclear whether abnormalities are restricted to motor neurons. Lastly, in all the three systems, no clear insights have emerged concerning the contributions to disease from ubiquitinated aggregates, TDP-43 cleavage, cytoplasmic mislocalization or nuclear clearance. More detailed review of these models can be found in reference [56].

Increased levels of FUS/TLS—To date one study in zebrafish has described expression of wild-type or ALS-linked mutants of FUS/TLS [51]. Unlike similar expression of wild-type or ALS-linked mutant TDP-43, no significant alterations in morphology or motor axon outgrowth were found with either wild-type or mutant FUS/TLS.

Disease modeling in rodents

Loss of TDP-43 in mice—TDP-43 is essential for early mouse embryogenesis as mice with homozygous disruption of the *TARBP* gene were found to die embryonically due to defective outgrowth of the inner cell mass [57–60]. Heterozygous *TARBP* gene disruption mice are phenotypically undistinguishable from control littermates, although one of the three reports describes modest motor behavioral abnormalities in aged mice [58]. To test a physiological role by systemic deletion of TDP-43 in adult mice, wide spread post-natal deletion of TDP-43 was achieved through inactivation of a conditional (floxed) TDP-43 allele by induction with tamoxifen of a Cre recombinase (produced from insertion of Cre coding sequences into the Rosa26 gene that is thought to be ubiquitously expressed). This did not lead to ALS- or FTLD-like symptoms, but instead produced dramatic loss of body fat and lethality within 9 days [57].

Loss of FUS/TLS in mice—Disruption of the *FUS/TLS* gene has been achieved by two independent groups by the insertion of “gene-trap” constructs inserted into exons 8 [61] or 12 [62]. Both insertions lead to complete loss of full length FUS/TLS protein. Heterozygous FUS/TLS gene disruption mice do not display any overt phenotype. The consequence of homozygous loss of FUS/TLS produces striking differences depending on the mouse genetic background. In the inbred C57BL/6 or 129 mouse strains, absence of FUS/TLS causes perinatal death [61,62], accompanied by major defects in B-lymphocyte development [61]. In an outbred background, mice with complete loss of FUS/TLS survive until adulthood, albeit with male sterility and reduced female fertility [62]. Interestingly, murine embryonic fibroblasts derived from the two sets of mice reveal high chromosomal instability and radiation sensitivity, suggesting that FUS/TLS plays a key role in the maintenance of genome integrity. That loss of FUS/TLS from outbred adult mice apparently does not lead to neurodegeneration offers strong evidence against loss of function(s) as a major contributor to FUS/TLS proteinopathies.

Consequences of increasing wild-type TDP-43 in mice—In the past two years, eight reports in mice or rats have described elevated levels of either wild-type or mutants of TDP-43 and one report for FUS/TLS (Table 2) [31,32,45–50,52]. All of the mouse models express a human TDP-43 cDNA (either wild-type or carrying ALS-linked mutations) under

the control of heterologous promoters (mouse prion, mouse Thy1 or calcium/calmodulin-dependent kinase II (CaMKII)) which are known to drive expression mostly in neuronal and non-neuronal cells of the central nervous system (CNS).

Expression of wild-type human TDP-43 in the CNS of mice has consistently been found to be toxic in a dose and threshold-dependent manner [31,32,45–47,49]. Reported phenotypes are divergent, including motor abnormalities [31,32,45,46,48–50], growth retardation [45,56,63] and cognitive impairments [47] and in some cases lethality [32,46,48–49]. The variability of the phenotypes observed does not always correlate with the promoter driving expression of the transgene. For example, a wide range of motor dysfunctions (such as gait abnormality or limb reflex impairment) were reported in mice expressing human wild-type TDP-43 under the control of Thy1 [45,49], prion [32] or CAMKII [31] promoters. On the other hand, prion-driven expression of comparable levels of TDP-43 (2.5–3 fold above that of the endogenous in total spinal cord extracts) either caused initial gait abnormalities and death by 60 days of age [32] or did not produce any overt phenotype [46]. The opposing results may be due to (1) divergence in the cells in which the transgene is expressed, (2) different genetic backgrounds (a mixed B6SJLF1xCD1 or C57BL/6xCD3heJ background or an inbred C57BL/6 or FVB strain) and/or (3) disruption of an endogenous locus by the random integration of the transgene. It is noteworthy that the severity of the phenotype was significantly increased in homozygous TDP-43 animals, compared to heterozygotes with similar total levels of transgene in the CNS. Homozygous expression of wild-type human TDP-43 driven either by Thy1 or prion promoters produced a severe motor phenotype and lethality, respectively [32,49], while no death was reported in heterozygous TDP-43 mice with apparently equivalent total levels of accumulated human TDP-43 in the CNS [45,46].

Early cognitive impairments accompanied with late motor defects, both of which are features of FTLD and primary lateral sclerosis, were found in transgenic mice expressing elevated levels of mouse TDP-43 under the control of the CaMKII promoter [47]. These transgenic mice developed learning and memory deficits at 2 months of age with motor dysfunctions (including gait abnormalities and reduced performance in rotarod) and reduction in brain mass observed at 6 months of age, concomitantly with cortical astrogliosis and neuronal loss. Untested is whether such cognitive defects develop in mice expressing comparable levels of wild-type human TDP-43 from the same promoter (such mice have so far only been reported to develop a late clasping phenotype [31]).

Hallmarks of neurodegeneration were also reported in some sets of transgenic mice (with varying promoters) to produce accumulated levels of wild-type TDP-43, estimated to range 1.7 to 4 fold above endogenous levels). Loss of cortical and/or anterior horn spinal cord neurons [47,49], or dentate gyrus neurons [31] was reported in mice where expression of wild-type TDP-43 accumulation was driven by either Thy-1 or CaMKII promoters, respectively. The neurons that degenerate are in most cases the ones in which expression was expected to be driven at highest levels by the specific transgene promoters. Astrogliosis was also found in the CNS of most of the transgenic mice [31,32,47,49]. However, axonal degeneration was only described in three sets of mice expressing TDP-43 through Thy1 [45,49] or prion [32] promoters. Altogether, expressing either wild-type human, or increased levels of mouse, TDP-43 predominantly in the CNS is toxic in mice, both when expression of the transgene is driven early during development (through the prion promoter) or post-natally (through either Thy1 or CaMKII promoters).

TDP-43 mutant expression in mice—Two studies have reported consequences in mice of expressing ALS-linked mutants of TDP-43 [46,48]. Accumulation of mutant human TDP-43 carrying the A315T mutation (TDP-43^{A315T}) to 3–4 fold above that of endogenous TDP-43 in the CNS (using the prion promoter) caused gait abnormalities and ultimately

death within 150 [46] or 75 days [48]. Despite wider expression of the transgene in the CNS when directed by the prion promoter, accumulation to 3 fold above the endogenous level of TDP-43^{A315T} led to a loss of cortical layer V pyramidal neurons in the frontal cortex, spinal cord motor neurons as well as upper motor axons, all of which are features that are reminiscent of both ALS and FTLD [48]. Not established is whether this loss is mutant selective, as loss of these neurons was also found in mice expressing human wild-type TDP-43 predominantly in neurons [49]. No reduction in the number of neurons was reported in transgenic mice accumulating higher levels of mutant TDP-43^{A315T} in the CNS [46]. The simplest explanation for this discrepancy is differences in cell types in which the transgene is accumulated at the highest levels, even though the expression of the transgene was driven by the same promoter (the same promoters in different lines of transgenic mice can lead to different patterns of transgene expression [63]). Settling what can be concluded from these efforts will require additional mice.

Two studies have proposed that expression of ALS-linked mutants (TDP-43^{A315T} [46,48]; TDP-43^{M337V} [46]) causes progressive motor neurodegeneration in mice. This is not established. Motor defects and/or loss of neurons have been reported only at one late time point of disease stage. With the exception of grip strength and gait posture, measurements have been reported at varying ages between 5 and 20 weeks; these findings reveal that 5 week old transgenic animals already have reduced motor capacities compared to their non-transgenic littermates [46]. Therefore, to determine age-dependent loss of motor neurons will require assessing motor defects at earlier ages.

Another key question not yet established is whether ALS-linked mutants of TDP-43 trigger higher toxicity than the wild-type protein in mice. Only one study has so far directly attempted to address this question. Analysis of transgenic mice expressing human wild-type, TDP-43^{A315T} or TDP-43^{M337V} under the same promoter (mouse prion) [46] led the authors to conclude that while expression of low levels of human ALS linked mutants of TDP-43 give rise to motor dysfunction, mice expressing the human wild-type protein do not display an overt phenotype. Nevertheless, although a large number of lines were obtained, no lines with matching levels of transgene accumulation in the CNS were identified for lines expressing mutant and wild-type TDP-43, thus preventing determination of whether TDP-43 toxicity is significantly enhanced in mice by ALS-causing mutations.

Transgenic mice with tetracycline-inducible expression in the forebrain of a human TDP-43 variant with a defective nuclear localization signal (TDP-43ΔNLS) and additional mice expressing wild-type protein (using CaMKII as a promoter) were reported in an effort to model the cytoplasmic mislocalization of TDP-43 reported in human disease [31]. High accumulation (~8–9 fold above the endogenous!) of TDP-43ΔNLS in the forebrain led to an abnormal motor clasping phenotype (a month after inducing the expression of the transgene) and a progressive loss of most dentate gyrus neurons (reaching a decrease of 90% after 6 months of induction of the transgene expression), as well as a decreased number of upper motor axons. Accumulation at much lower levels (1.7 fold above the endogenous) of wild-type TDP-43 in the forebrain caused similar neuronal and motor defects (although a longer period of induction of expression of the transgene was required). Whether either of these two transgenic mice also display cognitive/behavioral impairments or develop seizures (since most of the dentate gyrus neurons are lost) has not been reported.

A substantial down-regulation of endogenous mouse TDP-43 was found in mice expressing human wild-type TDP-43 [31]. This finding, which is also apparent in the analyses of transgenic TDP-43 mice built by other groups [19,32,48], confirms in an *in vivo* setting the auto-regulation mechanism for TDP-43 discussed above. Similarly, down-regulation of the endogenous TDP-43 was also found in mice expressing TDP-43ΔNLS. Although

TDP-43ΔNLS was designed to be exclusively in the cytoplasm, ~50% of the transgene product was actually found in nuclear cortical extracts. It is therefore likely that the decrease in the levels of the mouse endogenous TDP-43 is also caused by the nuclear pool of the TDP-43ΔNLS regulating the mRNA encoding endogenous TDP-43.

Mechanisms of TDP-43-mediated proteinopathies—Most of the features described in TDP-43 proteinopathies, including nuclear clearance and/or cytoplasmic mislocalization, ubiquitinated aggregates, phosphorylation or cleavage of TDP-43 [15] have been examined in the multiple transgenic mice outlined above. Surprisingly, although cytoplasmic TDP-43 inclusions have systematically been reported in neurons of ALS and FTLD-U (FTLD with ubiquitinated inclusions) patients, cytoplasmic ubiquitinated inclusions that are TDP-43 or phosphorylated-TDP-43 immunopositive were rarely [31,32,46,49] or never [45,48] found in the mouse models, indicating that at least in mice cytoplasmic inclusions are not required for neurodegeneration. In independent sets of mice expressing wild-type TDP-43 under the control two different promoters [32,45], TDP-43 negative cytoplasmic aggregates have been reported to be comprised of large perinuclear accumulation of mitochondria. With the implication of mitochondria in the mutant SOD1 rodent models of ALS [64], mitochondrial dysfunction may be a common feature of ALS pathogenesis occurring in both TDP-43 and SOD1-mediated disease. In the human context, TDP-43 cytoplasmic aggregation has been shown to be accompanied by nuclear clearance of the protein. Similarly, loss of nuclear localization of TDP-43 has been reported in neurons of most of the mouse models [31,32,47,48,49]. However, the frequency of nuclear TDP-43 depletion has not been reported, so it is not yet possible to conclude that loss of nuclear TDP-43 is a principal contributor to disease. Similarly, down-regulation of endogenous mouse TDP-43 has also been observed in multiple sets of transgenic mice [31,32,48]. Whether neuronal vulnerability results from perturbations of the normal mouse TDP-43 function is not established. The presence of carboxy-terminal truncated TDP-43 fragments (CTFs) has also been implicated in neurodegeneration from their appearance in human disease. In the mouse models, several studies have reported 25kDa and 35kDa CTFs in CNS extracts [32,46–49], but not in three other mice [31,45] despite a significant loss of neurons mediated by the expression of TDP-43 in one of these latter mice [31]. Therefore, how and why these fragments are generated (and their relationship to neurodegeneration) remains unclear and their direct toxic role in TDP-43 proteinopathies has not been established by the experimental evidence available to date.

Two studies using high-throughput sequencing (RNA-seq) or microarray approaches have identified changes occurring at the mRNA level in mice expressing wild-type TDP-43 [31,45] or TDP-43ΔNLS [31], all of which developed motor abnormalities. Alterations in both differential expression and alternative splicing of hundreds of transcripts, many encoding proteins involved in cellular architecture [45] or macromolecular complex organization (in particular DNA-protein assemblies) [31] have been reported. Neurofilament mRNAs are among those significantly down-regulated in some TDP-43 transgenic mice [31,45], similar to what happens after reduction of TDP-43 in an otherwise normal adult mouse CNS [19]. Since the mice expressing human wild-type or TDP-43ΔNLS down-regulate endogenous mouse TDP-43, it is likely that the reported decreased expression of neurofilaments is caused by loss of nuclear TDP-43 function. Finally, increased levels of wild-type TDP-43 in mice was also reported to lead to an increased number, in neuronal nuclei, of an intranuclear structure called GEMs (Gemini of coiled bodies) [45]. GEMs are nuclear structures enriched in SMN (Survival Motor Neuron) complex proteins which are widely thought to play a role in the biogenesis of small ribonucleoproteins required for mRNA splicing. (Reduction in SMN is causative of the juvenile recessive disease spinal muscular atrophy (SMA), in which spinal motor neurons degenerate, causing progressive

paralysis and muscular atrophy). It remains now to be determined if, and if so how, elevated numbers of these nuclear structures may contribute to damaging motor neurons.

TDP-43 model rats—Transgenic rats expressing human wild-type or an ALS-linked mutant form of TDP-43 (M337V) constitutively have also been generated [50]. To date, this is the only rodent model expressing human wild-type or mutant TDP-43 from the authentic human gene and its promoter (instead of TDP-43 cDNA). Wild-type TDP-43 expressing rats do not develop any overt phenotype. However, comparable levels of expression of the same construct but carrying the M337V substitution (TDP-43^{M337V}) were lethal and no lines could be established. This is the only evidence today in rodents for toxicity mediated in a TDP-43 mutant selective manner. An even more attractive model emerged from additional rats ubiquitously expressing TDP-43^{M337V} cDNA in a tetracycline dependent manner. While transgene expression when induced during development is lethal, induction at postnatal day 10 produces widespread neurodegeneration primarily in the motor system with fatal paralysis within 45–50 days of age. Of note, it is not known whether motor neuron disease from induced expression is selective for mutant TDP-43, as expression of wild-type TDP-43 using a similar promoter has not been examined.

FUS/TLS transgenic rats—Transgenic rats expressing human wild-type or an ALS-linked mutant form of FUS/TLS (R521C) conditionally have been generated [52]. Accumulation of mutant protein, but not wild-type, levels at three to six times above that of endogenous in the CNS led to degeneration of motor axons, loss of cortical and hippocampal neurons, increased denervation of neuromuscular junctions and ultimately paralysis in 30–70 days after induction of the transgene. Expression of wild-type FUS/TLS was accompanied by a mild loss of neurons in the cortex and hippocampus, as well as cognitive deficits at one year of age. Altogether, accumulation at high levels of mutant FUS/TLS in the CNS is more toxic than wild-type protein in rats.

Conclusion & Perspectives

It is early days in deciphering how mutations in the RNA/DNA binding proteins TDP-43 and FUS/TLS cause inherited ALS. TDP-43 normally functions in the maturation and splicing of thousands of pre-mRNAs. TDP-43 synthesis is controlled by an auto-regulatory mechanism mediated at least in part by direct binding to the 3′untranslated region (UTR) of its own RNA transcript. Modeling in rodent, zebrafish and invertebrate systems has produced imperfect replicas of ALS-like disease. Many of the transgenic animals reported so far develop motor and/or cognitive impairments at very young ages and progressiveness has in most cases not been established. As yet left unanswered are two of the most central questions concerning mutant TDP-43 (or FUS/TLS) mediated toxicity: 1) is pathogenesis caused by the loss of normal function(s), gain of one or more toxic properties or both, and 2) what are the crucial cell type(s) in which mutant TDP-43 or FUS/TLS acts to provoke toxicity?

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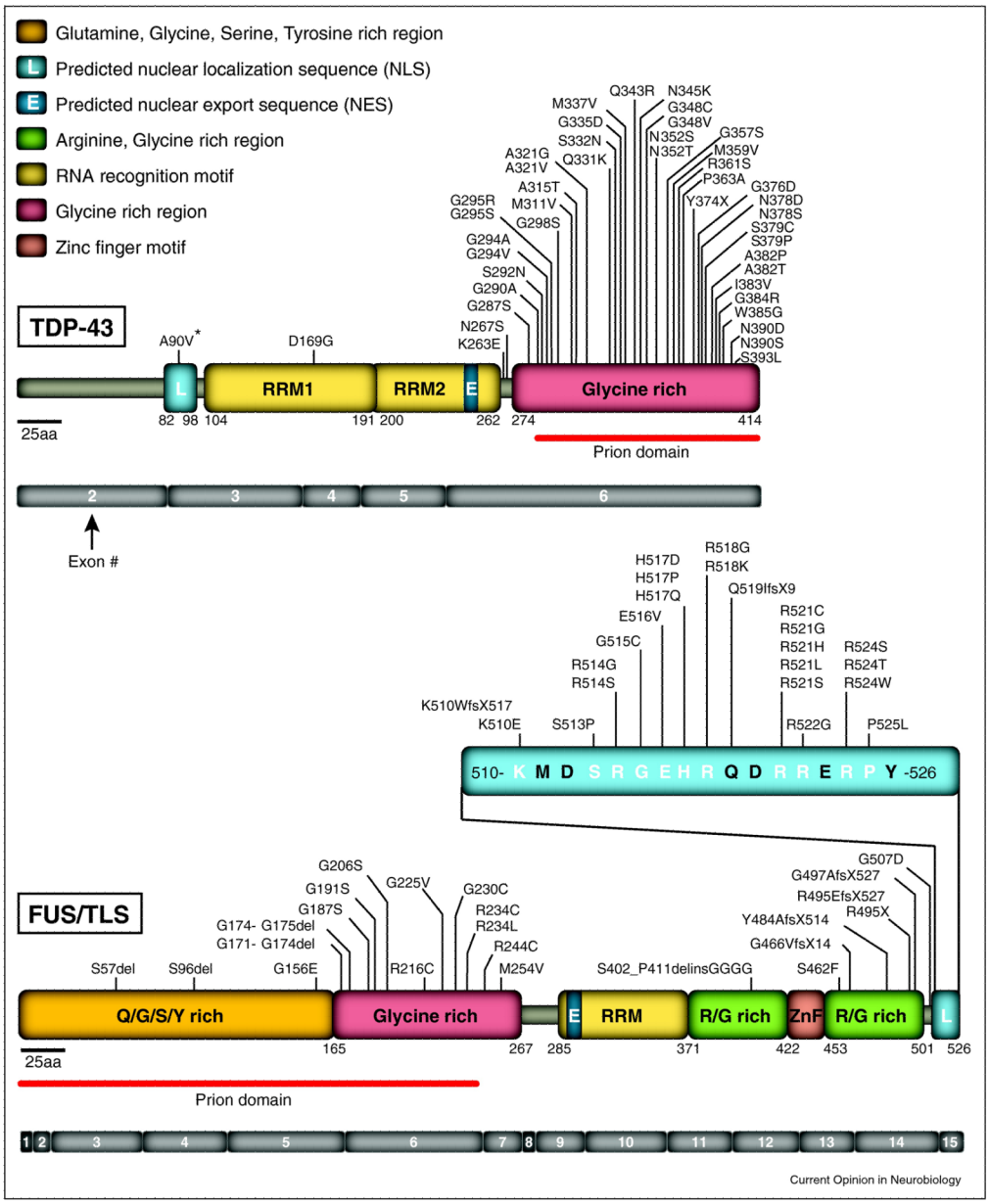

Figure 1. TDP-43 and FUS/TLS mutations in ALS and FTLD patients

(Upper panel) Forty-four mutations have been identified in TDP-43 in sporadic and familial ALS patients and in rare FTLD patients, with most lying in the C-terminal glycine-rich region. The putative prion domain comprises amino acids from 277–414. (Lower panel) Forty-three mutations have been identified in FUS/TLS in familial and sporadic ALS cases and in rare FTLD patients. Most mutations are clustered in the last 17 amino acids and in the glycine-rich region and the putative prion domain comprises amino acids 1–239.

Table 1

Genetics of human ALS.

Locus	Gene	Protein	Mutations	Proportion of inherited ALS	Discovery date	Reference
21q22.1	*SOD1*	Cu/Zn superoxide dismutase	>150*	20%	1993	[65]
9p13.2–21.3	*Unknown*	Unknown	Unknown	20%**	Unknown	[66–68]
1q36	*TARDBP*	TDP-43	>40*	5%	2008	[9,10••,11••]
16p11.2	*FUS*	FUS/TLS	>40*	4%	2009	[13••,14••]
9p13.3	*VCP*	Valosin-containing protein	5	1–2%+	2010	[70]
10p15-p14	*OPTN*	Optineurin	1	1–2%+	2010	[71]
6q21	*FIG4*	PI(3,5)P(2)5-phosphatase	5*	1%+	2009	[72]
12q24	*DAO*	D-amino acid oxidase	1	1%+	2010	[73]
14q11	*ANG*	Angiogenin	>10*	<1%	2006	[74]

Only classical adult onset ALS cases with autosomal dominant inheritance are included. Each gene, except for SOD1, accounts for <1% of all (familial + sporadic) ALS cases.

*Including sporadic ALS cases.

**The largest remaining cohort of inherited ALS, estimates of proportion of inherited ALS are up to 20% (reported to account for 38% of familial ALS in Finland [69]).

+To date, reported only in a single publication.

Table 2

mimics of TDP-43 and FUS/TLS in disease.

Promoter	Transgene	Mutations	# lines	Fold over expression	Phenotype	Death (days)	Overall pathology	Loss of endogenous TDP-43	Nuclear clearance	Aggregation	CTFs of TDP-43	Reference
Synaptobre-vin (snb-1)	hTDP-43 cDNA	WT	1	n.r.	Unco-ordinated movement	n.r.	Abnormal MN synapses	n.r.	n.r.	n.r.	n.r.	Ash *et al*., 2010 [38•]
		ΔRRM1 or −2 mutants	1	n.r.	Normal	n.r.	None	n.r.	n.r.	n.r.	n.r.	
Synaptobre- vin (snb-1)	hTDP-43 cDNA	WT	2	n.r.	• Unco-ordinated +slowed movement: more severe in mutants	None	None	n.r.	n.r.	NII TDP-43+	Yes	Liachko *et al*., 2010 [43•]
		G290A	1	n.r.	• Paralysis in mutants	Reduced lifespan	Degeneration of neurons in all the mutants	n.r.	n.r.	NII TDP-43+	Yes	
		A315T	1	n.r.								
		M337V	1	n.r.								
GMR-Gal4 (eye) D42-Gal4 (MN)	hTDP-43 cDNA	WT	1	n.r.	Eye depigmentation	n.r.	Degeneration of photoreceptor cells	n.r.	n.r.	Ubiquitination	n.r.	Hanson *et al*., 2010 [39•]
		WT	3	n.r.	• Movement defects • Paralysis	Reduced lifespan (50%)	n.r.	n.r.	n.r.	PhosphoTD P-43 localization	No	
GMR-Gal4	hTDP-43 cDNA	WT	1	n.r.	Abnormal eye morphology	n.r.	Degeneration of photoreceptor cells	n.r.	n.r.	n.r.	n.r.	Li *et al*., 2010 [42•]
OK107-Gal4 (MB) or OK371- Gal4 (MN)		ΔNLS+ RRM1- mutant	1	n.r.	Normal	n.r.	None	n.r.	n.r.	n.r.	n.r.	
		WT	1	n.r.	Reduced movement	n.r.	MN loss Aggregates in cell bodies and axons Reduced NMJ	n.r.	n.r.	Rare cyto TDP-43 localization	n.r.	
GMR-Gal4	hTDP-43 cDNA	WT	5	n.r.	Modest abnormalities in eyes	n.r.	n.r.	n.r.	n.r.	n.r.	No	Ritson *et al*., 2010 [45•]
		M337V	6	n.r.	Modest to severe abnormalities in eyes		Degeneration of photoreceptor cells			n.r.	Yes	
		ΔNLS-Mut	6	n.r.	Severe abnormalities in eyes		Severe degeneration of photoreceptor cells			Cyto TDP-43 localization	No	
		ΔNES-mut	5	n.r.	Normal							

Promoter	Transgene	Mutations	# lines	Fold over expression	Phenotype	Death (days)	Overall pathology	Loss of endogenous TDP-43	Nuclear clearance	Aggregation	CTFs of TDP-43	Reference
Gal4 221 (sensory neurons)	hTDP-43 cDNA	WT Q331K M337V	n.r.	n.r.	n.r.	n.r.	Increased number of small terminal dendritic branches (higher in WT)	n.r.	n.r.	n.r.	n.r.	Lu *et al*., 2009 [54•]
GMR-Gal4	hTDP-43 cDNA	WT	1	n.r.	Mild rough eye	n.r.	Loss of pigmentation	n.r.	n.r.	Nuclear and cyto TDP-43	n.r.	Miguel *et al*., 2010 [55•]
Elav-Gal4GS (neurons)		ΔNLS-Mut	1		Severe rough eye		Severe loss of pigmentation			Cyto TDP-43	n.r.	
		ΔNES-mut	1		Normal		None			TDP-43 + nuclear foci	n.r.	
		WT	1		Reduced lifespan	16					Yes	
		ΔNLS-Mut	1		Reduced lifespan	21					Yes	
		ΔNES-mut	1		Reduced lifespan	25 (/47)					Yes	
n.a.	hTDP-43 mRNA	WT	n.a.	n.r.	Motor defects	n.r.	• Axonal shortening	n.r.	n.r.	Nuclear TDP-43	n.r.	Laird *et al*., 2010 [41•]
		A315T	n.a.	n.r.	Motor defects	n.r.	• Aberrant branching • More severe axonal shortening • Aberrant branching • Axonopathy					
n.a.	hTDP-43 mRNA	WT A315T	n.a.	n.r.	Mild swimming deficits	n.r.	• Motor axonal shortening	n.r.	n.r.	n.r.	n.r.	Kabashi *et al*., 2010 [40•]
		A382T G348C	n.a.	n.r.	Swimming deficits	n.r.	• Excessive branching					
n.a.	hFUS/TLS mRNAs GFP tag	WT H517Q R521G	n.a.	n.r.	None	n.a.	None	n.r.	n.r.	Nuclear TDP-43	n.r.	Bosco *et al*., 2010 [51•]
		R495X G515X	n.a.	n.r.	n.r.	n.r.	n.r.	n.r.		Cyto TDP-43	n.r.	
Prnp	hTDP-43 cDNA Flag tag	A315T	1 het (/8 F0)	3×	• Gait abnormality (3–4 mo) • Weight loss (4.5 mo)	155	• Loss of CL5 +SC motor neurons • Loss of axons in DCT and LC • Astrogliosis in CL5 • SkM degeneration	Yes	Yes	NCI Ubi+, TDP-43−	Yes	Wegorzewska *et al*., 2009 [48••]

Promoter	Transgene	Mutations	# lines	Fold over expression	Phenotype	Death (days)	Overall pathology	Loss of endogenous TDP-43	Nuclear clearance	Aggregation	CTFs of TDP-43	Reference
Thy1	hTDP-43 cDNA	WT	2 het 2 homo (/ 23 F0)	<1× <2×	Normal • Gait abnormality • Reduced rotarod performance • Abnormal hindlimb reflex • Paralysis	n.a. 24–200	None • Loss of CL5 +SC motor neurons • Loss of axons in DCT and LC • Astrogliosis in CL5 • SkM degeneration	n.r. n.r.	No Yes	No Rare NII and NCI Ubi+, TDP-43+	No Yes	Wils *et al.*, 2010 [49••]
CaMKII	mTDP-43 cDNA	WT	3 homo (/ 10 F0)	2×	• Impaired learning/ memory capacities (2mo) • Reduced brain weight + rotarod performance (6mo) • Gait abnormality (6mo)	495 (/632 in non tg)	• Loss of cortical neurons (24%) • Astrogliosis in cortex/ hippocampus	n.r.	Rare	NCI Ubi+, TDP-43+	Yes	Tsai *et al.*, 2010 [47•]
Prnp	hTDP-43 cDNA	WT	2 het (/12)	3×	Normal	n.a.	• Mild astrogliosis	n.r.	No	Diffuse ubi+ neurons	No	Stallings *et al.*, 2010 [46••]
		A315T	3 het (/8 F0)	4×	• Gait abnormality (5w)	75	• Astrogliosis in SC	n.r.	n.r.	NCI Ubi+, TDP-43+	Yes	
		M337V	8 F0	>10×	• Reduced grip strength (5w) • Profound weakness, spasticity, reduced movements	16–42	• Mild SkM denervation • Astrogliosis in SC • Neuronal morphological abnormalities	n.r.	n.r.	NCI Ubi+, TDP-43+	Yes	
Prnp	hTDP-43 cDNA	WT	1 het	1.9×	Normal	n.a.	None	Yes	n.r.	n.r.	Yes	Xu *et al.*, 2010 [32••]
			1 homo (/ 6 F0)	2.5×	• Reduced body weight (14d) • Gait abnormality (21d) • Tremor (21d)	30–60	• Astrogliosis in SC • Axon degeneration	Yes	rare	• NCI TDP-43+ • NII TDP-43+ • Cyto aggregates with clusters of mitochondria	Yes	

Promoter	Transgene	Mutations	# lines	Fold over expression	Phenotype	Death (days)	Overall pathology	Loss of endogenous TDP-43	Nuclear clearance	Aggregation	CTFs of TDP-43	Reference
Thy1	hTDP-43 cDNA	WT	1 het (/3 F0)	2.3–4.6×	• Reduced body weight (4w) • Gait abnormality (14–18 d) • Tremor (14–18 d) • Abnormal hindlimb reflex	Normal (1 line was lethal)	• Abnormal NMJ morphology • Reduction in large-caliber motor axons	n.r.	n.r.	• Cyto aggregates with clusters of mitochondria • NII TDP-43+ • increased number of gemini bodies	No	Shan *et al.*, 2010 [45•]
CaMKII-tTA	tetO-hTDP-43 cDNA (Tet-off inducible)	WT	1 het (/4 F0)	1.7×	Abnormal limb clasping (1w*)	n.r.	• Loss of DG neurons (20% at 1mo)	Yes	Yes (mTDP-43)	Rare NCI ubi+, TDP-43+	No	Igaz *et al.*, 2010 [31••]
		(NLS mutant	1 het (/2 F0)	8–9×	Abnormal limb clasping (1–3 mo*)	n.r.	• Reduction in brain weight • Astrogliosis in CST • Axonal loss in CST • Loss of DG neurons (50% at 1mo) • Astrogliosis in CST • Axonal loss in CST	Yes	Yes (mTDP-43)	Rare NCI ubi+, TDP-43+	No	
TARDBP	22kb hTDP-43 gene	WT	2 het	2–4×	Normal	None	None	n.r.	n.r.	3 Cyto Phospho- TDP-43	Yes	Zhou *et al.*, 2010 [50••]
CAG-tTA	TRE-miniCMV- hTDP-43	M337V	3 F0	3–6×	Loss of mobility	10–29	n.r.	n.r.	n.r.	n.r.	n.r.	
	cDNA (Tet-off inducible)	M337V	2 het	2–4×	• Limb weakness (20–34 d) • Reduced rotarod performance (34d*) • Paralysis (35–49 d*)	35–49	• Loss of neurons in SC (15%) • Loss of L3 SC axons (40%) in CST • Neuronal degeneration in cortex, hippocampus and cerebellum • Astrogliosis in SC • Loss of NMJ (40%) • SkM denervation	n.r.	n.r.	• Cyto Phospho-TDP-43 • rare NCI TDP-43+	Yes	

Promoter	Transgene	Mutations	# lines	Fold over expression	Phenotype	Death (days)	Overall pathology	Loss of endogenous TDP-43	Nuclear clearance	Aggregation	CTFs of TDP-43	Reference
CAG-tTA	TRE-miniCMV- hFUS cDNA (Tet-off inducible)	WT	1 het (/12 F0)	3–6×	• Normal until 360d	None	• Loss of neurons in cortex (~15%) and hippocampus	n.r.	None	Ubi +, FUS−	n.r.	Huang *et al*., 2011 [52••]
		R521C	2 het (/14 F0)	3–6×	• Reduced spatial learning and memory (Barnes maze) • Reduced fore/hind grip strength (25–55d*) • Reduced body weight • Paralysis (30–60d*)	30–70	• Loss of L3 SC axons and in CST • Neuronal loss in cortex and hippocampus • Astrogliosis in SC and cortex • Loss of NMJ (40%)	n.r.	None	Ubi +, FUS−	n.r.	

gene above endogenous levels assessed by qRT-PCR either in cortex or spinal cord; CTFs: C-terminal fragments; n.r.: not reported; n.a.: not applicable; NLS: Nuclear localization signal; NES: Nuclear export signal; RRM1: RNA Recognition Motif; zygous; Homo: Homozygous; F0: Founder; Ubi+: Ubiquitin positive; NII: Neuronal Intranuclear Inclusions; NCI: Neuronal Cytoplasmic Inclusions; w: weeks; mo: months; d: days; NMJ: Neuromuscular junction; MN: motor neurons; SkM: Skeletal tract; LC: Lateral column; CL5: Cortical Layer 5; DG: Dentate Gyrus; MB: Mushroom Bodies; hTDP-43: human TDP-43; mTDP-43: mouse TDP-43; WT: Wild-type.