Partial loss of TDP-43 function causes phenotypes of amyotrophic lateral sclerosis

Chunxing Yang*, Hongyan Wang*, Tao Qiao*, Bin Yang*,1, Leonardo Aliaga*, Linghua Qiu*, Weiija Tan*, Johnny Salameh†, Diane M. McKenna-Yasek†, Thomas Smith*, Lingtao Peng*,e, Melissa J. Moore*,e,f, Robert H. Brown, Jr.†, Huabin Cai†, and Zuoshang Xu*a,e,g,h,2

Departments of *Biochemistry and Molecular Pharmacology, †Neurology, ‡Pathology, and ™Cell Biology, #RNA Therapeutic Institute, †Howard Hughes Medical Institute, and ‡Neuroscience Program, University of Massachusetts Medical School, Worcester, MA 01605; and ™Transgenics Section, Laboratory of Neurogenetics, National Institute on Aging, National Institutes of Health, Bethesda, MD 20892

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Amyotrophic lateral sclerosis (ALS) is a fatal neurological disease that causes motor neuron degeneration, progressive motor dysfunction, paralysis, and death. Although multiple causes have been identified for this disease, >95% of ALS cases show aggregation of transactive response DNA binding protein (TDP-43) accompanied by its nuclear depletion. Therefore, the TDP-43 pathology may be a converging point in the pathogenesis that originates from various initial triggers. The aggregation is thought to result from TDP-43 misfolding, which could generate cellular toxicity. However, the aggregation as well as the nuclear depletion could also lead to a partial loss of TDP-43 function or TDP-43 dysfunction. To investigate the impact of TDP-43 dysfunction, we generated a transgenic mouse model for a partial loss of TDP-43 function using transgenic RNAi. These mice show ubiquitous transgene expression and TDP-43 knockdown in both the periphery and the central nervous system (CNS). Strikingly, these mice develop progressive neurodegeneration prominently in cortical layer V and spinal ventral horn, motor dysfunction, paralysis, and death. Furthermore, examination of splicing patterns of TDP-43 target genes in human ALS revealed changes consistent with TDP-43 dysfunction. These results suggest that the CNS, particularly motor neurons, possess a heightened vulnerability to TDP-43 dysfunction. Additionally, because TDP-43 knockdown predominantly occur in astrocytes in the spinal cord of these mice, our results suggest that TDP-43 dysfunction in astrocytes is an important driver for motor neuron degeneration and clinical phenotypes of ALS.

Significance

Amyotrophic lateral sclerosis (ALS) is an incurable neurodegenerative disease that causes paralysis and death. TDP-43 is a protein that regulates gene expression. TDP-43 aggregation and depletion from cell nucleus are found in ALS. Therefore, TDP-43 may cause neurodegeneration by generating toxicity from its aggregation or by a loss of its function. Our experiments test the consequence of a partial loss of TDP-43 function in mice. The results demonstrate that a partial loss of TDP-43 function is sufficient to cause neurodegeneration and ALS symptoms. In addition, we have found evidence for TDP-43 dysfunction in human ALS. Therefore, we propose that TDP-43 dysfunction causes neurodegeneration in the human disease, and future therapy should aim to restore the normal function of TDP-43.


The authors declare no conflict of interest.

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1Present address: Division of Biology, California Institute of Technology, Pasadena, CA 91125.

2To whom correspondence should be addressed. E-mail: zuoshang.xu@umassmed.edu.

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and even cell death (21, 32, 33). Loss-of-function models in invertebrates and zebrafish have shown a wide range of defects, ranging from embryonic lethality to morphological and functional defects of the nervous system, vascular system, and muscle degeneration (27, 31, 34–37). In mammalian species, TDP-43 gene deletion leads to early embryonic lethality (38–41). Knockdown or knockout of TDP-43 in mammalian cells in vitro and in vivo also causes degenerative changes and cell death in some cases (21, 33, 38, 42, 43). Thus, the evidence suggests that TDP-43 dysfunction can lead to cellular dysfunction, degeneration, and death.

To further model TDP-43 dysfunction in vivo, a partial loss of function in mammalian species is desirable because it mimics the dysfunction but not a complete loss of function in the diseases. Additionally, experiments from selective knockout of TDP-43 in motor neurons have generated contradictory results, and it is not clear whether a lack of TDP-43 function in motor neuron alone is sufficient to cause motor neuron death (42, 43). Importantly, the motor neuron-selective knockout models do not address the possible contribution of TDP-43 dysfunction in glial cells. Given the fact that TDP-43 aggregation and nuclear depletion also occur in glial cells in human patients (10, 28, 29), TDP-43 dysfunction in glia may contribute to motor neuron toxicity and death. To address these questions, we created a transgenic (Tg) mouse line that expressed an artificial microRNA (amiRNA) targeting the TDP-43 mRNA (amiR-TDP43) for knockdown. We demonstrate that the transgene was expressed ubiquitously in both peripheral and CNS tissues. Strikingly, despite the widespread TDP-43 knockdown in various tissue and cell types, these mice predominantly developed age-dependent neurological symptoms, including hyperactivity, weakness, paralysis, and death. Concomitantly, pathological analysis revealed prominent degeneration of layer V cortical neurons and spinal motor neurons. These data suggest that motor neurons possess a heightened vulnerability to TDP-43 dysfunction. Because the transgene was expressed in both neurons and glia and mediated TDP-43 knockdown, our data also suggest that TDP-43 dysfunction in glia facilitates motor neuron degeneration. Finally, to determine whether TDP-43 dysfunction occurs in human ALS, we assayed alternative splicing of TDP-43–regulated genes and detected changes consistent with TDP-43 dysfunction. Our results support the hypothesis that TDP-43 dysfunction in both neurons and glia contributes to the neurodegeneration in ALS.

Results

Expression of an amiR-TDP43 Leads to ALS Phenotypes in Mice. We constructed Tg mice that expressed amiR-TDP43 using a Cre-loxP conditional expression strategy, which would permit temporal and spatial control of TDP-43 knockdown. The construct (amiR–TDP43u) would initially express GFP, but would switch to expressing a red fluorescent protein (RFP) and the amiR–TDP43 upon induction with Cre (44) (SI Appendix, Fig. S1A). However, although widely used in the literature, this strategy carried a technical artifact and caused microphthalmia when we attempted CNS-restricted induction of RFP and the amiRNA (45) (SI Appendix, Tables S1 and S2). Our previous studies have shown that this artifact was derived from tandem integration of inverted loxP-containing transgene copies into the genome (SI Appendix, Fig. S1B), which led to aberrant chromosome segregation (SI Appendix, Fig. S1C) and death of proliferating cells during brain development (45). Because of this artifact, we were precluded from inducing the transgene expression selectively in the CNS. To circumvent this problem, we crossed the amiR–TDP43u mice with a germ-line inducer, CMV–Cre, and converted the conditional-expression Tg mouse lines to the constitutive-expression ones, which expressed RFP and amiR–TDP43 constitutively and ubiquitously in a Cre-independent manner (SI Appendix, Figs. S1D, S2, and S6).

The converted line (amiR–TDP43i) expressed RFP, but no GFP, in the brain and spinal cord (SI Appendix, Fig. S2B–D) and consistently displayed age-dependent, progressive neurological phenotypes (Fig. 1). The mice initially behaved similarly to their nontransgenic (nTg) littermates but then developed head bobbing, unsteady gait, and discoordinated movements (Movies S1 and S2). Beginning at 4 wk of age, these mice displayed hyperactivity (Fig. 1A). However, the hyperactivity was transitory, peaking at ∼5 wk and ending at ∼10 wk of age, after which the mice became progressively weaker and less active compared with their nTg littermates (Movies S1 and S2 and Fig. 1A). Also, from ∼5 wk of age, the amiR–TDP43i mice started losing body weight (Fig. 1B) and showed reduced vertical behaviors (Fig. 1C). These abnormalities were progressive and eventually led to paralysis (Fig. 1D and Movies S1 and S2) and death (Fig. 1E). The motor weakness and paralysis suggested the presence of muscle degeneration. Indeed, needle electromyography (EMG) revealed that the amiR–TDP43i mice developed progressive active degeneration of muscles, as indicated by the presence of positive sharp waves (Fig. 1F and SI Appendix, Table S3). Notably, none of these abnormalities were observed in the parent, uninduced line (amiR–TDP43u) that expressed GFP. The amiR–TDP43u mice showed behavior and survival that were indistinguishable from their wild-type littermates, thus indicating that the phenotypes in the amiR–TDP43i mice were derived from the transgene induction and not from the transgene insertion into the mouse genome.

The amiR–TDP43i mice displayed three end-stage phenotypes: hindlimb paralysis, sudden death, and whole-body paralysis (SI Appendix, Tables S4 and S5). The mice that died with hindlimb paralysis were mostly males and had a relatively short lifespan (SI Appendix, Tables S4 and S5). Some developed hindlimb paralysis shortly after weaning. Some survived longer and went through a period of hyperactivity and weight loss before hindlimb paralysis (Movie S1 and Fig. 1). The mice with sudden death were also mostly males, which did not have significant weight loss but died suddenly and unpredictably. These mice also had relatively short lifespans (SI Appendix, Tables S4 and S5). A possible cause of death in these mice was cardiac failure because many Tg mice had an enlarged heart with TDP-43 knockdown (SI Appendix, Fig. S3). The mice with whole-body paralysis survived the longest among the three death phenotypes (SI Appendix, Table S5). This group had a stereotypic pattern of progression beginning with hyperactivity (Fig. 1J) and weight loss (Fig. 1B) before becoming totally paralyzed (Movie S2). A majority of both males and females had this phenotype (SI Appendix, Table S4). The average lifespan was slightly shorter in the males than in the females, but the difference was not statistically significant (Fig. 1E and SI Appendix, Table S5).

As a control, we also generated a line of Tg mice that constitutively expressed a scrambled amiRNA (amiR-SCR) using the same construct design and the same strategy to convert from the GFP-expressing parent line to the constitutive RFP-expressing and amiR-SCR–expressing line (45). Western blot analysis revealed that these Tg mice expressed a higher level of transgene than the amiR–TDP43 mice in the CNS (SI Appendix, Fig. S4A). However, the amiR-SCR mice did not develop any overt phenotypes up to >700 d of age, and there were no measurable changes in their behavior up to 10 mo of age (SI Appendix, Fig. S4B and C). Thus, it is unlikely that the expression of RFP and/or an exogenous miRNA nonspecifically caused the neurological phenotypes in the amiR–TDP43i mice. Rather, the phenotypes were likely linked to amiR–TDP43 expression and TDP-43 knockdown.

The Transgenes RFP and amiR–TDP43 Are Expressed in Multiple Tissues and in both Neurons and Glia in the CNS. To verify the TDP-43 knockdown, we first examined the transgene expression. We stained the mouse brain for the RFP distribution and found that...
the transgene was expressed broadly in the CNS, including forebrain, cerebellum, brainstem, and spinal cord (SI Appendix, Table S5). Additionally, Western blot analysis for RFP revealed that the transgene was expressed broadly in all tissues and particularly strongly in heart, muscle, and liver (SI Appendix, Fig. S6A). Furthermore, Northern blot analysis revealed similar pattern of expression for the amiR–TDP43 (SI Appendix, Fig. S6B). However, the difference in the amiR–TDP43 levels between the periphery and the CNS was not as wide as in the RFP levels (SI Appendix, Fig. S6C). Nevertheless, both RFP and amiR–TDP43 were expressed at higher levels in the peripheral tissues than in the CNS. This contrasts with the predominant neurological phenotypes in these mice and suggests that the CNS possesses a heightened vulnerability to the effects of the transgene expression.

To determine which CNS cell types express the transgene, we conducted double immunofluorescence analysis. The RFP was expressed in various neuronal types, including cortical neurons, cerebellar Purkinje cells, and spinal cord motor neurons. In addition, the RFP was expressed abundantly in astrocytes and in some oligodendrocytes, but not in microglia (SI Appendix, Fig. S7).

amiR–TDP43 Reduces TDP-43 Expression and Function in the CNS. Next we investigated TDP-43 knockdown by measuring TDP-43 levels and function. By bulk protein and mRNA measurements, we observed that TDP-43 was knocked down to modest degrees in the CNS but with large variations among individual animals (Fig. 2A–C and SI Appendix, Table S6). These variations might be caused by a variable number of cells that expressed transgene in different individual animals. We therefore sorted RFP-positive cells using fluorescence-activated cell sorting and found that only a small percentage of the cells from the cortex and the spinal cord were sorted as RFP-positive cells (Fig. 2D), where we detected a robust knockdown of TDP-43 mRNA (Fig. 2E). However, we also detected lower TDP-43 levels in RFP-negative cell populations (Fig. 2F). This suggested that the cell sorting could only separate the brightest RFP-positive cells, and the cells with modest levels of RFP were left with the RFP-negative cells. To test this possibility, we carried out immunofluorescence staining, which showed that ~30–50% of cells in the cortex were RFP-positive. These cells showed both neuronal and astrocytic morphology (Fig. 2F). In the RFP-positive cells, we detected a decrease in TDP-43 staining intensity compared with the neighboring RFP-negative cells (Fig. 2G). Thus, TDP-43 knockdown is correlated with the transgene expression.

To determine whether the TDP-43 knockdown compromised TDP-43 function, we examined changes in alternative splicing in genes that are regulated by TDP-43 (46–48). By PCR across the alternatively spliced exons, we detected the changes known to be caused by TDP-43 knockdown in sorted RFP-positive cells (Fig. 2H and I). Additionally, we consistently detected the change in alternative splicing of S6K1 Aly/REF-like target (Skar), also known as polymerase delta interacting protein 3 (Poldip3), in the CNS homogenates from amiR–TDP43i mice by both RT-PCR from the total RNA and Western blots of total proteins. Specifically, the exon 3-excluded isoform was increased in the brain and spinal cord homogenates (Fig. 2J).

Fig. 1. The amiR–TDP43i mice developed progressive neurological phenotypes. (A) The amiR–TDP43i mice went through a hyperactive stage before developing weakness and paralysis. (B) The body weight of the amiR–TDP43i mice peaked at ~50 d of age and then declined until the end stage of the disease. The values were averaged from 7 to 39 animals per group. (C) The amiR–TDP43i mice developed weak vertical activities, including rearing and jumping. These activities peaked at 5 wk of age and became progressively weaker thereafter. (D) An example of an amiR–TDP43i mouse with hindlimb paralysis. The mouse on the right was an nTg mouse, which was walking past the paralyzed amiR–TDP43i mouse on the left. (E) The amiR–TDP43i mice died prematurely (also see SI Appendix, Table S5). (F) EMG measurement showed a pattern of muscle denervation, as indicated by the presence of positive sharp waves (PSWs; also see SI Appendix, Fig. S7). The data in A and C were obtained by using a home cage scan system (SI Appendix, SI Materials and Methods) and from 16 nTg and 19 amiR–TDP43i mice. Both sexes were grouped together because there was no obvious difference in the pattern of progression in these behavioral parameters between the sexes.
To further characterize the pattern of TDP-43 knockdown in the spinal cord, we stained spinal cord sections for TDP-43 and choline acetyltransferase (ChAT) or TDP-43 and GFAP (Fig. 3 A and B). Nearly all ChAT-positive motor neurons and the GFAP-positive astrocytes were RFP-positive in the amiR–TDP43i mice (SI Appendix, Fig. S7). Therefore, we compared the TDP-43 fluorescence intensity in the ChAT- or GFAP-positive cells between the amiR–TDP43i and nTg mice. We found a significantly lowered level of TDP-43 in astrocytes, but not in motor neurons in the amiR–TDP43i mice (Fig. 3 A–C). The lack of detectable knockdown in motor neurons was likely a result of relatively weak transgene expression in motor neurons compared with the astrocytes (Fig. 3D). Thus, the transgene expression and TDP-43 knockdown predominantly occur in astrocytes in the spinal cord.

Mice Expressing amiR–TDP43i Develop Neurodegeneration in Layer V Cortex and Ventral Horn Spinal Cord. Hyperactivity is a common phenotype in many rodent models of forebrain neurodegeneration (49–53). Therefore, the hyperactivity of the amiR–TDP43i mice suggested the presence of neuronal degeneration in the forebrain.
Indeed, we detected a significantly reduced brain mass compared with controls (Fig. 4A) and robust astrogliosis in the deep layer of the frontal cortex (Fig. 4B). A quantitative analysis of the cortex using Nissl-stained sections revealed an ∼25% loss of large neurons in cortical layer V (Fig. 4C–E). The motor weakness and paralysis suggested the presence of motor neuron degeneration. To confirm this possibility, we examined the spinal cord of the amiR-TDP43i mice. We observed abundant astrogliosis and microgliosis in the ventral horn gray matter (SI Appendix, Fig. S8A). Additionally, at the paralysis stage there was an elevation in the expression of lipocalin 2, a factor that is induced in reactive astrocytes and is toxic to neurons (54) (SI Appendix, Fig. S8B). Furthermore, ChAT staining revealed dramatic motor neuron loss in the amiR–TDP43i mice (Fig. 5A and B), which showed ∼60% fewer motor neurons in the ventral horn compared with the nTg mice (Fig. 5C). Because TDP-43 knockdown can lower ChAT expression (46), to rule out the possibility that a reduced ChAT expression rather than an actual motor neuron loss accounted for the reduced motor neuron numbers, we analyzed the spinal cord nerve roots. There was an age-dependent and selective degeneration of axons in the ventral but not in the dorsal roots (Fig. 5D and E). This selectivity was not due to a lack of transgene expression in neurons in the dorsal root ganglia because the RFP expression could be readily detected in the dorsal root ganglia (SI Appendix, Fig. S8C).

A consequence of motor axon degeneration is denervation atrophy of skeletal muscles and compensatory hypertrophy of some muscle fibers. Data from our needle EMG measurements were consistent with the presence of active denervation (Fig. 1F and SI Appendix, Table S3). To confirm this morphologically, we examined the skeletal muscle. We noticed necrotic muscle fibers and fibers with centralized nuclei in some areas of the muscle (SI Appendix, Fig. S8D). These changes were likely caused by the transgene expression and TDP-43 knockdown in the muscle (SI Appendix, Fig. S8E). Nevertheless, both atrophic and hypertrophic muscle fibers that are characteristic of partially denervated muscle were readily observed (SI Appendix, Fig. S8D), thus confirming the presence of muscle denervation.

**Evidence for TDP-43 Dysfunction in Human ALS Spinal Cords.** Our data demonstrate that a partial loss of TDP-43 function can cause neurodegeneration and motor neuron disease phenotypes. This raises the question of whether TDP-43 dysfunction occurs in human ALS. To address this question, we selected 11 alternatively spliced genes that are highly regulated by TDP-43 (46, 48) (Figs. S9 and S10). We verified the presence of the same kind of TDP-43 pathology in these FALS cases and the more common cases—referring to the presence of TDP-43 pathology in these FALS cases and the more common cases—including the majority of FALS cases with non–TDP-43

**Discussion**

TDP-43 aggregation accompanied by its nuclear depletion is involved in >95% of ALS and 40% of FTLD cases (6, 10). In some ALS cases, TDP-43 proteinopathy and nuclear depletion are caused by mutations in the TDP-43 gene (7, 8). Although these are rare (<1%) FALS cases, the presence of the same kind of TDP-43 pathology in these FALS cases and the more common cases—including the majority of FALS cases with non–TDP-43
contains silent mutations in the amiR-TDP43 target region and possibilities is a rescue experiment, in which a TDP-43 gene that other genes (58, 59). The best approach to rule out these pos-

miRNA processing machinery and thereby interfered with the expression of exogenous miRNA, which overwhelmed the endogenous miRNA reads and <0.1% of the total endogenous miRNA reads. Additionally, the mice expressing a scrambled amiRNA, the amiR-SCRi mice, did not develop any overt phenotypes (SI Appendix, Fig. S4). Therefore, it is likely that knockdown of TDP-43 caused motor neuron degeneration and ALS phenotype in the amiR–TDP43i mice.

Previous studies attempted to model loss of TDP-43 function by deletion of TDP-43 gene specifically in motor neurons (42, 43). Wu et al. reported that motor neuron-selective TDP-43 knockouts driven by HB9–Cre developed weakness and premature death (43). However, the evidence for motor neuron degeneration driving the phenotype was equivocal. The data did not clearly differentiate motor neuron atrophy or loss of ChAT expression from motor neuron death. Indeed, Iguchi et al. selectively deleted the TDP-43 gene from motor neurons using a VACHT–Cre driver (42). They observed motor neuron atrophy and dysfunction, but no motor neuron loss and premature death.

mutations and the sporadic cases (55–57)—suggests that these ALS populations share the same pathogenic pathway, albeit the origin of the pathogenesis differs. However, the significance of neither the aggregation nor the nuclear depletion is known. Although the aggregation is postulated to generate cellular toxicity, the aggregation with its accompanying nuclear depletion of TDP-43 could also decrease the functional TDP-43, thus causing a partial loss of TDP-43 function. This raises the question of whether a partial loss of TDP-43 function causes motor neuron degeneration and ALS. We have addressed this question by knocking down TDP-43 using RNAi in the amiR–TDP43i Tg mouse model. Our studies have unequivocally demonstrated that this model had a partial loss of TDP-43 function (Figs. 2 and 3) and developed motor neuron degeneration and ALS symptoms (Figs. 1, 4, and 5).

Did the partial loss of TDP-43 cause motor neuron degeneration and ALS symptoms in the amiR–TDP43i model? To answer this question, we need to consider several possible sources of artifacts. First, the motor neuron degeneration might be caused by disruption of a vital endogenous gene from the transgene insertion. This possibility has been ruled out because the uninduced transgene amiR–TDP43u that was inserted into the same genomic location did not cause the disease. Second, the motor neuron degeneration might be caused by overexpression of exogenous miRNA, which overwhelmed the endogenous miRNA processing machinery and thereby interfered with the endogenous miRNA generation, or evoked off-target silencing of other genes (58, 59). The best approach to rule out these possibilities is a rescue experiment, in which a TDP-43 gene that contains silent mutations in the amiR-TDP43 target region and

is resistant to the RNAi mediated by the amiR–TDP43 is over-expressed to compensate for the lost TDP-43. However, this approach is impractical for several reasons. Overexpression of wild-type TDP-43 can generate toxicity and motor neuron degeneration (15). Even if Tg lines with low levels of overexpression may be obtained and thereby the neurodegeneration avoided, it would be difficult to know whether the expression level is sufficient for the rescue, and it would be impossible to guarantee that TDP-43 is overexpressed in the same cells where the amiR-TDP43 is expressed. Notwithstanding the absence of the rescue experiment, it is unlikely that a nonspecific effect caused the motor neuron degeneration and ALS phenotype because such an effect is observed only in extremely high levels of siRNA or shRNA expression conditions (58, 59). In the amiR–TDP43i mice, the amiR-TDP43 was produced at extremely modest levels in the CNS. By deep sequencing of the total miRNA from the cortices of three amiR–TDP43i mice, we found that the amiR–TDP43 comprised <1% of the highest endogenous miRNA reads and <0.1% of the total endogenous miRNA reads. Additionally, the mice expressing a scrambled amiRNA, the amiR-SCRi mice, did not develop any overt phenotypes (SI Appendix, Fig. S4). Therefore, it is likely that knockdown of TDP-43 caused motor neuron degeneration and ALS phenotype in the amiR–TDP43i mice.

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of the animals. Compared with these motor neuron-specific TDP-43 knockout mice, the amiR–TDP43i mice appeared to have developed more devastating motor neuron loss and clinical phenotypes (Figs. 1–5). This severe level of motor neuron loss in the knockdown mice cannot be explained by the degree of TDP-43 loss in the motor neurons because motor neurons completely lack TDP-43 in the knockout mice but have apparently normal levels in the amiR–TDP43i mice (Fig. 3). Instead, the reduced levels of TDP-43 function in astrocytes may play a key role (Fig. 3). Thus, our data, in conjunction with the data from Iguchi et al., suggest that TDP-43 dysfunction in glial cells plays a critical role in driving motor neuron death.

This conclusion is consistent with the strengthening notion that glia participate in driving motor neuron degeneration in ALS. Expression of mutant Cu/Zn superoxide dismutase (SOD1), the first identified ALS-causing gene mutation, in both motor neurons and glia, which include astrocytes and oligodendrocytes, drive motor neuron degeneration and ALS phenotype in the mutant SOD1 Tg mice (60, 61). Mutant SOD1 expression in astrocytes causes these cells to produce and secrete toxic factors that kill motor neurons (54, 62, 63). Transplanting astrocytes that express mutant SOD1 in wild-type animals causes motor neuron death (64). Recent evidence also supports an important role of astrocytes in TDP-43-mediated motor neuron toxicity. Overexpression of mutant TDP-43 in astrocytes causes motor neuron degeneration in vivo (65). Although mutant TDP-43 expression in motor neurons is sufficient to induce motor neuron death (66), astrocytes participate in this process by responding to signals from motor neurons and producing and secreting secretory factor lipocalin 2 (54), which was also elevated in the amiR–TDP43i mice (SI Appendix, Fig. S8B). The demonstration that astrocytes derived from neural stem cells from sporadic ALS patients, but not those from normal controls, mediated motor neuron toxicity suggests that astrocytes participate in pathogenesis of ALS (67). Notably, TDP-43 aggregates in both motor neurons and glial cells are a general hallmark in sporadic ALS (28, 29, 68–70). Together, our results suggest that TDP-43 dysfunction in glial cells contributes to motor neuron degeneration in ALS.

In the amiR–TDP43i mice, TDP-43 was knocked down in muscles, and there was muscle degeneration in addition to changes typical of denervation atrophy (SI Appendix, Fig. S8D). Therefore, it is possible that the muscle degeneration contributed to motor neuron dysfunction and degeneration. It has been reported that muscle degeneration can cause denervation and some degenerative changes in motor neurons, including axon terminal degeneration and microgliosis in the spinal cord (71–73). However, most studies also demonstrate that muscle degeneration is not a primary driver for motor neuron death to the degree observed in ALS (71, 72, 74, 75). Although one study suggested that mutant SOD1 expressed in muscles could drive motor neuron loss (73), the evidence was equivocal and inconsistent with the conclusions of other studies. Thus, although the muscle degeneration might have contributed to the degeneration and the overall neuromuscular phenotype, it is unlikely the driver for the motor neuron death in the CNS in amiR–TDP43i mice.

Given the broad expression of the amiR–TDP43 (SI Appendix, Figs. S5 and S6), it is striking that the predominant phenotype in the amiR–TDP43i mice is neurological (Fig. 1) and the predominant neuronal loss is concentrated in the large neurons with long projecting axons—i.e., the cortical layer V pyramidal neurons and spinal cord motor neurons (Figs. 4 and 5). Therefore, the key phenotypes and pathology in the amiR–TDP43i mice have replicated the core features of ALS, thus suggesting that the large neurons in the motor system of the CNS possess a heightened vulnerability to TDP-43 dysfunction relative to other cell types and that TDP-43 dysfunction plays a role in ALS.

Therefore, to explore whether TDP-43 dysfunction exists in humans, we evaluated TDP-43 function by measuring alternative splicing changes in the TDP-43–regulated genes. We have detected changes that are consistent with both an enhancement in TDP-43 function in some genes and a decrease in TDP-43 function in others (Fig. 6). These findings suggest that TDP-43 function is altered in ALS. However, most of the TDP-43 target genes remain unchanged, and those changed are simultaneously in the direction of an enhancement and a decrease in TDP-43 function, remains a conundrum. The fact that most of the target genes remain unchanged indicates that the signal for TDP-43 dysfunction is small. A possible scenario that results in a small overall change in TDP-43 function could be that the TDP-43 dysfunction develops gradually, and at each point in time only a minority of the cell populations has ongoing TDP-43 dysfunction. The observation that the human ALS tissue simultaneously display signals for an increase and a decrease of TDP-43 function could be a reflection of a complex state of TDP-43 function in ALS. It has been well established that the TDP-43 level is normally maintained constant by an autoregulatory mechanism (46, 76), and the observed alterations in alternative splicing may reflect a state of TDP-43 deregulation and dysfunction, which could have a major role in motor neuron degeneration and ALS phenotype. Indeed, it has been reported that TDP-43 levels in human ALS is modestly elevated (16, 77). Because TDP-43 functions in multiprotein/RNA complexes, an elevation in the TDP-43 level could disrupt the stoichiometry among the components in the complex, thereby causing dysfunction of the TDP-43 complexes (13). However, it remains to be investigated how functions of TDP-43 complexes change under modestly but persistently elevated TDP-43 levels. In complex tissues such as the CNS, the functional changes measured from total spinal cord RNA reflect averages from different cell populations, which
could also vary at different stages of neurodegeneration. The complexity of this condition could thereby produce varied changes in alternative splicing. For example, an elevated TDP-43 level in one cell population might enhance, whereas in another might inhibit, its normal function. It may also be possible that an elevated TDP-43 level initially enhances its function, but when the elevation exceeds a certain threshold at a late disease stage, an inhibition develops. Therefore, our results call for further investigation on the alterations in the TDP-43 levels and function in model systems for ALS and in human ALS tissues.

Materials and Methods

Tg Mice. The transgene amiR-TDP43u was built by using our published method (78). The preamir-TDP43cs sequence (21) was inserted into the pCAG-GFP/RFP-miRint-KX (78) (Addgene no. 19818). This transgene was a conditional construct that initially expressed GFP, but in the presence of Cre, the GFP sequence would be excised and the CAG promoter would drive expression of RFP and the amiR-TDP43 (SI Appendix, Fig. S1A). The Tg lines were created and screened as described (45). An artifact associated with multiple transgene integration into the mouse genome precluded a selective induction of transgene expression in the CNS (45) (SI Appendix, Fig. S1 B and C). Therefore, a GFP-expressing Tg line was converted to a line that constitutively and ubiquitously expressed RFP and amiR–TDP43 (SI Appendix, Fig. S1D and S2). As a control, a Tg line that constitutively expressed RFP and a scrambled amiRNA that does not target any known mouse genes (ami-SCR) was also established. See details in SI Appendix, SI Materials and Methods.

The preinduction parent Tg line (amiR-TDP3u or CAG-loxp-EGFP/3xpA-loxp-RFP-amiR-TDP43 mice) will be available from The Jackson Laboratory as stock no. 017919. The post-induction line (amiR-TDP43i mice) will be available from The Jackson Laboratory as stock no. 017934.

Behavioral Analysis and EMG. All mice were maintained at the University of Massachusetts Medical School animal facility according to the guidelines set forth by the Institutional Animal Care and Use Committee (IACUC), and all animal procedures were approved by the IACUC. The mice were maintained in pathogen-free environment and on a 12-h light-dark cycle. The Tg mice were monitored daily for their appearance and motor activity. They were weighed once or twice weekly. Their motor behavior was monitored at different disease stages by using an automated video monitoring system. Their muscle activity was monitored by EMG. See details in SI Appendix, SI Materials and Methods.

Measurement of Transgene Expression and TDP-43 Knockdown. Transgene expression was measured in tissue homogenates by immunoblotting for GFP and RFP and by Northern blots for amiR–TDP43. The tissue expression pattern was further confirmed by immunohistochemistry of brain and spinal cord sections. TDP-43 knockdown was measured in tissue homogenates and RFP-positive cells sorted from the cortex and the spinal cord by using immunoblotting and reverse-transcriptase quantitative PCR. See details in SI Appendix, SI Materials and Methods.

Measurement of Alternative Splicing in Human Cells and Spinal Cord. Total RNA was extracted from human cells transfected with plasmid expressing TDP-43 or with siRNA targeting TDP-43 for knockdown. The alternative splicing was assayed by PCR using primers across the alternatively spliced exons. The PCR products were resolved on agarose gels, and the bands were quantified to determine the relative ratios of the exon-excluded band to the exon included band. See details in SI Appendix, SI Materials and Methods.

Statistics. Quantitative data on TDP-43 knockdown and pathology in amiR–TDP43i mice were compared with those from the nTg mice by using Student’s t test. Genotype frequencies were compared by using the χ² test.

ACKNOWLEDGMENTS. We thank Drs. Jemeen Sreedharan and Daryl Bosco for critically reading the manuscript; Drs. Alonzo Ross and Phillip D. Zamore for sharing equipment; Dr. Hong Cao for assisting with confocal imaging; the UM/Johns Hopkins Transgenic Animal Core for pronuclear injection; and the technical support from the Core Electron Microscopy Facility, which receives support from the National Center for Research Resources (5R02RR027897). This work was supported by National Institutes of Health/National Institute of Neurological Disorders and Stroke Grants R21NS062230 and R01NS059708, the ALS Association, ALS Therapeutic Alliance, and the Packard Center for ALS Research at Johns Hopkins (to X.Z.), and in part by National Institute on Aging/National Institutes of Health Intramural Research Program AG000943.


Supporting Information (SI) Appendix
SI Materials and Methods

Transgenic mice

The amiR-TDP43u transgene construct was constructed as described previously (1). Briefly, we inserted the DNA encoding the pre-amiR-TDP43b sequence (2) into the pCAG-GFP/RFP-miRint-KX (1) (Addgene #19818). This construct initially express GFP, but in the presence of Cre, the GFP sequence is excised and the CAG promoter drives expression of RFP and the amiR-TDP43 (Fig. S1A). After the sequence verification and tests in cultured cells, the amiR-TDP43u (u denotes “uninduced”) construct was used to make the transgenic mice by pronuclear injection. The founder mice were screened by PCR using the primers complementary to the RFP DNA sequence: forward AGTGGGAGCGCGTGATGAACTTCGA and reverse CTGCTCCACGATGGTGTAGTCCTCGT. The positive founders where bred with wild type mice and the offspring were sacrificed and characterized for their expression of GFP in the CNS. Their brain and spinal cord were examined under a fluorescence microscope. The lines were terminated if 3 to 5 animals from the line showed no detectable GFP fluorescence. The lines survived this screen were further analyzed for their GFP expression levels by immunoblots (see below) and for their transgene copy numbers as described previously (3).

Attempts were made initially to induce expression of the RFP and the amiR-TDP43 selectively in the CNS by crossing the amiR-TDP43u lines with the Nestin-Cre driver (Jackson Lab, stock# 003771). The offspring were genotyped by PCR using primers for the RFP and Cre genes. The Cre primers were forward GCAAGTTGAATAACCGGAAAT and reverse TGCCAGATTACGTATATCCTG. The brains from the offspring were dissected and weighed after the mice were sacrificed. This confirmed the presence of microcephaly in the amiR-TDP43u/Nestin-Cre double transgenic (dTg) mice. Since the microcephaly was an artifact associated with multiple transgene integration into the genome as shown previously (3) (Fig. S1B, C), we took steps to circumvent this technical problem by converting the GFP-expressing transgenic line to a line that constitutively and ubiquitously expressed RFP and amiR-TDP43 (Fig. S1D, S2A). The amiR-TDP43u mice were crossed with the CMV-Cre driver (Jackson Lab, stock# 003465). The resulting male double transgenic (dTg) mice were further crossed with wild type F1 females that were generated from crosses between FVB/NJ and C57BL/6J strains. The male single transgenic amiR-TDP43i (i denotes “induced”) mice were used to propagate the line by always crossing with females on a FVB/NJ and C57BL/6J F1 hybrid background (Fig. S2A).

To generate the mice that constitutively express RFP and a scrambled artificial miRNA that does not target any known mouse genes (amiR-SCR), we crossed the CAG-G/R-amiR-Scr line 109 that was described previously (3), with the CMV-Cre mice. The double transgenic mice that expressed RFP were segregated from the CMV-Cre mice by crossing with wild type mice. The RFP-expressing amiR-SCR single transgenic mice were maintained in successive generations by crossing with wild type mice as described above.

Behavioral analysis using an automated video system

To monitor the mouse behavior in their home cage continuously, single mice were housed in polycarbonate cages with minimal bedding (~200 ml). A digital video camera was mounted on one side of the wall. Each mouse was recorded for 24h with 12h daylight and 12h dim red light, and then returned to its cage with its littermates. Video data were analyzed by HomeCageScan software (Clever Systems, Reston, VA, USA) to quantify travel and verticals. Travel measures the overall motor/muscle functions by recording distance travelled in meters. Verticals measures rearing up, jumping and coming down, which require strong hind limbs to support the body and were used to assess the hind limb function.
**Electromyography**

Mice were anaesthetized by inhalation of isoflurane. Animals were placed immediately on a heating pad to maintain their core temperature at 37°C. Measurements were performed using a Cardinal Synergy electromyography (EMG) machine. A ground self-adhesive gelled surface electrode was placed over the tail. Potentials were recorded from several sites of the muscles of all four limbs with a concentric needle electrode (30G) using a gain of 50 µV/division and a band pass filter with low and high cut-off frequency settings of 20 and 10,000 Hz, respectively. The entire recording process took 15-20 minutes per mouse, after which the mice were euthanized by isoflurane overdose or used for tissue collection as described below.

**Immunoblotting**

Mice under deep anesthesia were decapitated and spinal cord, brain, and other tissues were quickly harvested, snap-frozen in liquid N\_2, and stored at -80°C. For protein preparation, frozen tissues were homogenized in a homogenization solution containing 25 mM phosphate pH 7.2, 1 mM EGTA, 1% SDS, 0.05% Triton X-100 and protease inhibitor mixture (Thermo Scientific) and heated at 95°C for 5 min. After clearing by centrifugation, protein concentration was measured using BCA assay (Pierce, Rockford, IL). The samples were heated in Laemmli buffer, and equal amounts of protein were loaded and resolved by SDS-PAGE. After transfer to nitrocellulose membranes, blots were blocked with 5% nonfat dry milk in PBST (0.25% Triton X-100 in PBS, pH 7.4) for 1 h, and then incubated with primary antibodies overnight at 4°C and then again with horseradish peroxidase–linked secondary antibodies to rabbit or mouse (GE Healthcare) in PBST with 5% dry milk for 1 hour at RT. Membranes were washed three times and proteins were visualized after ECL (Pierce) treatment and by the LAS-3000 imaging system (Fujifilm).

**RT-PCR and qRT-PCR**

For total RNA extraction, frozen tissues or sorted cells were homogenized in cold TRIzol reagent (Invitrogen) following the manufacturer’s protocol. RNA was then reverse transcribed to cDNA using SuperScript III (Invitrogen). To test candidate splicing targets, RT-PCR amplification using between 33 and 37 cycles were performed from at least three nTg mice and three Tg mice. Products were separated on 2% agarose gels and visualized by staining with ethidium bromide and photographed. For qRT-PCR measurements of TDP-43, real time PCR was performed on the cDNA using the primer pair forward TGGTGTGACTGTAAACTTCCC and reverse GACATCTACCATTCTCCATACTG. The PCR cycles were carried out in a Bio-Rad Real-Time PCR system (C1000 Thermal Cycler, Biorad) and PCR product was detected using Sybr Green. The levels of TDP-43 were standardized to the housekeeping gene GAPDH in individual animals and then further normalized to the mean △CT of the wild type mice. For qRT-PCR measurement of lipocalin 2 (lcn2), real time PCR was performed as described above using the primer pair forward GCAGGTGGTACGTTGTGGG and reverse CTCTTGTAGCTCAGATGGTG.

**Northern blots**

Northern blots were used to detect the amiR-TDP43 expression. Twenty µg of total RNA was mixed with equal volume of loading buffer (95% formamide, 0.1% TBE, bromophenol blue and xylene cyanol), denatured at 65°C for 5 min, loaded onto the 15% acrylamide urea gel and run for 2h at 150 volts at room temperature. A $^{32}$P-labeled RNA ladder (Decade-Markers; Applied Biosystems) was used as a molecular weight marker. The RNA was transferred to a Nylon membrane (Amersham) with a semi-dry apparatus. The blotted membrane was cross-linked in a UV crosslinker (Stratalinker) and hybridized with $^{32}$P-labeled probes for amiR-TDP43 (GCAATGCTGAACCTAAGCATAA) and U6 snRNA.
(AAAATATGGAACGCTTCACGAATTTGCG) overnight. The membrane was rinsed with 2X SSC twice then washed three times for 10 minutes each at 37°C. The membrane was wrapped in Saran wrap and exposed overnight to a phosphorimager screen. The blot image was obtained by scanning the screen in a Fujifilm FLA-5100 scanner.

**Cell sorting by Flow Cytometry**

To collect the RFP-positive cells from cortex or spinal cord of amoR-TDP43i mice, the cortex or spinal cord was first treated with Neural Tissue Dissociation Kit (P) (Miltenyi Biotec Inc.) to separate the tissue to single cells, which were sorted by fluorescence-activated cell sorting (FACS). The sorted cells were stored at -80°C until being used for RNA preparation.

**Immunofluorescence and immunohistochemistry**

Mice under deep anesthesia were transcardially perfused with cold PBS followed by 4% paraformaldehyde in PBS. The perfused mice were then immersed in the same fixative at 4°C for another 24-48h. After fixation, tissues were immersed in PBS containing 30% sucrose at 4°C for 2–3 days. Tissues were then frozen in OCT freezing media (Sakura, Torrance, CA) and stored at -20°C. Frozen sections were cut at 20 µm using a cryostat. For immunostaining, sections were incubated in the blocking solution (5% normal serum in PBS, pH 7.4) for 1 hour at room temperature (RT) and then incubated with a primary antibody in the blocking solution overnight at 4°C. The dilutions and source of primary antibodies were as follows: NeuN (Millipore MAB377, 1:200), calbindin (Millipore AB1778, 1:500), GFAP (Abcam Ab7260, 1:1000), IBA1 (BioCare Medical CP290AB, 1:200), APC (EMD Bioscience OP80-100UG, 1:200), GFP (Invitrogen G10362, 1:333), RFP (Abcam AB34771, 1:500), ChAT (Millipore AB1044P, 1:200), rabbit polyclonal antibody raised to amino acids 394-414 of human TDP-43 (custom made) and TDP-43 (Encor biotechnology MCA-3H8, 1:250). Sections were then washed 3 times for 5 minutes each and incubated in the appropriate secondary antibody at room temperature for 90 minutes. For immunofluorescence, the sections were washed 3 times in PBS for 5 minute each and mounted with Vectashield mounting medium containing 4,6-diamidino-2-phenylindole (DAPI, Vector Laboratories) and sealed with nail polish. Images of the brain and spinal cord sections were taken with a Nikon Eclipse Ti Widefield fluorescence microscope equipped with a Retiga-2000RV cooled-CCD camera or a confocal microscope (Leica).

For quantification of TDP-43 signal intensity in various cell types, sections were double stained for TDP-43 and one of the following markers: RFP, ChAT and GFAP. After staining, the cells were visualized and photographed using a confocal microscope. The cells in the cortex or ventral horn of spinal cord were measured for their fluorescence intensity using the Nikon NIS Elements software. For each cells, the average fluorescence intensity was calculated. Cells on at least 5 different sections from each of the three or more mice per genotype were measured.

For immunohistochemistry, sections were washed 3 times in PBS containing 0.25% Tween 20 and then stained following the manufacturer’s instructions for Vectastain ABC kit and Elite PK-6100 standard ImmPact tm DAB peroxidase Substrate kit SK-4105 (Vector Lab). The sections were then mounted on slides and dried overnight at 55°C. After soaking in Xylenen 2 times for 2 minutes each, the slides were sealed with Permount (Vector Lab).

**Visualization and quantification of neurodegeneration**

For visualization and quantification of the cortical neurons, whole mouse brains were placed in 30% sucrose solution for 2 days, frozen, and sectioned sagittally at 50 µm thickness. Layer V pyramidal neurons were counted using every ninth section, with a total of nine sections per half brain. Sections were mounted onto gelatin coated slides and Nissl-stained with cresyl violet. Stereological counting was performed using Stereo Investigator software (MBF Bioscience, Williston VT). Counting was performed within the motor area of cortical layer V.
Only pyramidal neurons with a soma greater than 15 µm in diameter were included. A single experimenter who was blinded to the genotype performed all counts.

For visualization of ventral root axons, mice were fixed by transcardial perfusion using 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1M sodium phosphate (pH 7.6). Tissues were further fixed by soaking in the same fixative at 4°C for 24 hours. L4 and L5 roots attached to dorsal root ganglia were dissected and postfixed with 2% osmium tetroxide in 0.1 M phosphate (pH 7.6), dehydrated in a graded ethanol series, and embedded in Epon-Araldite resin. One-micron sections were stained with toluidine blue and examined and photographed by light microscopy.

For quantification of ventral horn motor neurons, lumbar enlargement of the fixed spinal cords were sectioned on a cryostat at 20µm thickness. Every other section was collected until a total of ten sections were collected from each spinal cord. The sections were stained with goat ChAT antibody at 4°C overnight. A secondary donkey anti-goat biotinylated antibody and a Vectastain ABC and DAB peroxidase Substrate kit (Vector Lab) were used to reveal motor neurons. Images of the spinal cord sections were taken using a Nikon microscope and motor neuron numbers in the ventral horn region were counted manually from each section.

For muscle histology, isopentane in a container was pre-chilled with liquid nitrogen until the isopentane started to solidify at the bottom of the container. Fresh specimen isolated from gastrocnemius muscle was placed on a cork disc with a drop of OCT, which kept the muscle in the desired orientation. The specimen was frozen by immersion into the isopentane for ~5 seconds and then stored at -20°C. The frozen tissue was sectioned using a cryostat and stained with Hematoxylin & Eosin (H&E).

**Measurement of alternative splicing in human cells and spinal cord**

We chose 11 human genes for assaying changes in alternate splicing. Ten of these (POLDIP3, RWDD1, RABGEF1, BCL2L11, DDR1, RGL1, OGT, MIAT, CACNA1C and REEP6) were reported to have significant changes in their alternative splicing after TDP-43 knockdown in human SH-SY5Y cells (4). One (EIF4H) was reported to change after TDP-43 knockdown in mouse brain (5). This gene was selected because its alternatively spliced exon-intron structure is conserved between human and mouse and the change after TDP-43 knockdown was confirmed in HEK293 cells (Fig. S9C).

For verifying changes in alternative splicing after TDP-43 knockdown in human cells, HEK293 cells were transfected with siTDP43 (5’GATGAGAACGATGAGCCCA), which targets TDP-43 for knockdown, and a scrambled siRNA (siSCR: 5’AACAGTCGGTTTCGACTGG 3’) using Lipofectamine 2000 (Invitrogen). Seventy two hours later, cells were harvested for RNA preparation. Total RNA was extracted from HEK293 cells cold TRIzol reagent (Invitrogen) following the manufacturer’s protocol. PCR detection for the alternatively spliced exons is described below.

For testing changes in alternative splicing after TDP-43 overexpression in human cells, HEK293 TRex cells (Invitrogen) were transfected with pcDNA5/FRT/TO-TDP-43 construct, which express a FLAG-tagged human TDP-43 under the control of a tetracycline-inducible promoter. The construct was made as follows: Human wild type TDP-43 ORF was amplified by PCR and a FLAG tag was put at its N-terminal using primers (5’GGGAAGCTTTGACCAAGAGGACGATGACAAGATGCGCTCAAACGATTATACCCCAAC3’ and 5’CCGGATCCTTGAATACGAGCGCTTTCGATC3’). The PCR product was digested and inserted into the HindIII and BamHI sites of pcDNA5/FRT/TO (Invitrogen). To generate the stable cell line, the cells were grown in 6-well plates with Dulbecco’s modified eagle medium containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Two µg of
pcDNA5/FRT/TO-TDP-43 plasmid and 0.2 μg of pOG44 plasmid expressing Flp-recombinase were transfected using TransIT-293 transfection reagent (Mirus) following manufacturer’s instruction. Cells were selected under Blasticidin (15 mg/ml) and Hygromycin (100 mg/ml) to get stable cell lines with chromosomal integrated FLAG-TDP-43. Doxycycline (Dox, 100ng/ml) (Sigma) was added into stable cells to induce expression of FLAG-TDP-43. RNA extraction and alternative splicing measurement were carried out as described below.

For measurement of alternative splicing in human cells and spinal cords, total RNA was extracted from HEK293 cells or the anterior horn of frozen human spinal cord by cold TRIzol reagent (Invitrogen) following the manufacturer’s protocol. RNA was then reverse transcribed to cDNA using SuperScript III (Invitrogen). Candidate splicing targets were amplified by RT-PCR between 33 to 37 cycles. Products were separated on a 2% agarose gel and visualized by staining with ethidium bromide. Quantification of the different isoforms was performed with ImageJ software (US National Institutes of Health). The intensity ratios between the exon-included and excluded products were calculated. The value from each spinal cord was an average from duplicate measurements.


### Table S1. amiR-TDP43u transgenic lines

<table>
<thead>
<tr>
<th>Transgene</th>
<th>GFP expression</th>
<th>Transgene copy number</th>
<th>microcephaly status</th>
</tr>
</thead>
<tbody>
<tr>
<td>amiR-TDP43u6</td>
<td>2+ to 4+</td>
<td>15.03 ± 1.11</td>
<td>Yes converted to amiR-TDP43i</td>
</tr>
<tr>
<td>amiR-TDP43u49</td>
<td>3+ to 4+</td>
<td>7.05 ± 0.78</td>
<td>Yes maintaining</td>
</tr>
<tr>
<td>amiR-TDP43u22</td>
<td>1+ to 2+</td>
<td>3.37 ± 0.56</td>
<td>Yes terminated</td>
</tr>
</tbody>
</table>

Fourteen transgene-positive founder lines were generated. By examining the levels of GFP expression in the CNS using fluorescence microscopy, we screened and found three Tg lines that expressed modest levels of GFP in the brain and the spinal cord. Based on a semi-quantitative assessment of the GFP fluorescence, the line amiR-TDP43u-49 expressed the highest level, which was followed by lines 6 and 22. Transgene copy numbers were measured using real-time quantitative PCR as previously described (3). All three lines carried multiple copies of the transgene as shown. To induce the RFP and amiR-TDP43 expression in the CNS, all three lines were crossed with nestin-Cre and the brain size was assessed by comparing the brain weight with those of the non-transgenic or single transgenic littermates. All three lines developed microcephaly in the double transgenic mice (see Table S2). As demonstrated previously (3), this was a technical artifact generated from chromosome integration of multiple copies of loxP-containing transgenes (Fig. S1B, C). Because of the unknown consequence of this artifact on the phenotypes, it would be difficult to differentiate phenotypes from this artifact from those derived from transgene expression. Therefore, we did not pursue this approach further but decided to convert these transgenic lines to those that would constitutively express the RFP and amiR-TDP43.

### Table S2

Brain weight in amiR-TDP43u/Nestin-Cre dTg mice

<table>
<thead>
<tr>
<th>AGE</th>
<th>Brain weight (g)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>24.1+/-2.0</td>
<td>7</td>
</tr>
<tr>
<td>Nestin-Cre</td>
<td>24.6+/-1.9</td>
<td>9</td>
</tr>
<tr>
<td>amiR-TDP43u</td>
<td>27.0+/-4.6</td>
<td>5</td>
</tr>
<tr>
<td>dTg</td>
<td>25.3+/-4.5</td>
<td>4</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>25</strong></td>
<td></td>
</tr>
</tbody>
</table>

The brain weights in the double transgenic mice were substantially lower compared with the single transgenic mice as a result of Cre-mediated recombination of inverted loxP-containing transgenes (see Fig. S1). * t test comparing dTg with WT, p = 5.8x10⁻³; with Nes-Cre, p = 4.7x10⁻⁵; with STG, p = 7.5x10⁻⁴.
Table S3. EMG Measurements and symptomatic progression

<table>
<thead>
<tr>
<th></th>
<th>Age (days)</th>
<th>Stage</th>
<th>PSW</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>miTD-P43i</td>
<td>20-22</td>
<td>presymptomatic</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>24-31</td>
<td>presymptomatic, head bobbing</td>
<td>1+ to 2+</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>45-71</td>
<td>symptomatic, end stage</td>
<td>3+ to 4+</td>
<td>3</td>
</tr>
<tr>
<td>WT</td>
<td>65-80</td>
<td>normal</td>
<td>0</td>
<td>3</td>
</tr>
</tbody>
</table>

0 = no fibrillations and positive sharp waves (PSWs)
+1 = occasional fibrillations and PSWs in 2 or more areas probed
+2 = frequent fibrillations and PSWs but not in all areas probed
+3 = frequent fibrillations and PSWs all areas probed
+4 = extensive fibrillations and PSWs masking the baseline

Table S4. Clinical causes of death

<table>
<thead>
<tr>
<th></th>
<th>Death with hindlimb paralysis (%)</th>
<th>Sudden death without paralysis (%)</th>
<th>Death with whole body paralysis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>16(17.4)</td>
<td>23(25.0)</td>
<td>53(57.6)</td>
</tr>
<tr>
<td>Males</td>
<td>92</td>
<td>23</td>
<td>53</td>
</tr>
<tr>
<td>Females</td>
<td>32</td>
<td>5</td>
<td>27</td>
</tr>
<tr>
<td>Total</td>
<td>124</td>
<td>28</td>
<td>80</td>
</tr>
</tbody>
</table>

Table S5. Lifespan of amiR-TDP43i mice (days)

<table>
<thead>
<tr>
<th></th>
<th>Death with hindlimb paralysis</th>
<th>Sudden death without paralysis</th>
<th>Death with whole body paralysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>All</td>
<td>70.3 ± 26.4</td>
<td>38.9 ± 11.6</td>
<td>41.3 ± 16.7</td>
</tr>
<tr>
<td>Males</td>
<td>80.7 ± 19.5</td>
<td>33.6 ± 9.6</td>
<td>80.7 ± 19.5</td>
</tr>
<tr>
<td>Females</td>
<td>N/A</td>
<td>41.3 ± 16.7</td>
<td>80.7 ± 19.5</td>
</tr>
<tr>
<td>p (t test)</td>
<td>0.114</td>
<td>0.102</td>
<td>0.621</td>
</tr>
</tbody>
</table>

Table S6. TDP-43 levels in CNS regions

<table>
<thead>
<tr>
<th>Region</th>
<th>nTg</th>
<th>amiR-TDP43i</th>
<th>p (t test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forebrain</td>
<td>1.00 ± 0.14</td>
<td>0.78 ± 0.02</td>
<td>0.005</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>1.00 ± 0.03</td>
<td>0.90 ± 0.03</td>
<td>0.015</td>
</tr>
<tr>
<td>Brainstem</td>
<td>1.00 ± 0.11</td>
<td>0.83 ± 0.03</td>
<td>0.260</td>
</tr>
<tr>
<td>Cervical spinal cord</td>
<td>1.00 ± 0.03</td>
<td>0.84 ± 0.04</td>
<td>0.033</td>
</tr>
<tr>
<td>Lumbar spinal cord</td>
<td>1.00 ± 0.14</td>
<td>0.85 ± 0.01</td>
<td>0.001</td>
</tr>
</tbody>
</table>

TDP-43 and tubulin band densities were measured from Western blot shown in Fig. 2A. The density ratio between TDP-43 and tubulin was calculated for each animal. The average ratios and the standard deviations in each region were calculated from 3 nTg and 3 amiR-TDP43i mice. All values were then normalized to the average of the nTg mice.
Fig. S1. Schematic representations of the transgene construct and possible recombination scenarios. (A) The amiR-TDP43 was inserted into the backbone of pCAG-EGFP/RFP-miRNAint-KX (1) to generate the amiR-TDP43 transgene. The construct is composed of the following elements in linear succession: the CAG promoter, a loxP site, the EGFP gene with 3 consecutive SV40 poly A signals, a second loxP site, the RFP gene, a 3' UTR carrying an intron containing the pre-miRNA hairpin and a poly A signal. This construct (amiR-TDP43u) would initially express GFP, but would switch to expressing a red fluorescent protein (RFP) and the amiRNA upon induction by Cre (6). The amiR-TDP43 was designed to match TDP-43 mRNA completely, thereby mediating the knockdown by RNAi, i.e. by destroying the TDP-43 mRNA. The amiR-TDP43 was coexpressed with RFP by the same CAG promoter, thereby enabling tracking of the transgene expression spatially and temporally. We chose this strategy because of its apparent advantages in controlling transgene expression and its reported success in the literature (7-11). (B) However, pronuclear injection leads to multiple transgenes being integrated into the genome in a tandem array. Because each transgene copy can be integrated in either orientation, inverted loxP sites between the neighboring genes as illustrated are common (3). (C) During cell proliferation and chromosome replication, Cre-driven loxP recombination may occur between the two sister-chromosomes before their segregation. Some scenarios are benign (Scenario 1), but others will be harmful (Scenario 2), which is expected to prevent normal chromosome segregation and cause cell death (12, 13). The harmful effect can cause microcephaly if the Cre-driven recombination occurs in developing brain (3) (Tables S1, S2). The left and right strands of the DNA between the two inverted loxP sites are colored differently to allow an easy tracking of the DNA strands following recombination. (D) When the amiR-TDP43u mice are crossed with a ubiquitous driver CMV-Cre, recombination occurs in early embryos (14). Most double transgenic (dTg) animals are predicted to die due to the harmful recombination events shown in C, scenario 2. However, some recombination events could stochastically occur across the inverted transgene copy, leading to its removal. With the inverted transgene removed, the recombination could go to completion and the RFP and amiR-TDP43 genes would be induced. Mice with this kind of recombination should be able to survive and breed if the induced transgene expression does not cause embryonic lethality. Crossing the surviving, induced transgenic mice (amiR-TDP43i) with the wild type mice can segregate the amiR-TDP43i transgene from the CMV-Cre transgene. The actual genotype percentage in these crossings verifies this prediction (see Fig. S2).
Fig. S2. Generation of transgenic mice that constitutively express RFP and amiR-TDP43 (amiR-TDP43i mice). (A) Steps in generation of the constitutively induced amiR-TDP43i transgenic mice and genotype composition in each of these steps. In step 1, amiR-TDP43u line 6 mice were crossed with CMV-Cre mice. Notice the significantly lowered genotype frequency of the dTg mice ($X^2$ test, $p = 0.0005$). In step 2, one surviving dTg male was crossed with wild type females. A significant reduction in dTg births was still present ($X^2$ test, $p = 0.0024$). In step 3, the single transgenic RFP-expressing males were crossed with wild type females. The genotype frequencies of transgenic and non-transgenic matched with the Mendelian ratio ($X^2$ test, $p = 0.71$). Similar crossings were carried out for other two amiR-TDP43u lines but were not successful. Crossing line 22 with CMV-Cre did not yield any dTg pups in ~40 births and the line was terminated. Crossing line 49 with CMV-Cre also yielded dTg mice at low frequency. The dTg mice also developed severe neurological phenotypes and all died before breeding except one male, which produced a few single transgenic (sTg) amiR-TDP43i offspring, all of which developed neurological phenotypes similar to the line 6. However, the neurological phenotypes in line 49 developed earlier than line 6 and all the mice died before breeding. (B) Conversion from the amiR-TDP43u mice to the amiR-TDP43i mice changed the brain from expressing GFP to expressing RFP. The whole brain was visualized directly under the fluorescence microscope. Notice that the amiR-TDP43u mice expressed GFP but not RFP throughout the brain. Conversely, the amiR-TDP43i mice expressed RFP but not GFP throughout the brain. (C) Conversion from the amiR-TDP43u mice to the amiR-TDP43i mice changed the spinal cord from expressing GFP to expressing RFP. The lumbar spinal cord was cross-sectioned and stained for GFP and RFP. Notice that similar to (B), the amiR-TDP43u mice expressed GFP but not RFP in the spinal cord. Conversely, the amiR-TDP43i mice expressed RFP but not GFP throughout the spinal cord. (D) Western blot analysis showed the GFP and RFP expression in the forebrain from three non-transgenic, three amiR-TDP43u and three amiR-TDP43i mice. All samples were run on the same gel, transferred and probed with antibodies on the same membrane. The bands were cropped and arranged in the same order as the panels above for illustration purpose. Notice that GFP was only expressed in amiR-TDP43u but not in nTg and amiR-TDP43i mice, and that RFP was only expressed in amiR-TDP43i but not in nTg and amiR-TDP43u mice.
**Fig. S3.** The transgene was expressed and TDP43 expression was knocked down in the heart of amiR-TDP43i mice. (A) Western blots showing RFP expression and TDP-43 knockdown in the heart. (B) The heart was enlarged in the amiR-TDP43i mice.
Fig. S4. Mice expressing a scrambled amiRNA (amiR-SCR) (3) did not develop neurodegenerative phenotypes. (A) The transgene was expressed at higher levels in the CNS of the induced amiR-Scr mice than in the amiR-TDP43i mice. (B, C) The induced amiR-SCR mice did not develop behaviors that were significantly different from the wild type littermate controls up to 10 months old.
Fig. S6. The amiR-TDP43i mice express the transgene ubiquitously and at relatively higher levels in the periphery than in the CNS. (A) Western blot analyses of RFP expression in different tissues from three amiR-TDP43i mice. Each lane was loaded with 15 µg of total protein. Notice the relatively faint signal in the CNS tissues. At higher loading amount (30 µg of total protein per lane), the RFP signal was clearly detected. SC = spinal cord. (B) Northern blot for amiR-TDP43 confirmed the relatively higher levels of the amiRNA expression in the peripheral tissues than in the CNS of the amiR-TDP43i mice. U6 snRNA was detected as a positive control for the loading of total RNA. (C) A quantitative plot of the relative RFP levels (band intensities) from the Western blots for RFP and the Northern blots for amiR-TDP43. All the band intensities were normalized to the level in the brainstem. The bottom plot plots the same Northern blot data that is plotted in the top plot so that the relative levels of amiR-TDP43 can be seen more clearly. Notice that, in general, the peripheral tissues accumulated higher levels of RFP and amiRNA than the CNS. However, the levels of RFP did not entirely reflect the levels of amiR-TDP43. The RFP accumulated to much higher levels than the amiR-TDP43 in the peripheral tissues, particularly in the heart, spleen, muscle and liver, compared with the CNS tissues. (D) Western blots for TDP-43 in different tissues. Notice that the transgene expression in the amiR-TDP43i mice did not alter the relative levels of TDP-43 among the different tissues compared with the nTg mice.
**Fig. S7.** The amiR-TDP43i mice expressed transgenes in multiple different cell types, including from the top to bottom rows: spinal motor neurons, cortical neurons, Purkinje cells, spinal cord astrocytes, some oligodendrocytes but not microglia. Sections were double immunostained for RFP and various cellular marks as indicated. Scale bars represent 20 µm.
Fig. S8. Neuropathology associated with motor neuron degeneration. (A) Astrogliosis and microgliosis in the spinal cord of amiR-TDP43i mice. Notice that GFAP and Iba1 staining intensity was greatly increased in the ventral horn of the amiR-TDP43i mice compared with the nTg mice. (B) Astrocyte-generated lipocalin 2 that is toxic to neurons was elevated in the spinal cord of the paralyzed amiR-TDP43i mice. The mRNA levels were measured using real time PCR from 5 non-transgenic (nTg) and 7 amiR-TDP43i mice at both the pre-hyperactive (pre-HA) and the hyperactive (HA) stages, and from 16 nTg and 16 amiR-TDP43i mice at the paralyzed stage. Student t-tests showed that the differences between nTg and amiR-TDP43i mice were not significant at the pre-HA (p=0.58) and HA (p=0.94) stages but were significant (p=0.0001) at the paralyzed stage. (C) The transgene was expressed in dorsal root ganglion (DRG) cells in the amiR-TDP43i mice. The DRGs were freshly dissected and squashed on a glass slide and then photographed using fluorescence microscopy. (D) Muscle pathology and TDP-43 knockdown in amiR-TDP43i mice. Compared with muscle of the nTg mice (a), amiR-TDP43i mice showed typical denervation changes, including atrophic fibers (arrows) and hypertrophic fibers (arrowheads) (c). Some areas showed fiber degeneration (b), which was likely a result of TDP-43 knockdown in muscles. (E) The transgene was expressed in skeletal muscle where TDP-43 was knocked down. Proteins from muscle homogenates of three amiR-TDP43i and three nTg mice were resolved by SDS-PAGE, blotted to nitrocellulose membrane and probed with TDP-43 and RFP antibodies. Tubulin was detected as a loading control.
Fig. S9. Changes in TDP-43-regulated genes in human cells after TDP-43 knockdown or overexpression. (A) Induction of TDP-43 with doxycycline in HEK293 cells that were stably transfected with a tet-inducible plasmid expressing FLAG-tagged human TDP-43. Notice that the tagged TDP-43 was fully induced at 24h after addition of doxycycline. Mock means no plasmid was included in the transfection. (B) HEK293 cells were transfected with Mock (no plasmid), siSCR (siRNA with scrambled sequence) and siTDP43 (siRNA targeting TDP-43). The cells were harvested and RNA extracted at ~48 hours after transfection. Notice that TDP-43 was knocked down in the siTDP43-transfected cells. (C) Changes in alternative splicing in the eleven genes that are regulated by TDP-43. Based on the changes, the genes are divided into four groups. Group 1 had one gene REEP6 that did not show changes. Group 2 also had one gene EIF4H that did not show changes after TDP-43 overexpression but showed change after TDP-43 knockdown. Group 3 had seven genes that showed changes in opposite directions in overexpression and knockdown. Group 4 had two genes that showed changes in the same direction in both overexpression and knockdown. (D) Six TDP-43-regulated alternatively spliced genes did not show significant changes in human ALS spinal cords.
SI Video Legends

**Video S1.** Motor neuron disease progression in the amiR-TDP43i mice—hindlimb paralysis. The transgenic mouse was marked with red ink in its tail. Other mice in this video were control non-transgenic littermates. Notice the mouse went through stages from normal, wobbly-gait, hyperactive to finally paralysis.

**Video S2.** Motor neuron disease progression in the amiR-TDP43i mice—whole body paralysis. The transgenic mouse (an agouti mouse at the right lower corner) was indistinguishable from others at 5 weeks, hyperactive at 7 weeks, less active (on the left) at 9 weeks and paralyzed at 11 weeks. The other mice were nTg controls.
Supporting Information

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**Movie S1.** Motor neuron disease progression in the artificial microRNA (amiR)–TDP43i mice—hindlimb paralysis. The transgenic mouse is marked with red ink in its tail. Other mice in this video are control nontransgenic littermates. Notice the mouse goes through stages from normal, wobbly-gait, hyperactive to, finally, paralysis.

**Movie S2.** Motor neuron disease progression in the amiR–TDP43i mice—whole body paralysis. The transgenic mouse (an agouti mouse at the right lower corner) is indistinguishable from others at 5 wk, hyperactive at 7 wk, less active (on the left) at 9 wk, and paralyzed at 11 wk. The other mice are nontransgenic controls.

**Other Supporting Information Files**

*SI Appendix (PDF)*