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Accessibility
Toxic gain of function from mutant FUS protein is crucial to trigger cell autonomous motor neuron loss

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Abstract

FUS is an RNA-binding protein involved in amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD). Cytoplasmic FUS-containing aggregates are often associated with concomitant loss of nuclear FUS. Whether loss of nuclear FUS function, gain of a cytoplasmic function, or a combination of both lead to neurodegeneration remains elusive. To address this question, we generated knockin mice expressing mislocalized cytoplasmic FUS and complete FUS knockout mice. Both mouse models display similar perinatal lethality with respiratory insufficiency, reduced body weight and length, and largely similar alterations in gene expression and mRNA splicing patterns, indicating that mislocalized FUS results in loss of its normal function. However, FUS knockin mice, but not FUS knockout mice, display reduced motor neuron numbers at birth, associated with enhanced motor neuron apoptosis, which can be rescued by cell-specific CRE-mediated expression of wild-type FUS within motor neurons. Together, our findings indicate that cytoplasmic FUS mislocalization not only leads to nuclear loss of function, but also triggers motor neuron death through a toxic gain of function within motor neurons.

Keywords amyotrophic lateral sclerosis; frontotemporal dementia; FUS; motor neuron degeneration; PY-NLS

Subject Categories Molecular Biology of Disease; Neuroscience

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Introduction

Mutations in several aggregation-prone RNA-binding proteins (RBPs) are increasingly linked to various neurodegenerative diseases. Such mutations constitute a major cause of amyotrophic lateral sclerosis (ALS), the most frequent adult-onset motor neuron disease, with mutations in TDP-43 (Gitcho et al, 2008; Kabashi et al, 2008; Sreedharan et al, 2008) and FUS (Kwiatkowski et al, 2009; Vance et al, 2009) accounting each for about 5% of familial ALS cases. Even in the absence of mutations, abnormal cytoplasmic inclusions of TDP-43 represent a pathological hallmark of sporadic ALS, non-SOD1 familial ALS, and frontotemporal dementia (FTD) (Neumann et al, 2006,
control cellular levels of RBPs. For instance, both TDP-43 (Ayala et al., 2011; Polymenidou et al., 2011; D’Alton et al., 2015) and FUS (Lagier-Tourenne et al., 2012; Zhou et al., 2013; Dini Modigliani et al., 2014) control their own levels by binding to their mRNAs. As a consequence, the levels of endogenous TDP-43 or FUS proteins are strikingly reduced in animal models overexpressing wild-type or mutant TDP-43 and FUS (Wegorzewska et al., 2009; Huang et al., 2011; Arnold et al., 2013; Mitchell et al., 2013; Sephton et al., 2014).

Here, we use homologous recombination techniques to circumvent issues inherent to overexpression animal models. We systematically compare the pathological and molecular features of two novel mouse models either expressing a truncated FUS protein that lacks the NLS and localizes within the cytoplasm (knockin mice) or harboring a genomic mutation associated with complete absence of FUS protein (knockout mice). Both mice expressing mutant cytoplasmic FUS and mice completely devoid of FUS died at birth of respiratory insufficiency. Using genomic approaches, we determined that FUS knockin mice display expression and splicing alterations consistent with loss of FUS nuclear function. However, mice expressing truncated cytoplasmic FUS, but not FUS knockout mice, exhibit perinatal motor neuron loss, which can be rescued by motor neuron-restricted reversal of the mutant FUS gene to wild-type FUS. These findings demonstrate that cytoplasmic FUS leads to loss of nuclear FUS function, yet exerts a toxic gain of function within the cytoplasm of motor neurons necessary to trigger neuronal death.

**Results**

**Cytoplasmic mislocalization of mutant FUS in Fus<sup>NLS/INLS</sup> mice**

With the aim to investigate in vivo consequences of altered FUS localization, we generated a mouse model with targeted deletion of the PY-NLS, encoded by the last exon of the Fus gene (exon 15). This mutation closely mimics ALS-causing truncating mutations of FUS (Fig EV1). We opted for a strategy that would not only result in ablation of exon 15, but also allow for CRE-mediated reversal to the wild-type locus. Due to the small size of intron 14, we engineered the Fus locus to include, in intron 12, a floxed cDNA encoding exons 13 and 14 of Fus, followed by 3 transcription stop cassettes and a polyadenylation signal (Fig 1A). Germ line transmission of the recombinant allele was obtained (Fig 1B) and mice heterozygous and homozygous for the targeted allele will hereafter be referred to as Fus<sup>NLS/+</sup> and Fus<sup>NLS/ANLS</sup>, respectively. Homozygous Fus<sup>NLS/ANLS</sup> mice died shortly after birth, and tissues dissected at birth (P0) were analyzed for expression and localization of Fus<sup>NLS</sup> mRNA and protein. The ANLS mRNA could be detected by RT–PCR in tissues of Fus<sup>NLS/+</sup> and Fus<sup>NLS/ANLS</sup> mice (Figs 1C and EV2A). FUS protein was detected by immunoblotting in Fus<sup>NLS/+</sup> and Fus<sup>NLS/ANLS</sup> brain, spinal cord, and muscle protein extracts using antibodies targeting the internal or N-terminal parts of FUS (Figs 1D and EV2B). Contrastingly, no signal was found in Fus<sup>NLS/ANLS</sup> protein extracts when using two different antibodies that recognize the C-terminal NLS of FUS (Figs 1D and EV2B), demonstrating that the engineered Fus gene leads to the generation of a FUS protein devoid of NLS. FUS protein levels were heterogeneous in Fus<sup>NLS/+</sup> brains (Fig 1D). As expected, FUS protein localized to the nucleus in cultured mouse embryonic fibroblasts...
Figure 1. FUS mislocalization in Fus<sup>ΔNLS/ΔNLS</sup> mice.

A Schematic representation of the Fus gene locus (upper panel). Lower panels depict exons 11–15 in the wild-type allele (left) and ΔNLS allele (right) with localization of PCR primers used for genotyping (gDNA, used in B) and for RT–PCR (Total and ΔNLS, used in C). Arrow: translational start site. STOP cassettes are indicated in red, loxP sites as black triangles; coding regions are in dark blue and UTRs in light blue. Location of the region encoding the nuclear localization signal (NLS) is indicated in exon 15.

B Representative PCR genotyping results from 2 Fus<sup>+/+</sup>, 2 Fus<sup>ΔNLS/+</sup>, and 2 Fus<sup>ΔNLS/ΔNLS</sup> knockin mice using primers designed around the distal loxP site of the Fus<sup>ΔNLS</sup> allele and shown as gDNA in (A). The expected size of the PCR product of the ΔNLS allele is 240 bp; the size of wild-type allele is 160 bp.

C RT–PCR analysis of brain from 2 Fus<sup>+/+</sup>, 2 Fus<sup>ΔNLS/+</sup>, and 2 Fus<sup>ΔNLS/ΔNLS</sup> knockin P0 mice using primers located in the STOP cassette, and thus specific to the ΔNLS mRNA (ΔNLS, upper panel), or primers located in exon 11, that is, upstream of the floxed cDNA insertion, and thus amplifying total Fus-derived mRNA (Total, middle panel). PCR amplification of 18S rRNA is shown as a standard gene (lower panel).

D Immunoblot analysis of FUS protein in cerebral cortex of 2 Fus<sup>+/+</sup>, 2 Fus<sup>ΔNLS/+</sup>, and 2 Fus<sup>ΔNLS/ΔNLS</sup> knockin mice using a combination of two different antibodies targeting either the C-terminal (C-ter. 1 and C-ter. 2) NLS, the N-terminal part (N-ter. 1), or an internal part (N-ter. 2) of FUS. Molecular weight markers are shown on the left, and apparent MW is indicated.

E Double immunostaining for the motor neuronal marker ChAT and Fus (N-terminal part) in the ventral horn of spinal cord.

F Double immunostaining for nuclei (DAPI, blue) and Fus (N-terminal part) in the cerebral cortex.

Source data are available online for this figure.
(MEFs) of Fus+/+ mice. In striking contrast, FUS redistributed from the nuclear compartment to the cytoplasm in FusANLS/ANLS MEFS (Fig EV2C) and was detected in both the nucleus and the cytoplasm of FusANLS/+ MEFS (Fig EV2C). Consistently, in FusANLS/ANLS newborn mice, immunostaining revealed that mutant FUS is localized to the cytoplasm of spinal motor neurons and cortical neurons, contrasting with the normal nuclear localization in Fus+/+ neurons (Fig 1E and F). In FusANLS/+ mice, FUS is detected in both the nucleus and the cytoplasm of motor neurons (Fig 1E). Thus, the FusANLS allele effectively leads to the expression of a truncated FUS protein that lacks the NLS and localizes predominantly to the cytoplasm.

Both cytoplasmic mislocalization of FUS and complete loss of FUS result in perinatal lethality

FusANLS/ANLS mice were born alive, but died within minutes after birth, while FusANLS/+ mice survived the perinatal period. The body length and weight of FusANLS/ANLS pups were slightly but significantly reduced as compared to Fus+/+ and FusANLS/+ newborn mice (Fig 2A–C). The cause of death appeared to be respiratory insufficiency, as FusANLS/NLS animals showed poor respiratory movements and cyanosis, and H&E staining of sections through the lung revealed uninfated lungs with complete alveolar atelectasis (Fig 2D).

Cytoplasmic FUS mislocalization could have detrimental effects either through toxicity resulting from increased cytoplasmic FUS levels or through loss of its normal nuclear function. To distinguish between these possibilities, we investigated whether complete loss of FUS recapitulates the perinatal lethality phenotype of FusANLS/ANLS mice. Mice with a gene trap insertion in exons 8 or 12 of the Fus gene have been described previously (Hicks et al., 2000; Kuroda et al., 2000), yet both of these Fus gene trap lines express low amounts of truncated FUS protein, thus precluding their use to discriminate between loss- versus gain-of-function mechanisms. To tackle this issue, we generated a novel Fus knockout model (hereafter referred to as Fus–/–) that was systematically compared to FusANLS/NLS mice in the same C57Bl6 background. In this new Fus knockout allele, a trap cassette was inserted in intron 1 to completely disrupt transcription of the endogenous Fus gene (Fig 3A). Southern blot and direct sequencing confirmed the position and orientation of the gene trap insertion, and excluded additional insertion events elsewhere in the genome (not shown). FUS protein was undetectable in the central nervous system of Fus–/– newborn mice by Western blot using the above-described antibodies against the N-terminal and C-terminal parts of the protein (Fig 3B and C) and Fus mRNA could not be detected by quantitative real-time PCR (Fig 3D). Consistently, immunostaining for FUS on spinal cord sections of E18.5 Fus–/– mice did not detect FUS protein (Fig 3E), confirming that Fus–/– mice are FUS protein null. Depending on the genetic background, previously described Fus gene trap mice were reported either to be adult viable, to die before the age of weaning or within 16 h after birth (Hicks et al., 2000; Kuroda et al., 2000; Kino et al., 2015). In contrast, Fus–/– mice died within 30 min after birth due to respiratory insufficiency. Similar to FusANLS/NLS pups, the body weight and body length of Fus–/– newborn mice were significantly reduced as compared to littermate controls (Fig 3F and G). Thus, both complete loss of FUS and its cytoplasmic mislocalization trigger a similar perinatal phenotype in C57Bl6 mice.

Extensive overlap of RNA expression changes induced by cytoplasmic FUS mislocalization and complete loss of FUS

The phenotypic similarity of FusANLS/ANLS and Fus–/– newborn mice is consistent with FUS cytoplasmic mislocalization leading to loss of FUS nuclear function. FUS has been involved in the regulation of gene expression and alternative splicing of its mRNA targets (Polymenidou et al., 2011; Tollervey et al., 2011; Ishigaki et al., 2012; Lagier-Tourenne et al., 2012; Rogelj et al., 2012). In addition, FUS interacts with several proteins, including U1-snRNP (Yamazaki et al., 2011; Tollervey et al., 2011; Ishigaki et al., 2012), Drosha (Morlando et al., 2012), SMN (Yamazaki et al., 2012; Groen et al., 2013; Tsuji et al., 2013; Sun et al., 2015), HDAC1 (Wang et al., 2013), Drosha (Morlano et al., 2012), RNA polymerase II (Schwartz et al., 2012), and PRMT1 (Tibshirani et al., 2014), known to have profound effects on splicing and gene expression. Hence, the expression of a truncated cytoplasmic form of FUS may primarily lead to loss of nuclear FUS function and defective regulation of direct FUS RNA targets. In addition, cytoplasmic accumulation of FUS may also alter the function and/or
Figure 3. Generation of a complete Fus<sup>−/−</sup> loss-of-function mouse model.

A. Schematic representation of the Fus gene locus (upper panel). Lower panels depict exons 1–3 in the wild-type allele (left) and loss-of-function allele (right). Arrow: translational start site; SA: splice acceptor; βgeo: β-galactosidase/neomycin phosphotransferase fusion gene; pA: polyA.

B. Representative immunoblot for FUS on protein extracts of E18.5 brain. Histone 3 is used as a loading control.

C. Quantification of FUS protein levels from immunoblots.

D. Quantitative real-time PCR for Fus transcript in Fus<sup>+/−</sup> and Fus<sup>−/−</sup> mice. ND: not detected.

E. Immunostaining for the neuronal marker NeuN and FUS on the spinal cord ventral horn of E18.5 Fus<sup>+/−</sup> and Fus<sup>−/−</sup> mice. ND: not detected.

F, G. Body weight (F) and length (G) of Fus<sup>+/−</sup>, Fus<sup>+/−</sup>/C0, and Fus<sup>−/−</sup>/C0 pups at birth; N = 14 Fus<sup>+/−</sup>, N = 36 Fus<sup>+/−</sup>/C0, and N = 13 Fus<sup>−/−</sup>/C0 for body weight; N = 6 per genotype for body length.

Data information: Data represent mean ± SEM. **P < 0.01 versus Fus<sup>+/−</sup>, ##P < 0.01 versus Fus<sup>−/−</sup>/C0; one-way ANOVA followed by Tukey’s post hoc test.

Source data are available online for this figure.
subcellular localization of FUS-interacting proteins and result in additional gene expression and splicing alterations. To discriminate between gain- and loss-of-function changes, we systematically compared RNA profiles in brains from Fus<sup>ANLS/ANLS</sup> and Fus<sup>+/−</sup> mice. First, we used strand-specific, genomewide sequencing of RNAs (Parkhomchuk et al, 2009) (RNA-seq) to evaluate RNA expression levels in brains of Fus<sup>ANLS/ANLS</sup> and Fus<sup>+/−</sup> mice. Total RNA was extracted from E18.5 embryonic brains of Fus<sup>ANLS/ANLS</sup> and Fus<sup>+/−</sup> mice (N = 5), and wild-type littermates (N = 4), as well as homozygous Fus<sup>−/−</sup> (N = 5) and their control Fus<sup>+/+</sup> littermates (N = 5). Expression levels for each annotated protein-coding gene were determined by the number of mapped fragments per kilobase of exon, per million mapped reads (FPKM) (Mortazavi et al, 2008; Trapnell et al, 2012). The FPKM ratio of the Fus gene confirmed that Fus was expressed in Fus<sup>ANLS/ANLS</sup> mice, but not in Fus<sup>+/−</sup> mice (Fig EV3A). Inspection of the reads mapped on the Fus gene demonstrated the absence of reads throughout all exons for the Fus<sup>−/−</sup> mice, while only exon 15 was not integrated in Fus transcripts of Fus<sup>ANLS/ANLS</sup> mice (Fig 4A). Fus transcript levels as determined by FPKM ratio were not significantly different in Fus<sup>ANLS/ANLS</sup> and Fus<sup>+/−</sup> mice (Fig EV3A). Unsupervised hierarchical clustering with all genes reliably discriminated mutant genotypes from their controls (Fig EV3B and C), indicating that both Fus mutation and Fus deletion displayed RNA expression profiles divergent from wild type. Statistical comparison of FPKM values identified 237 genes upregulated and 549 genes downregulated in Fus<sup>ANLS/ANLS</sup> mice (Dataset EV1 and Fig 4B). Of note, only nine genes and 56 genes were upregulated and downregulated more than 1.5-fold, respectively (Dataset EV1). Identical analysis in Fus<sup>−/−</sup> mice identified 669 upregulated genes (29 genes more than 1.5-fold) and 889 downregulated genes (72 genes more than 1.5-fold) (Dataset EV2 and Fig 4C). Comparison of both models identified 353 genes that were altered in the same direction, consistent with loss of FUS nuclear function underlying the altered levels of these transcripts (Fig 4D and Dataset EV3).

Downregulation of selected genes in both mouse models was confirmed by qRT–PCR (Appendix Fig S1A) yielding data similar to the RNA-seq results (Fig 4E). Out of these, several genes have been previously involved in neurological diseases such as the Abelson helper integration site 1 (Ahi1) gene mutated in the neurodevelopmental Joubert syndrome (Ferland et al, 2004), the Dystrophia myotonica protein kinase (Dmpk) gene implicated in myotonic dystrophy type 1, the low and medium molecular weight neurofilament subunits (Nef1 and Nefm) (Bergeron et al, 1994), and the gene encoding tubulin alpha 4A (Tuba4a) that was recently implicated in ALS (Smith et al, 2014). Similarly, selected upregulated genes were confirmed by qRT–PCR (Fig 4E and Appendix Fig S1A), including Taf15, a FUS family member also mutant in ALS (Courthouis et al, 2011). Increased levels of Taf15 in Fus<sup>ANLS/ANLS</sup> and Fus<sup>+/−</sup> mice are consistent with the presence of FUS binding sites on the Taf15 transcript (Lagier-Tourenne et al, 2012) and may illustrate a mechanism of compensation induced by loss of FUS nuclear function. Overall, an extensive overlap in RNA expression changes was found in Fus<sup>ANLS/ANLS</sup> and Fus<sup>−/−</sup> brains consistent with loss of FUS nuclear function in knockin mice.

**Cytoplasmic mislocalization of FUS leads to unique RNA expression changes**

A subset of transcripts were altered uniquely in Fus<sup>ANLS/ANLS</sup> animals (Fig 4F and Appendix Fig S1B), including the Vitronectin (Vtn) gene, the small nuclear ribonucleoprotein poly peptides B and B1 (Snrbp) gene, the Trove2 gene encoding for the 60 kDa SS-A/ Ro ribonucleoprotein, and the U2AF homology motif kinase 1 (Uhmk1) gene encoding for the kinase interacting with stathmin (KIS) protein that was implicated in schizophrenia and the regulation of splicing (Manceau et al, 2008) and local translation in neuritic projections (Cambray et al, 2009; Pedraza et al, 2014). Interestingly, Fus<sup>ANLS/ANLS</sup> animals displayed increased mRNA levels of Ephb3, a member of Eph/ephrin signaling pathways crucial in synaptogenesis and previously involved in Alzheimer’s disease (Sheffler-Collins & Dalva, 2012), while EphA4, another member of Eph/ephrin system, has been recently involved in ALS (Van Hoecke et al, 2012) (Fig 4F and Appendix Fig S1B). Thus, Fus<sup>ANLS/ANLS</sup> expression profiles similarly recapitulated expression profiles of Fus<sup>−/−</sup> brains, yet a subset of genes was found specifically associated with the expression of cytoplasmic truncated FUS in Fus<sup>ANLS/ANLS</sup> brains.

Gene ontology analysis showed that transcripts whose expression was upregulated in Fus<sup>ANLS/ANLS</sup> animals were enriched for genes involved in mRNA translation and extracellular matrix constituents (Dataset EV4). In contrast, transcripts upregulated in Fus<sup>−/−</sup> brains revealed enrichment for nuclear and nuclear proteins involved in the regulation of transcription, DNA replication, or regulation of RNA metabolic processes (Dataset EV5). Gene ontology
Figure 4.

A. RNA-seq results showing the expression levels of Fus Exons 1-23.

B. Expression alterations in Fus^NL/S/NLS

C. Expression alterations in Fus^−/−

D. Venn diagram comparing Fus^NL/S/NLS and Fus^−/−

E. mRNA levels by RNAseq (fraction of +/+ mice)

F. mRNA levels by RNAseq (fraction of +/+ mice) for various genes
analysis revealed a similar enrichment for synaptic activity and function among transcripts downregulated in FusANLS/ANLS and Fus−/− animals (Datasets EV4 and EV5), supporting that cytoplasmic mislocalization of FUS alters the expression of genes involved in synaptogenesis, as a direct consequence of FUS loss of function. Contrastingly, gain of function elicited by cytoplasmic FUS might be more related to alterations in genes related to mRNA translation and extracellular matrix.

Widespread splicing alterations induced by cytoplasmic mislocalization and loss of FUS

To further compare the molecular changes elicited by cytoplasmic FUS mislocalization and FUS loss of function, we asked whether regulation of mRNA splicing was similarly affected in E18.5 brains of FusANLS/ANLS mice and Fus−/− mice. To characterize mRNA splicing, we exploited the RNA-mediated oligonucleotide annealing, selection, and ligation with next-generation sequencing (RASL-seq) method (Li et al., 2012; Zhou et al., 2012). This approach allowed us to quantitatively profile 3,859 unique alternative splicing events that correspond to exon inclusion or skipping events conserved between mouse and human (Fig EV4). Ratios of shorter to longer mRNA isoform counts were calculated and used to statistically compare the splicing changes between different groups. Unsupervised hierarchical clustering for all splicing events showed that mutant and deleted Fus mice clustered apart from their controls (Fig EV4B and C). In FusANLS/ANLS brains, 9.3% (173 events) of the 1,852 detected splicing events were different from controls, with 101 increased long splicing isoforms and 72 enhanced short isoforms (defined by t-test with P < 0.05 and average fold change > 1.5) (Fig 5A and Dataset EV6). Heterozygous FusANLS/+ mice clustered with wild-type mice demonstrating that their splicing profile is not significantly altered (Appendix Fig S2). Nevertheless, heat map of the 173 events significantly changed in homozygous FusANLS/ANLS mice showed an intermediate pattern in FusANLS/+ heterozygous littersmates supporting a partial loss of FUS function in these mice (Appendix Fig S2). In Fus−/− brains, 17.9% (252 events) of the 1,406 detected splicing events were different from control littersmates, with 118 increased long isoforms and 134 enhanced short isoforms (Fig 5B and Dataset EV7).

Comparison of the splicing changes between FusANLS/ANLS and Fus−/− mice revealed a striking overlap between both models. Seventy-five splicing events were commonly regulated in FusANLS/ANLS and Fus−/− mice, 100% of which were differentially included or excluded in the same direction (Fig EV4D and E and Dataset EV8).

Semi-quantitative RT–PCR of selected RNAs confirmed these FUS-dependent splicing changes (Figs 5C and EV4F), including transcripts previously implicated in neurodegenerative diseases such as the N-myel downstream-regulated gene 2 (Ndrg2) that is misaccumulated in Alzheimer’s disease (Mitchemore et al., 2004), the microtubule-associated protein tau (Mapt) gene mutated in frontotemporal dementia (Hutton et al., 1998), the ataxin 2 (Atxn2) gene mutated in ALS (Elden et al., 2010), spinocerebellar ataxia type 2 (SCA2) (Imbert et al., 1996), and the pro-neurotrophin receptor sortilin 1 (Sort1) (Hu et al., 2010). Interestingly, inclusion of sortilin 1 exon 17b [also referred to as exon 18 in Polymenidou et al (2011)] was previously associated with low levels of TDP-43 (Polymenidou et al., 2011; Prudencio et al., 2012) and found significantly increased in cortex of FTD patients with TDP-43 proteinopathy (Prudencio et al., 2012). Here, we observe that Fus mutation and deletion have an opposite effect on sortilin 1 as compared to TDP-43, with decreased inclusion of exon 17b in embryonic brains from both FusANLS/ANLS and Fus−/− mice (Figs 5C and EV4F).

Despite expected developmental differences in the alternative splicing patterns of embryonic and adult brains, 57 splicing events found misregulated by RASL-seq in FusANLS/ANLS and Fus−/− mice were also identified by Affymetrix microarrays in striatum from adult wild-type mice with antisense oligonucleotide-mediated depletion of Fus (Lagier-Tourenne et al., 2012), or in embryonic brains from another Fus knockout model that expresses low levels of truncated Fus protein (Hicks et al., 2000; Lagier-Tourenne et al., 2012). Among FUS-dependent alterations, abnormal splicing of Mapt exon 10 (Figs 5C and EV4F) is of particular relevance for disease pathogenesis as mutations enhancing exon 10 inclusion are linked to frontotemporal dementia (Liu & Gong, 2008). While in embryonic mouse brains the predominant mRNA isoforms of Mapt do not include exon 10 and encode for tau protein with three microtubule binding repeats (3R-tau) (McMillan et al., 2008; Dillman et al., 2013), we observed increased inclusion of exon 10 encoding for the 4-repeat tau isoform (4R tau) in both FusANLS/ANLS and Fus−/− mice. In all, splicing alterations caused by cytoplasmic FUS mislocalization are largely overlapping with those elicited by complete loss of FUS. Nevertheless, a subset of events were uniquely found in FusANLS/ANLS (Fig EV4D), which may be the consequence of functional disruption of other RNA-binding proteins by abnormal accumulation of FUS in the cytoplasm.

Cytoplasmic mislocalization of FUS leads to increased perinatal motor neuron apoptosis

We next asked whether perinatal death of FusANLS/ANLS and Fus−/− mice was accompanied by loss of motor neurons in the lumbar spinal cord. Immunostaining for choline acetyltransferase (ChAT), which specifically labels large motor neurons in the spinal cord ventral horn, revealed that the number of motor neurons was reduced by approximately 30% in FusANLS/ANLS mice as compared with both FusANLS/+ and FusANLS−/ mice (Fig 6A and B). To exclude
Figure 5.
the possibility that this reduced number of ChAT-positive motor neurons reflected downregulation of ChAT expression rather than loss of motor neurons, we also performed Nissl staining, a histochemical stain that does not rely on the expression level of a specific marker. Quantification of neurons with an area of ≥ 80 µm² revealed a 50% reduction in the number of large motor neurons in the ventral horn of Fus^ANLS/ANLS^ newborn mice (Fig 6C). Interestingly, Fus^ANLS/+^ mice displayed a smaller but statistically significant
loss of large Nissl-stained cells, suggesting that motor neurons were also affected in these mice despite showing normal numbers of ChAT-positive cells. In striking contrast with the situation in Fus<sup>ANLS/ANLS</sup> mice, spinal motor neuron counts were similar between Fus<sup>−/−</sup> mice and their wild-type littermates (Fig 6A, D and E), demonstrating that the mutant FUS protein expressed in Fus<sup>ANLS/ANLS</sup> mice is toxic to motor neurons during development. Besides motor neuron degeneration, neither Fus<sup>ANLS/ANLS</sup> nor Fus<sup>−/−</sup> mice displayed gross developmental abnormalities of the brain. Indeed, cortical thickness appeared normal in both mouse strains (Appendix Fig S3). In addition, the thickness of layers II-IV and layers V-VI defined by CUX1 and CTIP2 immunofluorescence, respectively, was normal in both mouse strains (Appendix Fig S4).

At birth, mouse motor neurons are still in the developmental period, and motor neurons that did not efficiently create synaptic contacts with muscles undergo apoptosis until P10 (Kanning et al., 2010). Supporting an increased perinatal motor neuron apoptosis in Fus<sup>ANLS/ANLS</sup> mice, the number of apoptotic cells detected by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) was threefold higher in lumbar spinal cord sections of Fus<sup>ANLS/ANLS</sup> than in wild-type littermates (Fig 7A and B). Furthermore, double immunostaining for ChAT and active caspase-3, labeling motor neurons actively undergoing apoptosis, revealed that motor neuron apoptosis was three times more frequent in Fus<sup>ANLS/ANLS</sup> than in wild-type littermates (Fig 7C–E). Together, these data indicate that cytoplasmic mislocalization of FUS leads to increased motor neuron death resulting in reduced numbers of lower motor neurons. The absence of motor neuron loss in Fus<sup>−/−</sup> mice indicates that cytoplasmic FUS accumulation leads to motor neuron death through a toxic gain-of-function mechanism.

Cytoplasmic mislocalization of FUS alters SMN and HDAC1 localization and induces elf2α phosphorylation

We then asked whether cytoplasmic mislocalization of FUS in Fus<sup>ANLS/ANLS</sup> mice would affect the subcellular localization of some of its interaction partners. Immunostaining for SMN1 revealed that SMN1-positive nuclear gems were lost in motor neurons of Fus<sup>ANLS/ANLS</sup> mice (Fig 8A), as in cells from ALS patients and mouse models (Yamazaki et al., 2012; Tsuchi et al., 2013; Sun et al., 2015; Yu et al., 2015) and consistent with enhanced interaction between mutant FUS and SMN1 (Yamazaki et al., 2012; Gerbino et al., 2013; Groen et al., 2013; Sun et al., 2015; Yu et al., 2015). Furthermore, HDAC1, another FUS interaction partner (Wang et al., 2013), showed profoundly abnormal immunoreactivity in Fus<sup>ANLS/ANLS</sup> mice. Indeed, HDAC1 was diffusely nuclear in cells from wild-type spinal cords, while in Fus<sup>ANLS/ANLS</sup> animals HDAC1 immunoreactivity was condensed in 1–3 nuclear foci per cell (Fig 8B). Double immunofluorescence in spinal cords from control animals found a weak HDAC1 staining in ChAT-positive motor neurons contrasting with a strong nuclear signal in neighboring cells. The pattern was strikingly different in Fus<sup>ANLS/ANLS</sup> animals with HDAC1 aggregation into nuclear foci in ChAT-positive motor neurons (Fig 8C). In contrast to SMN1 and HDAC1, the binding partner of FUS TAF15 remained mostly nuclear in motor neurons from Fus<sup>ANLS/ANLS</sup> mice (Appendix Fig S5). Thus, cytoplasmic mislocalization of FUS perturbs the localization, and potentially the function, of specific interaction partners in motor neurons.

In autopsy material of ALS-FUS patients, FUS-containing cytoplasmic aggregates are found in neurons and glial cells. These aggregates are usually also immunopositive for ubiquitin (Vance et al., 2009; Baumer et al., 2010; Huang et al., 2010; Kobayashi et al., 2010; Tateishi et al., 2010). Despite motor neuron loss in Fus<sup>ANLS/ANLS</sup> mice, we did not observe FUS-positive (Fig 1E and F) aggregates in motor neurons or cortical neurons of newborn Fus<sup>ANLS/ANLS</sup> mice. We also did not observe ubiquitin-positive, poly-ubiquitin-positive, or neurofilament-positive aggregates in the ventral spinal cord of Fus<sup>ANLS/ANLS</sup> mice (Fig EV5A and B). As FUS is recruited to stress granules, and since neuropathology revealed that cytoplasmic protein aggregates in ALS-FUS patients stain positive for stress granule markers (Baumer et al., 2010; Dormann et al., 2010; Liu-Yesucievitz et al., 2010), we evaluated the distribution of TIAR, a stress granule marker in the spinal cord ventral horn in Fus<sup>ANLS/ANLS</sup> mice. TIAR displayed a similar diffuse cytosolic pattern in motor neurons of Fus<sup>ANLS/ANLS</sup> and control mice (Fig EV5). As an additional marker for cellular stress, we evaluated eIF2α phosphorylation, which occurs in response to cellular stress and results in decreased global protein translation (Holcik & Sonenberg, 2005; Sonenberg & Hinnebusch, 2009). Immunostaining for the phosphorylated form of eIF2α revealed a diffuse cytoplasmic staining that was substantially more intense in spinal motor neurons of Fus<sup>ANLS/ANLS</sup> mice as compared to controls (Fig 8D) reflecting cellular stress in Fus<sup>ANLS/ANLS</sup> motor neurons, with consequent repression of protein translation.

Taken together, cytoplasmic mislocalization of FUS in motor neurons led to altered subcellular localization of several of its binding partners accompanied by stress-induced translational repression, but without the induction of protein aggregation or stress granule formation.

Cytoplasmic FUS mislocalization is intrinsically toxic to motor neurons

We subsequently asked whether FUS mislocalization within motor neurons is necessary to induce motor neuron loss in Fus<sup>ANLS/ANLS</sup> mice and therefore evaluated whether restricted expression of wild-type FUS in motor neurons could rescue their survival despite accumulation of the mutant protein in neighboring cells. We exploited the presence of isoP sites flanking the STOP cassette (Fig 1A) to selectively revert the ΔNLS allele to wild type in motor neurons. For this purpose, Fus<sup>ANLS/+</sup> mice were crossed to mice expressing the CRE recombinase from the ChAT locus, which leads to CRE recombinase activity in virtually all cholinergic neurons (Rossi et al., 2011; Saxena et al., 2013). We expected that Fus<sup>ANLS/ANLS</sup>/ChAT-CRE mice would express truncated FUS protein ubiquitously, except for cholinergic neurons. Double immunostaining for FUS and ChAT on spinal cord sections of Fus<sup>ANLS/ANLS</sup>/ChAT-CRE newborn mice revealed that FUS nuclear localization was indeed largely restored in cholinergic neurons in the ventral spinal cord, but not in other neighboring cells, consistent with motor neuron selective CRE expression (Fig 9A). The rescue of FUS nuclear localization was either complete or only partial, with in the latter case motor neurons showing mixed cytoplasmic/nuclear FUS localization (Fig 9A), consistent with CRE having successfully recombined at least one Fus<sup>ANLS</sup> allele to wild type. Neonatal lethality of Fus<sup>ANLS/ANLS</sup> mice was not rescued by the
Figure 7. Motor neuron apoptosis in Fus<sup>DLNLS/DLNLS</sup> mice.

A  Representative images of TUNEL assay in spinal cord of Fus<sup>DLNLS/DLNLS</sup> mice (A<sup>4</sup>-A<sup>6</sup>) and Fus<sup>DLNLS</sup> mice (A<sup>1</sup>-A<sup>3</sup>).

B  Quantification of the total number of TUNEL and DRAQ5 (blue) double-positive cells in Fus<sup>DLNLS/DLNLS</sup> and Fus<sup>DLNLS</sup> per spinal cord section. Mean ± SEM, N = 3 per genotype. *P < 0.05, by Student’s unpaired t-test.

C, D  Immunofluorescence microscopy of spinal cord of Fus<sup>DLNLS</sup> (C) and Fus<sup>DLNLS/DLNLS</sup> (D) mice showing active caspase-3 (green), ChAT (red), and DNA (cyan, DRAQ5).

E  Quantification of caspase-3 (Cas3)/ChAT/DRAQ5 triple-positive cells in Fus<sup>DLNLS/DLNLS</sup> mice. Mean ± SEM, N = 7 per genotype. **P < 0.01, by Student’s unpaired t-test.
Figure 8. Alterations of SMN, HDAC1, and eIF2α in Fus\textsuperscript{ANLS/ANLS} mice.

A Representative images of SMN (green) immunofluorescence in spinal cord. Nuclear gems, corresponding to SMN-immunoreactive foci in nuclei, are marked by arrows.

B HDAC1 immunoreactivity in spinal cord sections of Fus\textsuperscript{+/+} and Fus\textsuperscript{ANLS/ANLS} mice. Arrows point to HDAC1-immunoreactive nuclear foci.

C Representative images of immunofluorescence staining of motor neurons, labeled with ChAT (red) and HDAC1 (green). Examples of HDAC1 immunoreactive foci in motor neurons are indicated by arrows.

D Representative images of immunofluorescence staining of motor neurons labeled with ChAT (red) and phosphorylated eIF2α (green), a general translational stress response marker. DRAQ5 (cyan) was used to label nuclei.
Figure 9.
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A

Fus^{+/-}

ChAT-CRE

DRAQ5

ChAT

FUS N-ter. 2

Merge

Spinal Cord ( Ventral Horn)

Fus^{+/-}

ChAT-CRE

A1

A2

A3

A4

A5

A6

A7

A8

Fus^{+/-}

ChAT-CRE

A9

A10

10μm

A11

A12

B

Fus^{+/-}

ChAT-CRE

Fus^{ANLS/ANLS}

Fus^{ANLS/ANLS}

ChAT-CRE

Nissl

B1

B2

B3

ChAT

B4

B5

B6

C

Nissl+

Number/ventral horn

ChAT-CRE +

ChAT-CRE -

Fus^{+/-}

Fus^{+/-}

Fus^{+/-}

E

ChAT+/Cas3+

Number/section

ChAT-CRE +

ChAT-CRE -

Fus^{+/-}

Fus^{+/-}

Fus^{+/-}
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ChAT-CRE allele, suggesting that expression of mutant FUS in motor neurons is not the main contributor of neonatal lethality in homozygous mice. Last, we evaluated whether restoration of FUS nuclear localization in motor neurons would prevent motor neuron loss in Fus\(^{\text{NL5/NL5}}\) mice. This approach revealed that the presence of a ChAT-CRE allele was indeed sufficient to fully rescue motor neuron loss (Fig 9B–D), as well as restore a normal number of caspase-3-positive motor neurons (Fig 9E). These findings establish that cytoplasmic FUS mislocalization within motor neurons is required to induce their loss in Fus\(^{\text{NL5/NL5}}\) mice.

Discussion

FUS and other related RBPs form cytoplasmic inclusions associated with nuclear clearance in affected cells from ALS and FTD patients. An outstanding question is whether disease is caused by gain of cytoplasmic toxicity or by loss of the nuclear function of the respective RBP. To gain insight into this conundrum, we targeted the FUS locus to generate two novel mouse models. In the first model, the last exon of Fus is no longer transcribed, resulting in the production of a truncated FUS protein that lacks the NLS and localizes almost exclusively to the cytoplasm (Fig 1). The second model results in complete loss of FUS expression (Fig 3). A thorough comparison of these two models allowed us to determine whether a gain of a toxic cytoplasmic function is required for the observed phenotypes.

Interestingly, this approach revealed that Fus\(^{\text{NL5/NL5}}\) and Fus\(^{-/-}\) mice share several features: both mouse models die shortly after birth due to respiratory insufficiency and exhibit reduced body weight and size. Transcriptomic analysis also revealed overlapping alterations in RNA levels and splicing. However, a reduced number of spinal motor neurons associated with increased perinatal motor neuron apoptosis and a subset of expression and splicing changes were uniquely found in Fus\(^{\text{NL5/NL5}}\) mice (Figs 4, 5, 6 and 7). Thus, FUS mislocalization to the cytoplasm with reduced levels of nuclear FUS is associated with phenotypes linked to loss of FUS function, but also with motor neuron death induced by a gain of toxicity mechanism that is not recapitulated in the knockout mouse model (Fig 6). The notion that loss of FUS function may not be sufficient to induce motor neuron degeneration is also supported by two recent reports. First, in an outbred genetic background, homozygous Fus gene trap mice reached 2 years of age without manifesting ALS-like phenotypes or motor neuron loss (Kino et al., 2015). Second, selective inactivation of the FUS homolog cabeza in neurons of adult Drosophila did not affect motor performance or life span, indicating that cabeza is not required for maintenance of neuronal function in adults (Frickenhauser et al., 2015). Here, we demonstrate that loss of FUS function is not sufficient and that gain of function by cytoplasmic redistribution of FUS is necessary to elicit motor neuron apoptosis. Notably, RNA-seq and RASL-seq experiments showed that the ANLS mutation triggered a partial loss of FUS function in splicing and gene expression regulation. Loss of function, although not sufficient, may be necessary in Fus\(^{\text{NL5/NL5}}\) mice to induce motor neuron death. Indeed, a number of genes involved in synaptogenesis and/or in neurodegenerative diseases show altered expression (Aahi1, Dmpk, Nefl, Nefm, Tub4a1, Taf15) or splicing (Ndr2, Mapt, Atxn2, Sort1) in Fus\(^{\text{NL5/NL5}}\) and Fus\(^{-/-}\) mice (Figs 4 and 5), and these alterations linked to loss of FUS nuclear function may weaken the motor neuron and sensitize it to the toxic effects of cytoplasmic FUS accumulation.

Importantly, we showed that motor neuron death in Fus\(^{\text{NL5/NL5}}\) mice could be prevented by selectively restoring FUS nuclear import in motor neurons (Fig 9), demonstrating that FUS cytoplasmic mislocalization within motor neurons is required to induce motor neuron loss. The observation that cytoplasmic mislocalization of FUS is intrinsically toxic to motor neurons contrasts with the non-cell-autonomous contribution to motor neuron degeneration demonstrated for SOD1 mutations (Boilee et al., 2006). Indeed, abrogation of mutant SOD1 expression in motor neurons delayed, but did not prevent, motor neuron degeneration, and a contribution of neighboring cells to SOD1 mediated toxicity is well established (Iliev et al., 2009). Moreover, our data support a degenerative rather than developmental origin of motor neuron loss in newborn Fus\(^{\text{NL5/NL5}}\) mice. The reduced number of motor neurons could have a developmental origin, either by impaired proliferation of motor neuron progenitors or defects in acquisition of motor neuron fate. However, this scenario is unlikely since motor neuron loss in Fus\(^{\text{NL5/NL5}}\) mice can be prevented by ChAT-CRE-induced reversal of the Fus\(^{\text{NL5}}\) locus to wild type (Fig 9). Expression of the ChAT gene, which encodes choline acetyltransferase, an enzyme essential for the biosynthesis of acetylcholine, is detectable in postmitotic motor neurons from E11.5 onwards, thus after exit from the cell cycle and acquisition of motor neuron fate at E9.5 (Chen & Chiu, 1992; Alaynick et al., 2011; Cho et al., 2014). Therefore, the pathogenic events driving motor neuron loss in Fus\(^{\text{NL5/NL5}}\) mice occur after motor neuron specification. It should be noted that a large proportion of motor neurons showed an incomplete rescue of FUS...
nuclear localization. This could be either due to the rescue of one single allele or to the persistence of truncated FUS produced before CRE-mediated recombination. In any case, only a partial rescue of FUS subcellular localization was sufficient to abrogate motor neuron loss.

An attractive hypothesis to explain motor neuron loss in Fus\textsuperscript{ANLS/ANLS} mice is that excessive motor neuron apoptosis happens during the so-called natural cell death period. During development, motor neurons are generated in excess and approximately 40% of the initially generated motor neurons are progressively removed. This process ensures the generation of the appropriate number of motor neurons and guarantees the elimination of motor neurons that did not establish proper neuromuscular junctions. According to the “neurotrophin hypothesis”, developing motor neurons compete for limited amounts of neurotrophic factors produced by the muscle targets. Only motor axons that establish stable and functional neuromuscular junctions receive sufficient survival signals and are maintained (Oppenheim, 1991; Kanning et al., 2010). Importantly, we found that motor neuron loss elicited by cytoplasmic FUS mislocalization is associated with increased apoptosis of spinal motor neurons (Fig 7). Several motor neuron intrinsic mechanisms may underlie increased motor neuron apoptosis in Fus\textsuperscript{ANLS/ANLS} mice and future studies are needed to investigate these possible mechanisms. For instance, expression of axon guidance receptors may be deregulated in Fus\textsuperscript{ANLS/ANLS} mice, leading to defects in motor axon targeting and failure to receive neurotrophic support. Alternatively, Fus\textsuperscript{ANLS/ANLS} motor axons may reach their targets, but reduced expression of receptors for muscle-derived neurotrophic factors may render a fraction of motor neurons insensitive to survival signals.

Previous work suggested various potential toxic mechanisms for cytoplasmic FUS, and our study sheds light on several of these candidate pathways. First, cytoplasmic FUS could generate toxic FUS aggregates in the cytosol, as shown in yeast models of FUSopathy. For instance, expression of axon guidance receptors may be deregulated in Fus\textsuperscript{ANLS/ANLS} mice, leading to defects in motor axon targeting and failure to receive neurotrophic support. Alternatively, Fus\textsuperscript{ANLS/ANLS} motor axons may reach their targets, but reduced expression of receptors for muscle-derived neurotrophic factors may render a fraction of motor neurons insensitive to survival signals.

An interesting observation is that both Fus\textsuperscript{ANLS/ANLS} and Fus\textsuperscript{−/−} mice die shortly after birth of respiratory insufficiency, presumably caused by loss of FUS function. A concerted action of several physiological systems is required for newborn mice to breathe normally, and defects in any of these systems can lead to neonatal lethality (Turgeon & Meloche, 2009). Firstly, the respiratory rhythm is generated in the respiratory center in the brainstem and transmitted through the spinal motor neurons to the respiratory muscles (diaphragm and intercostals muscles). Therefore, structural or functional defects of neurons in the respiratory center, motor neurons, neuromuscular junctions, and/or respiratory muscles can lead to respiratory distress. Our observation that neonatal lethality of Fus\textsuperscript{ANLS/ANLS} mice is not rescued by restoration of FUS nuclear import in motor neurons indicates that loss of FUS function in cell types other than motor neurons is sufficient to trigger neonatal lethality. Alternatively, the expression of CRE under the ChAT promoter may occur too late in the development to provide a complete rescue of the motor neurons functionality. The remaining cytoplasmic FUS observed in rescued motor neurons could result either from a long half-life of FUS protein in motor neurons or from CRE-mediated recombination of only one allele. Thus, rescued motor neurons, despite being histologically normal, could remain functionally altered due to residual cytoplasmic FUS. This would be analogous to results observed in Nova double knockout animals rescued with neuronal agrin (Ruggiu et al., 2009). Electrophysiological characterization of neuromuscular transmission could help discriminate between these two possibilities. Apart from neuromuscular alterations, defects in lung morphogenesis or maturation, cardiovascular defects, hematological defects, or skeletal defects can all result in poor blood oxygenation and the characteristic cyanosis observed in Fus\textsuperscript{ANLS/ANLS} and Fus\textsuperscript{−/−} neonates. It will be important to investigate these possible causes of neonatal lethality due to loss of FUS function in future studies. In particular, generation of conditional Fus knockout mice would provide a valuable tool to evaluate whether selective FUS inactivation in neurons, muscle, lung epithelium, or the vasculature would result in respiratory insufficiency and neonatal death. Possibly, loss of FUS function in a combination of these tissues is necessary to induce this phenotype.

While a few FUS mutations have a recessive pattern of inheritance (Kwiatkowski et al., 2009; Bertolin et al., 2014), the vast majority are dominantly inherited, and most ALS patients are affected in
their 5th to 7th decade. Thus, our observation of perinatal death is not fully representative of the disease state as occurs in ALS patients and some changes observed here might be deleterious through developmental rather than degenerative effects. In this respect, it will be crucial to further analyze heterozygous Fus<sup>ANLS/+</sup> mice that closely mimic the genetic situation in the vast majority of ALS-FUS patients. These mice are viable and show no obvious motor phenotype until 6 months of age (Scekic-Zahirovic, unpublished results). Nevertheless, Fus<sup>ANLS/+</sup> mice showed cytoplasmic mislocalization of FUS and subtle alterations in mRNA splicing at birth. Aging might exacerbate these defects, consistent with a late-onset disease such as ALS, and it will be highly interesting to characterize a potential age-dependent phenotype of Fus<sup>ANLS/+</sup> mice and Fus<sup>−/−</sup> mice.

The very severe phenotype of Fus<sup>ANLS/ANLS</sup> mice resembles spinal muscular atrophy, which is caused by mutations in the SMN1 gene, whose product SMN interacts directly with FUS and is an integral component of the spliceosome (Yamazaki et al., 2012; Gerbino et al., 2013; Groen et al., 2013; Tsuji et al., 2013; Sun et al., 2015; Yu et al., 2015). This is further reinforced by the strong splicing defects observed in Fus<sup>ANLS/ANLS</sup> mice and is consistent with juvenile onset in many ALS-FUS patients with C-terminal truncation mutations. These similarities strengthen the links between ALS and SMA, and support that SMA, ALS, and FTD may be part of the same disease spectrum with common molecular mechanisms leading to neuronal death.

In conclusion, this study provides in vivo genetic evidence that cytoplasmic mislocalization of FUS triggers apoptotic motor neuron degeneration and demonstrates a crucial role for a gain of toxic function in this process. Motor neuron loss occurs at least partially through a cell autonomous gain-of-function mechanism, since complete loss of FUS is not associated with motor neuron death, and rescue of nuclear FUS within motor neurons prevents neuronal death.

Materials and Methods

**Generation of conditional knockin Fus<sup>ANLS/ANLS</sup> and Fus<sup>−/−</sup> mice**

Knockin Fus mice with the conditional ablation of exon 15 were generated in the Institut Clinique de la Souris (ICS, Illkirch, Strasbourg) using standard procedures. The Fus locus was engineered to include, in between the exons 12 and 13 of the gene, an inserted floxed cDNA encoding exons 13 and 14 of Fus, followed by 3 STOP cassettes. We obtained germ line transmission of the recombinant allele. Homozygous Fus<sup>ANLS/ANLS</sup> mice were generated by intercrossing Fus<sup>ANLS/+</sup> animals.

For generation of Fus<sup>−/−</sup> mice, the mouse ES cell clone EUC0131_G08 was obtained from the European Conditional Mouse Mutagenesis Consortium (EUCOMM) (Friedel et al., 2007). Southern blotting and sequencing of PCR-amplified genomic sequences confirmed a single gene trap insertion event in the first intron of Fus. Blastocyst injection of ES cells resulted in chimeric mice, which allowed for germ line transmission of the mutant Fus allele. As the ES cells were generated in a 129P2 background, the resulting offspring was backcrossed at least five times to C57Bl6 mice. The genetic background of all mice used in this study is C57Bl6.

**Western blot**

Western blotting and antibodies used are presented in Appendix Supplementary Methods.

**Histology and motor neuron counts**

Spinal cords were removed and immersed in fixative for 2 h at 4°C. Samples were transferred overnight into 30% sucrose in 0.1 M phosphate buffer (PB) at 4°C for cryoprotection, embedded in medium (Tissue-Tek® O.C.T. Compound, SAKURA#4583), and cut with a cryostat (Leica CM 3050S). P0 spinal cords were cut in serial 25-μm-thick sections and mounted onto 2% gelatin-coated slides to be processed for immunostaining.

For Nissl staining, slides were air-dried overnight. Sections were then hydrated through 100% and 95% alcohol to distilled water, immersed in 0.1% Cresyl violet acetate (Ceristain®, MERCK®5235), and coverslipped with Roti-Histokitt (Roth, 6638.1).

For immunohistochemistry (IHC), unspecific binding sites were blocked with 5% horse serum (HS), 0.5% Triton X-100 for 30 min at room temperature (RT), immersed in 3% hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) to remove the endogenous peroxidase activity, rinsed in phosphate buffered saline (PBS), and incubated with goat polyclonal anti-choline acetyltransferase (ChAT) antibody (Millipore, AB144-P; diluted 1:50) overnight at RT in a humidified chamber. After rinsing in PBS, sections were incubated with biotinylated donkey anti-goat IgG (Jackson, 705-066-147; 1:250) for 1.5 h, rinsed in PBS, and then incubated with ABC kit (Vector, PK7200; 1:4,000) for 1 h. All antibodies were diluted in 0.01 M PBS, 0.1% Triton X-100. Peroxidase staining was obtained by incubating the sections in the 0.075% 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma Aldrich) and 0.002% H<sub>2</sub>O<sub>2</sub> in 50 mM Tris–HCl pH 7.5. Sections were dehydrated, air-dried, and coverslipped with Roti-Histokitt (Roth, 6638.1).

Motor neurons were counted at L1–L5 on both cresyl violet- and ChAT-stained sections at 20× magnification (for each genotype: exact numbers of animals per group are provided in figure legends). The counting was performed per ventral horn in every tenth section for ten sections in total per animal. In Nissl-stained sections, only neurons with an area ≥ 80 μm<sup>2</sup> and located in a position congruent with that of motor neuron groups were counted (d’Errico et al., 2013). All ChAT<sup>+</sup> profiles located in the ventral horns of immunostained sections clearly displayed on the plane of the section were counted. Total estimated motor neuron numbers were obtained using a computer-assisted microscope (Nikon Eclipse E800) and the software (Nis Elements version 4.0). Cells were counted on the computer screen using a digital camera (Nikon Digital Sight DS-U3) mounted on a microscope. The soma size of Nissl+ motor neurons was also analyzed by measuring cross-sectional areas at 20× magnification using ImageJ software (Schneider et al., 2012).

Other histological techniques are described in Appendix Supplementary Methods.

**Analysis of expression changes by RNA-seq**

Total RNA from brains of Fus<sup>ANLS/ANLS</sup>, Fus<sup>−/−</sup>, and their control littermates were extracted with TRIzol (Invitrogen). RNA quality was measured using the Agilent Bioanalyzer system or RNA ScreenTape (Agilent technologies) according to the manufacturer’s
recommendations and processed using the Illumina TruSeq Stranded mRNA Sample Preparation Kit according to manufacturer’s protocol. Generated cDNA libraries were sequenced using an Illumina HiSeq 2000 sequencer with 4–5 biological replicates sequenced per condition using single read, 50 cycle runs. Quality of sequencing reads was assessed using FastQC (Babraham Bioinformatics) and then aligned to a mouse reference genome (mm9, UCSC Genome Browser) using TopHat (version v2.0.10). Sequencing yielded, on average, 15 million non-redundant reads per sample with a 48.4–58.7% mapping rate. Cufflinks (version v2.1.1) was used to generate transcript abundance for each annotated protein-coding gene as fragments per kilobase of transcript per million mapped reads (FPKM), and statistical analysis and comparison of FPKM values were calculated using Cuffdiff (version v2.1.1). Genomewide unsupervised clustering analysis and heat maps with significant changes between different groups were generated using R (Bioconductor). qRT–PCR techniques used to confirm RNA-seq changes are described in Appendix Supplementary Methods and oligonucleotides sequences are provided in Dataset EV9.

Analysis of splicing alterations by RASL-seq

RNA-mediated oligonucleotide annealing, selection, and ligation with next-generation sequencing (RASL-seq) analysis of alternative splicing changes was carried out as already described elsewhere (Li et al., 2012; Zhou et al., 2012). In brief, a pool of oligonucleotides was designed to detect 3,859 alternative splicing events in mice. One hundred fmol of RASL-seq oligos were annealed to 1 μg of total RNA isolated from brains of Fas+/+, Fas−/−, and their control littermates. After ligation, 5 μl eluted ligated oligos were used for 16–18 cycles of PCR amplification, and the barcoded PCR products were sequenced using an Illumina HiSeq 2000 sequencer with 24–30 samples per lanes. Sequencing data were decoded allowing no mismatch with each barcode, and target sequences were mapped with RASL-seq oligo pool sequences using the short read alignment software Bowtie allowing for 1 mismatch at both the left and right side of the ligated oligos. An average of ~5 million reads from each sample was mapped, with events with <4 counts in one of the isoforms removed. Ratios of the counts of shorter to longer isoforms were calculated. The significantly changed events were identified by t-test and average fold change. Unsupervised clustering analysis and heat maps with significant changes between different groups were generated using R (Bioconductor). Semi-quantitative PCR techniques used to confirm RASL-seq changes are described in Appendix Supplementary Methods. Oligonucleotides sequences are provided in Dataset EV9.

Statistics

For the animal experiments, the values from each animal were averaged for each genotype group and analyzed by Student’s unpaired two-tailed t-test. Comparison of three or four groups was performed using one-way ANOVA and Tukey’s post hoc test. Data were analyzed by using the Graphics Prism Program (Graph Pad Software, San Diego, CA) and expressed as mean ± SEM (standard error of the mean) and differences were considered significant when \( P \leq 0.05 \).

Data accession

RNA-seq and RASL-seq data have been deposited in GEO, under entry GSE78730.

Expanded View for this article is available online.

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Author contributions

JSZ, OS, ES, CLT, and LD designed research; JSZ, OS, MJ, SY, KD, SDG, KD, NM, MCB, JQ, CR, TS, and ES performed research; SY, JQ, YZ, HL, XDF, ES, CLT, and LD analyzed data and made figures; and JSZ, ES, CLT, and LD wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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