Scekic-Zahirovic and collaborators
Toxic gain of function from mutant FUS protein is crucial to trigger cell autonomous motor neuron loss

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Animal breeding and genotyping

Fus\textsuperscript{ΔNLS} and ChAT-CRE mice were housed in the animal facility of the Faculty of medicine from Strasbourg University, with 12/12 hours of light/dark cycle. The animals had unrestricted access to standard diet and water. Fus\textsuperscript{+/+}, Fus\textsuperscript{ΔNLS/+} and Fus\textsuperscript{ΔNLS/ΔNLS} mice were produced by interbreeding Fus\textsuperscript{ΔNLS/+} mice, and littermates were systematically used as controls. Fus\textsuperscript{ΔNLS/ΔNLS}/ChAT-CRE mice were generated by a two-step breeding strategy. First, heterozygous Fus\textsuperscript{ΔNLS/+} mice were crossed with ChAT-CRE mice. Fus\textsuperscript{ΔNLS/+}/ChAT-CRE mice were then crossed with heterozygous Fus\textsuperscript{ΔNLS/+} mice and these F2 litters were used in experiments. Fus\textsuperscript{ΔNLS} mice were genotyped by PCR on tail DNA using following primers: GAT TTG AAG TGG GTA GAT AGA TAG TGC AGG and CCT TTC CAC ACT TTA GGT TAG TCA CAG. ChAT-CRE mice were genotyped by PCR on tail DNA using following primers: CCA TCT GCC ACC AGC CAG and TCG CCA TCT TCC AGC AGG. Fus\textsuperscript{−/−}, Fus\textsuperscript{+/−} and Fus\textsuperscript{+/+} mice were generated by crossing Fus\textsuperscript{+/−} mice and PCR genotyped using a combination of three primers: (i) a common forward primer (CTC TCC TGG CCC GGT CAC) which anneals upstream of the gene trap insertion, (ii) a reverse primer (GCC AGA GGA GCG CGT GC) which anneals downstream of the gene trap insertion and gives rise to a 150 bp band for wild type Fus, but no band for the gene trap allele under the cycling conditions used, and (iii) a reverse primer (CTG GAC TAC TGC GCC CTA C) which anneals in the gene trap and gives rise to a 715 bp band when the gene trap allele is present.

All experiments were approved by the local ethical committees of Strasbourg and Muenster universities.

Western blotting

For western blotting, tissue powder (brain, spinal cord and gastrocnemius muscle) was homogenized in lysis buffer (250 mM Sucrose solution, 1 mM EDTA, 2% SDT, 1 mM DTT, 10 mM Tris HCl pH 7.4) containing protease inhibitor (Sigma P8340) and phosphatase inhibitor cocktail (Sigma 8345) and centrifuged at 12000 x rpm for 15 minutes at room temperature. Protein concentration was measured using BCA Protein Assay. Equal amounts of protein (20µg) were separated by SDS-PAGE 10% and blotted onto a nitrocellulose membrane. Membranes were saturated with 10% non-fat milk and then incubated with the primary
antibodies against the internal or N-terminal part of FUS (Proteintech\textsuperscript{TM}, 11570-1-AP; 1:1000 and Bethyl A303-839A; 1:1000) diluted in 3% non-fat milk, and antibodies against the C-terminal part of FUS (Bethyl A300-294A; 1:10000 and Bethyl A300-302A; 1:10000), followed by anti-rabbit (P.A.R.I.S.; BI2413) or anti-goat (Sigma A5420) secondary antibody diluted 1:5000. Of note, the Bethyl antibody A300-302A is reported by the company as raised against the N-terminal part of the protein, but evidence supports either that this antibody recognizes the C-terminal NLS of FUS or that its binding to FUS is altered by the presence of mutation. An antibody against Histone 3 (Cell signaling, #9715; 1:1000) was used as loading control. All blots were analyzed with chemiluminescence (ECL; Luminata Forte Kit, Millipore WBLUF0500) using the Molecular Imager Chemidoc XRS (Biorad) as detection system.

**Immunodetection of FUS in mouse embryonic fibroblasts, motor neurons and cerebral cortex**

Localization of FUS protein was analyzed in spinal cord motor neurons on cryosections prepared as described below and double stained for FUS (Proteintech\textsuperscript{TM}; 11570-1-AP; 1:100), and ChAT (Millipore, AB144-P; 1:50) or for FUS (Proteintech\textsuperscript{TM}; 11570-1-AP; 1:100) and NeuN (Millipore, ABN78; 1:100). Localization of FUS in the cerebral cortex was analyzed on 30 µm coronal cryosections of the brain of newborn $\text{Fus}^{\Delta\text{NLS}/\Delta\text{NLS}}$ and $\text{Fus}^{+/+}$ mice using a rabbit anti-FUS antibody (Bethyl A300-302A, 1:150). Fus immunoreactivity was visualized with a confocal microscope (LSM 510; Carl Zeiss, Thornwood, NY), within mouse embryonic fibroblasts (MEF) double stained with antibodies against the internal or N-terminal part of FUS (Proteintech\textsuperscript{TM}, 11570-1-AP; 1:100 and Bethyl A303-839A; 1:100) and Draq5 (Cell Signaling, 4084; 1:1000) followed by fluorescent secondary antibodies donkey anti-rabbit Alexa 488 (Jackson, A21206) and donkey anti-goat Alexa 594 (Molecular Probes, A11058) diluted 1:500. This experiment was repeated three times; data shown are from one representative experiment.

**Quantification of cortical thickness**

For quantification of cortical thickness, brains of newborn $\text{Fus}^{\Delta\text{NLS}/\Delta\text{NLS}}$, $\text{Fus}^{+/+}$ and their respective $\text{Fus}^{+/+}$ littermate controls were dissected and fixed for two hours in 4% paraformaldehyde at room temperature. Brains were subsequently cryoprotected in 30% sucrose at 4°C overnight, washed in PBS and embedded in cryo medium. 30 µm coronal cryosections were made, air-dried for several hours, and stored at -80°C or directly used for histochemical or immunostaining. Cortical thickness was quantified on images of Nissl-
stained sections, using the first section on which the hippocampus could be identified for quantification. ImageJ software was used to measure the cortical thickness, which was normalized to the area of the cerebral hemisphere to take brain size into account. The ratio of cortical thickness to hemisphere area was determined for each animal and compared between genotypes.

Immunostaining for Cux1 and Ctip2 was performed to visualize cortical layers II-IV and V-VI, respectively. For antigen retrieval, 30 µm coronal cryosections were incubated in hot sodium citrate buffer pH 6.0, followed by blocking and overnight incubation at 4°C with primary antibodies against Cux1 (Santa Cruz Biotechnology, sc-13024, 1:250) or Ctip2 (Abcam, 25B6, 1:500). After incubation with Alexa488-labeled secondary antibodies for two hours, a cover slip was mounted onto the sections using mounting medium with DAPI. The first section on which the hippocampus could be identified was selected for confocal imaging, and ImageJ was used to generate maximal intensity Z-projections of the resulting confocal sections. The thickness of the cortical layers was measured and normalized to the hemisphere area which had been determined on Nissl stained sections.

In situ detection of apoptosis in spinal cord cells by TUNEL assay

For TUNEL staining, the spinal cord cryosections prepared as described above were treated with TUNEL reagent (Trevigen, 4812-30-K) according to the kit instructions. The tissue sections were permeabilized with Cytonnin™. All TUNEL-positive cells were counted and examined for the typical pathological feature of apoptosis under a fluorescent microscope (Nikon Eclipse E800) at a 20x magnification. The numbers in each set of sections were summed up and divided by number of the sections in a set. The mean number of TUNEL-positive cells for each genotype group was calculated.

Caspase 3 immunostaining and apoptotic events

Apoptotic bodies across the lumbar spinal cord cross-sectional area were determined by fluorescence microscopy. For detection of caspase-positive motor neurons sections were double-stained with goat-anti-ChAT (Millipore, AB144-P; diluted 1:50) and rabbit-anti-cleaved caspase-3 (R&D Systems, AF835; 1:100) and combination of secondary antibodies Alexa 594 anti-goat (Molecular Probes, A11058; 1:500) and Alexa 488 anti-rabbit (Jackson, A21206; 1:500) and Draq5 (Cell Signaling, 4084; 1:1000). Total number of apoptotic bodies was counted all over the cross sectional area for every tenth section for 10 sections in total.
per animal. Apoptotic motor neurons were counted as cells triple positive for ChAT, caspase and condensed chromatin by Draq5. Information on the total numbers of apoptotic counts and further details are presented along with the description of the results. This experiment was repeated two times for both groups of animals $Fus^{\Delta NLS}$ and $ChAT-CRE$ mice; images shown are from one representative experiment.

**Lung histology**

Lungs were harvested and fixed with 4% formaldehyde solution (Sigma 47608) for 24 h and embedded in paraffin; Sections of 6 µm were stained with hematoxylin (Vector H-3401) and eosin (Eosin Y-solution 0.5%; Roth X883.1) for light microscope observation (Nikon Eclipse E800) at a 40x magnification.

**Other immunostainings**

For detection of protein aggregation spinal cord sections were double immunostained with N-terminal part of FUS (Bethyl A303-839A; 1:100) and anti-Sqstm1 (p62, Abcam 56416; 1:100) followed by fluorescent secondary antibodies donkey anti-mouse Alexa 488 (Jackson, 715-545-150) and donkey anti-goat Alexa 594 (Molecular Probes, A11058) diluted 1:500. Anti Neurofilament (Abcam 24574; 1:100), anti Ubiquitin (Cell signaling 3933; 1:100) and anti Ubiquitin (Millipore, MAB1510; 1:100) were used for studing corresponding proteins. For stress granule markers we used following antibodies anti eIF2α phosphorylated (Cell signaling 9722; 1:100), and anti TIAR (Santa Cruz 1751; 1:100).

Imunoreactivity for SMN positive germs was studied using anti SMN (BD 610646; 1:100) and HDAC1 was visualized by anti HDAC1 (Bethyl A300-713A; 1:100).

Double immunostaining with anti ChAT (Millipore, AB144-P; diluted 1:50) was performed at the spinal cord for analyzing the localization of TAF15 (Abcam 134916; 1:100).

**Imaging**

Single-layer images (except for mouse embryonic fibroblasts (MEF) were acquired using a laser-scanning microscope (confocal Leica SP5 Leica Microsystems CMS GmbH) equipped with 63xoil objective (NA1.4). Excitation rays are sequential argon laser 488nm, diode 561nm, Helium Neon laser 633nm. Emission bandwidths are 500-550nm for Alexa488, 570-620nm for Alexa594, and 650-750nm for Draq5.
Confirmation of expression changes by quantitative RT-PCR

RNA samples from brains of $\text{Fus}^{\Delta\text{NLS}/\Delta\text{NLS}}$, $\text{Fus}^{-/-}$ and their control littermates were treated with DnaseI (Invitrogen) and converted to cDNA using SuperScript III kit (Invitrogen) with random hexamers or the Iscript Reverse Transcriptase (Bio-Rad). qRT-PCR reactions were performed with 3-5 mice for each group and two technical replicates using the iQ SYBR green Supermix (Bio-Rad) on either the IQ5, the CFX96 Touch or the CFX384 Touch Real-Time PCR detection system (Bio-Rad). Analysis was performed using the iQ5 optical system software (Bio-Rad; version 2.1) or the CFX manager system software (Bio-Rad; version 3.1). Expression values were normalized to the control gene Rsp9, and were expressed as a percentage of the average expression of the control samples. Primer sequences were designed using Primer3 software (http://frodo.wi.mit.edu/primer3/) and are available in Dataset S9.

Confirmation of splicing changes by semi-quantitative RT-PCR

Semi-quantitative RT-PCR (25-30 cycles) was used to validate alternative splicing changes. Isoform products were separated on 10% polyacrylamide gels and stained with SYBR gold (Invitrogen) and quantified with ImageJ software to record the intensity of the bands corresponding to different splicing isoforms. Intensity ratios of long and short isoforms were averaged from three biological replicates per group. Primer sequences were designed using Primer3 software (http://frodo.wi.mit.edu/primer3/) in exons flanking the alternatively spliced exon. PCR primer sequences are shown in Dataset S9.
Fig S1: Validation of expression changes identified by RNA-seq in $Fus^{\Delta NLS/\Delta NLS}$ and $Fus^{-/}$ mouse brain

(A) Quantitative RT-PCR (qRT-PCR) for genes identified by RNA-seq to be significantly downregulated ($Ahi1$, $Kcnip1$, $Nefm$, $Nefl$, $Tuba4a$, $Dmpk$, $Rad9b$, $Stac3$, $Hist1h2bc$, $Hist1h1c$) or upregulated ($Fam193b$, $Pmm2$, $Bphl$, $Taf15$) in both $Fus^{\Delta NLS/\Delta NLS}$ (blue bars) and $Fus^{-/}$ (red bars) compared to their control littermates ($Fus^{+/+}$, black bars). Error bars represent SEM in 3-5 biological replicates.

(B) qRT-PCR for $Trove2$, $Uhmk1$, $Ssh3$, $Vtn$, $Snrpb$ and $Ephb3$ in brains from $Fus^{\Delta NLS/\Delta NLS}$ (blue bars), $Fus^{-/}$ (red bars) and control littermates ($Fus^{+/+}$, black bars), showing genes identified by RNA-seq to be associated with the presence of truncated FUS in $Fus^{\Delta NLS/\Delta NLS}$ animals and not modified by loss of FUS in $Fus^{-/}$ mice. Error bars represent SEM in 3-5 biological replicates.
Fig S2: Intermediate splicing pattern in heterozygous $Fus^{ANLS/+}$ mice

Heatmap with hierarchical clustering of RASL-seq data from biological replicates of $Fus^{ANLS/ANLS}$ (N=4), $Fus^{ANLS/+}$ (N=4) and control littermates (N=4), showing 173 alternative splicing alterations associated with expression of cytoplasmic FUS in knock-in animals (defined by t-test with p<0.05 and average fold change >1.5).
Fig S3: Normal cortical development in Fus knock-in and knock-out mice

(A) Representative Nissl staining of cerebral cortex of Fus\textsuperscript{-/-} mice and Fus\textsuperscript{ΔNLS/ΔNLS} mice, and their respective controls at birth. Note that the cerebral cortex appear grossly normal in all 4 groups.

(B-C) Quantification of cerebral cortical thickness, relative to wild type littermates in Fus\textsuperscript{ΔNLS/ΔNLS} mice (n=6 for Fus\textsuperscript{+/+} and n=8 for Fus\textsuperscript{ΔNLS/ΔNLS}; B) and Fus\textsuperscript{-/-} mice (n=9 for Fus\textsuperscript{+/+} and n=9 for Fus\textsuperscript{-/-}; C). Scale bar: 100\(\mu\)m
Fig S4: Normal cortical layering in *Fus* knock-in and knock-out mice

(A, B) representative immunofluorescent staining of Cux1 (A, marker of neurons from layers II-IV) and Ctip2 (B, marker of neurons from layers V-VI) in the cerebral cortex of *Fus*−/− mice and *Fus*ΔNLS/ΔNLS mice, and their respective controls at birth.

(C, D) Measurement of the thickness of Cux1 positive layers (B) and Ctip2 positive layers (D), in the cerebral cortex of *Fus*−/− mice and *Fus*ΔNLS/ΔNLS mice, and their respective controls at birth.

Scale bar: 100µm.
Fig S5: Normal subcellular distribution of TAF15 in \( Fus^{\text{ANLS/ANLS}} \) mice

Representative images of immunofluorescent staining of motor neurons, labelled with DRAQ5 (nuclei, blue), ChAT (red) and TAF15 (green), a binding partner of FUS, that is also a FET family member. Scale bar: 10\( \mu \)m.