Supplementary material for Wächter N. et al. Human TDP-43 and FUS selectively affect motor neuron maturation and survival in a murine cell model of ALS by non-cell-autonomous mechanisms. Amyotrophic Lateral Sclerosis and Frontotemporal Degeneration. 2015; doi:10.3109/21678421.2015.1055275.

Supplementary material

Wächter et al., Human TDP43 and FUS selectively affect motor neuron maturation and survival in a murine cell model of ALS by non-cell-autonomous mechanisms

Embryonic stem cells

Murine ESCs harbouring a bacterial artificial chromosome (BAC)-based eGFP reporter construct under control of the Hb9 promoter (Hb9::GFP) were kindly provided by Jared Sternecker (24). The cells were cultured in ES-cell media (KO DMEM, Gibco) supplemented with 15% knockout serum replacement (KOSR, Gibco) and 5% foetal bovine serum (FBS, Gibco), 1% L-glutamine, 1% non-essential amino acids, 1% penicillin/streptomycin, 0.2% β -mercaptoethanol and 1000 units/ml of leukaemia inhibitory factor (LIF, Millipore) and passaged every 2–3 days.

Plasmids and PCR

Expression constructs containing the human cDNA of wild-type *TDP-43*, missense mutant *TDP43A315T*, wild-type *FUS*, missense mutants *FUS R514S*, *FUS R521C* or *FUS P525L* were kindly provided by Robert H. Baloh (*TDP-43*) and Daisuke Ito (*FUS*). The cDNAs were cut out via KpnI/ApaI (Fermentas), restriction digested and ligated into the pDsRed-Monomer-C1 expression vector (Clontech Laboratories, Inc.). To proof the correct insertion site and orientation of cDNA, the plasmid constructs were sequenced and additionally analysed by PCR (Taq DNA polymerase, 5 Prime) using the following primers: sequencing primer (also used as forward primer for PCR, Clontech Laboratories, Inc.), 5'-AGCTGG ACA TCA CCA ACC ACA ACG-3'; hTDP43-dsRed fusion primer, 5'-CAG ACTTAT CGT CGT CGT CCTTGT A-3', 160 bp product; hFUS-dsRed fusion primer, 5'-ACC ACA CTG GAC TAG TGG ATC CGA-3', 162 bp product; neomycin resistance gene primers, 5'-CAC CAA GCG AAA CAT CGC ATC-3' and 5'-ATG GGT CAC GAC GAG ATC C-3', 160 bp product (all primers from MWG Operon). Correct constructs were amplified in *E. coli*, purified by anion-exchange (Plasmid Kit, Qiagen) and linearized with ApaLI (Fermentas) for stable transfection of Hb9::GFP mESCs.

Stable transfection

Murine Hb9::GFP ESCs were transfected with linearized plasmids by electroporation using the

Amaya™ Mouse ES cell Nucleofector™ Kit (Lonza). To ensure a stable integration of the plasmid into the murine stem cell genome, transfected cells were treated with a lethal concentration of 600–1000 μ g/ml geneticin disulfate (G418, Carl Roth) utilizing the presence of a neomycin resistance gene in the pDsRed-Monomer-C1 backbone. Two weeks after inducing selection, the single grown mESC colonies were subcultivated for further expansion without G418 for one week. Re-cloning of those single colonies was induced by retreatment with G418 for an additional 1–2 weeks. Stable integration of the plasmid into the murine genome was proven by PCR analysis and immunofluorescence imaging (Figure 1B, C).

Motor neuron differentiation

For long-term experiments, non-transfected mESCs (non-transgenic control, nt-control) and the stably transfected mESCs were differentiated into the neural lineage by embryoid body (EB) formation in DMEM/F12 medium (Gibco) containing 10% KSR (Gibco), 1% penicillin/streptomycin, 1% non-essential amino acids (NEAA), 1% L-glutamine and 0.2% β -mercaptoethanol (all from Gibco). After three days of EB formation the neural differentiation was induced by media supplementation with 1 μ M retinoic acid (Sigma), 1 μ g/ml purmorphamine (Cayman Chemical) for an additional three days as previously described by (24). Subsequently, neural differentiation for six days was initiated. EBs were enzymatically digested with 0.05–0.25% trypsin/EDTA (Gibco) and plated as monolayer (day 0; 0d) onto poly-D-lysine (Sigma) and laminin (Roche Diagnostics) coated cell culture dishes in DMEM/F12 medium supplemented with 2% horse serum, 1x B27, 1x N2, 1% Glutamax, 1% NEAA and 1% penicillin/streptomycin (all from Gibco). Plated cells were matured over a time-period of 14 days (14d) and analysed at day 5 (d5) and d14 post plating (see Figure 1A for a scheme of the experimental design).

Muscle cell differentiation

Induction of muscle differentiation for coculture analysis was achieved by EB formation of mESCs using the hanging drop technique (1000 cells/25 μ l) in muscle cell differentiation medium (DMEM high glucose) supplemented with 15% FBS, 1% L-glutamine, 1% NEAA, 1% penicillin/streptomycin (all from Gibco) for three days. EBs were then cultivated in suspension for an additional three days

and subsequently digested with 0.05% trypsin/EDTA (Gibco). Muscle cell maturation was enabled by plating the cells on Matrigel™ (BD Biosciences) for 2–3 weeks in differentiation medium (DMEM, high glucose) containing 2% FBS, 1% L-glutamine, 1% NEAA, 1% penicillin/streptomycin (all from Gibco).

Motor neuron/muscle cell cocultures (chimeric cultures)

For a defined coculture with motor neurons, non-transfected mESCs were differentiated into motor neurons as described above and sorted for GFP fluorescence as single cell suspension in FACS buffer [PBS (Gibco), 1% BSA (Serva), 5 mM EDTA (Sigma)]. Sorted GFP⁺ cells were plated on matured muscle cells and cultivated for 24 h in medium (DMEM/F12, Gibco) supplemented with 2% horse serum and 10 ng/ml GDNF (Sigma).

Immunocytochemistry

Cell cultures were fixed with 4% paraformaldehyde (Sigma) for 12 min at room temperature. Immunofluorescence staining was carried out using standard protocols. Cell nuclei were stained with Hoechst (Invitrogen) according to manufacturers' protocols. The following primary antibodies were used: rabbit-anti-TARDBP (Abcam, 1:400), rabbit-anti-Neuronal

Class III β -Tubulin (TUJ1) (Covance, 1:3000) or mouse-anti-Neuronal Class III β -Tubulin (Covance, 1:1000) or chicken-anti-Neuronal Class III β -Tubulin (TUJ1) (Chemicon, 1:500), rabbit-anti-DsRed (Clontech, 1:300), rabbit-anti-GFP (Abcam, 1:500) or chicken-anti-GFP (Abcam, 1:1000), chicken-anti-GFAP (Abcam, 1:500) or mouse-anti-GFAP (Chemicon, 1:500), mouse-anti-FUS (Santa Cruz Biotechnology, 1:300), mouse-anti-MAP2 (BD Pharmingen, 1:1000) or chicken-anti-MAP2 (Abcam, 1:2000), Mouse-anti-Smooth muscle actin (Dako Cytomation, 1:500). Primary antibodies were detected by fluorescence labelled secondary antibodies (Alexa Fluor, Invitrogen). Fluorescence images were captured using a fluorescence microscope (Leica DM IRE2).

Cell counting, neurite length measurement and statistics

Quantification of the percentage of red fluorescent transgenic cells and marker⁺ cells was carried out by counting the number of positive cells from the total number of Hoechst stained nuclei. Neurite length analysis was performed by the use of NeuronJ (plugin of ImageJ software). Statistical comparisons were either made by one-way and two-way ANOVA with Bonferroni post hoc *t*-test or Student's *t*-test (SPSS version 19.0). All data are presented as mean $\pm$ S.E.M. if not indicated otherwise.

Supplementary Figure 1. Protein mislocalization, neurodegeneration and astrocytosis of neurally differentiated non-transgenic and hTDP-43- and hFUS transgenic mESCs. Representative images are depicted. (A) Visualization of GFP⁺ motor neurons and Tuj1 stained neurons growing close to hFUS R514S and R521C transgenic cells resulted in normal neuron morphology after 14 days of maturation in mixed monolayer cultures. (B) Visualization of GFAP⁺ cells growing close to non-transgenic and transgenic cells or even with transgene colocalization resulted in normal astrocyte morphology after 14 days of maturation in all analysed monolayer cultures. The cell nuclei were stained with Hoechst. Scale bar, 100 μ m.

Supplementary Figure 2. Non-transgenic and DsRed Monomer-C1 transgenic neural cultures were analysed towards their quantitative presence of Tuj1⁺, Map2⁺ and GFP⁺ cells after five days of maturation. There was no difference in the amount of Tuj1⁺ immature neurons and GFP⁺ motor neurons. The number of Map2⁺ mature neurons was slightly decreased ($p = 0.031$). Statistics: two-sided unpaired t -test. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Supplementary Table 1. Statistics determined for the amount of GFP⁺ motor neurons for the different ES-hTDP43 cells lines and days of differentiation (Figure 2A). Two-way ANOVA with post hoc t -test and Bonferroni adjustment with ESC lines and differentiation day as fixed factors revealed that there is no significant interaction effect of fixed factors on GFP⁺ motor neurons ($p = 0.964$, F-value = 0.036) and no significant differences among differentiation days ($p = 0.846$, F-value = 0.039), but significant differences among cell lines ($p = 0.032$, F-value = 4.28). Displayed are the Bonferroni-adjusted p -values of significances among the different cell lines.

	Day 5	Day 14
nt-Ctr vs. hTDP43 wt	1.000	0.814
nt-Ctr vs. hTDP43 A315T	0.043	0.048
hTDP43 wt vs. hTDP43 A315T	0.609	1.000

Supplementary Table 2. Statistics determined for the amount of Tuj1⁺ early neurons for the different ES-hTDP43 cells lines and days of differentiation (Figure 2A). Two-way ANOVA with post hoc t -test and Bonferroni adjustment with ESC lines and differentiation day as fixed factors revealed that there is no significant interaction effect of fixed factors on Tuj1⁺ early neurons ($p = 0.878$, F-value = 0.131) and no significant differences among differentiation days ($p = 0.284$, F-value = 1.226), but significant differences among cell lines ($p = 0.016$, F-value = 5.423). Displayed are the Bonferroni-adjusted p -values of significances among the different cell lines.

	Day 5	Day 14
nt-Ctr vs. hTDP43 wt	0.598	0.292
nt-Ctr vs. hTDP43 A315T	0.207	0.049
hTDP43 wt vs. hTDP43 A315T	1.000	1.000

Supplementary Table 3A, B. Statistics determined for the absolute amounts of mature neurons (Map2⁺ cells) for the different ES-hTDP43 cells lines and days of differentiation (Figure 2A). Two-way ANOVA with post hoc t -test and Bonferroni adjustment with ESC lines and differentiation day as fixed factors revealed that ESC lines and differentiation day have no significant interaction effect on Map2⁺ neuron count ($p = 0.388$, F-value = 1.004), but significant differences among ESC lines ($p < 0.001$, F-value = 12.814) and differentiation day ($p = 0.010$, F-value = 8.591). Displayed are the Bonferroni-adjusted p -values. (A) Significances among the different ESC lines. (B) Significances among the differentiation days. Bold values indicate significant differences.

A.

	Day 5	Day 14
nt-Ctr vs. hTDP43 wt	0.067	0.899
nt-Ctr vs. hTDP43 A315T	0.011	0.006
hTDP43 wt vs. hTDP43 A315T	1.000	0.034

B.

	nt-Ctr	hTDP43 wt	hTDP43 A315T
Day 5 vs. Day 14	0.075	0.539	0.023

Supplementary Table 4. Statistics determined for the ratio of GFP⁺ motor neuron and Map2⁺ total neuron counts for the different ESC lines and days of differentiation (Figure 2A). Two-way ANOVA with post hoc t -test and Bonferroni adjustment with ESC lines and differentiation day as fixed factors revealed that there is no significant interaction effect of fixed factors on GFP⁺/Map2⁺ cell ratios ($p = 0.447$, F-value = 0.848) and no significant differences among differentiation days ($p = 0.214$, F-value = 1.677), but significant differences among cell lines ($p = 0.001$, F-value = 11.055). Displayed are the Bonferroni-adjusted p -values of significances among the different cell lines.

	Day 5	Day 14
nt-Ctr vs. hTDP43 wt	1.000	0.775
nt-Ctr vs. hTDP43 A315T	0.013	0.434
hTDP43 wt vs. hTDP43 A315T	0.012	0.030

Supplementary Table 5. Statistics determined for the amount of GFP⁺ motor neurons for the different ES-hFUS cells lines and days of differentiation (Figure 2B). Two-way ANOVA with post hoc t -test and Bonferroni adjustment with ESC cell lines and differentiation day as fixed factors revealed that ESC lines and differentiation day have no significant interaction effect on GFP⁺ motor neuron count ($p = 0.133$, F-value = 2.000) and no significant differences among ESC lines ($p = 0.969$, F-value = 0.132), but significant differences among differentiation day ($p < 0.001$, F-value = 30.211). Displayed are the Bonferroni-adjusted p -values for significances among the differentiation days. Bold values indicate significant differences.

	nt-Ctr	hFUS wt	hFUS R514S	hFUS R521C	hFUS P525L
Day 5 vs. Day 14	0.992	0.013	0.004	0.002	0.013

Supplementary Table 6. Statistics determined for the amount of Tuj1⁺ early neurons for the different ES-hFUS cells lines and days of differentiation (Figure 2B). Two-way ANOVA with post hoc *t*-test and Bonferroni adjustment with ES cell lines and differentiation day as fixed factors revealed that ESC lines and differentiation day have no significant interaction effect on Tuj1⁺ early neuron count ($p = 0.237$, F-value = 1.512) and no significant differences among ESC lines ($p = 0.285$, F-value = 1.135), but significant differences among differentiation day ($p < 0.001$, F-value = 20.222). Displayed are the Bonferroni-adjusted *p*-values for significances among the differentiation days. Bold values indicate significant differences.

	nt-Ctr	hFUS wt	hFUS R514S	hFUS R521C	hFUS P525L
Day 5 vs. Day 14	0.828	0.107	0.003	0.010	0.076

Supplementary Table 7. Statistics determined for the amount of Map2⁺ early neurons for the different ES-hFUS cells lines and days of differentiation (Figure 2B). Two-way ANOVA with post hoc *t*-test and Bonferroni adjustment with ES cell lines and differentiation day as fixed factors revealed that ESC lines and differentiation day have no significant interaction effect on Map2⁺ early neuron count ($p = 0.605$, F-value = 2.681) and no significant differences among ESC lines ($p = 0.299$, F-value = 1.313), but significant differences among differentiation day ($p < 0.001$, F-value = 33.297). Displayed are the Bonferroni-adjusted *p*-values for significances among the differentiation days. Bold values indicate significant differences.

	nt-Ctr	hFUS wt	hFUS R514S	hFUS R521C	hFUS P525L
Day 5 vs. Day 14	0.248	0.011	0.003	0.008	0.018

Supplementary Table 8. Statistics determined for the ratio of GFP⁺ motor neurons and Map2⁺ total neuron counts for the different ES-hFUS cells lines and days of differentiation (Figure 2B). Two-way ANOVA with post hoc *t*-test and Bonferroni adjustment with ES cell lines and differentiation day as fixed factors revealed that ESC lines and differentiation day have no significant interaction effect on GFP⁺/Map2⁺ ratio ($p = 0.146$, F-value = 1.992) and no significant differences among ESC lines ($p = 0.815$, F-value = 0.387), but significant differences among differentiation day ($p < 0.001$, F-value = 60.474). Displayed are the Bonferroni-adjusted *p*-values for significances among the differentiation days. Bold values indicate significant differences.

	nt-Ctr	hFUS wt	hFUS R514S	hFUS R521C	hFUS P525L
Day 5 vs. Day 14	0.293	0.001	0.001	<0.001	<0.001