**FUS Mutant Human Motoneurons Display Altered Transcriptome and microRNA Pathways with Implications for ALS Pathogenesis**

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**SUMMARY**

The FUS gene has been linked to amyotrophic lateral sclerosis (ALS). FUS is a ubiquitous RNA-binding protein, and the mechanisms leading to selective motoneuron loss downstream of ALS-linked mutations are largely unknown. We report the transcriptome analysis of human purified motoneurons, obtained from FUS wild-type or mutant isogenic induced pluripotent stem cells (iPSCs). Gene ontology analysis of differentially expressed genes identified significant enrichment of pathways previously associated to sporadic ALS and other neurological diseases. Several microRNAs (miRNAs) were also deregulated in FUS mutant motoneurons, including miR-375, involved in motoneuron survival. We report that relevant targets of miR-375, including the neural RNA-binding protein ELAVL4 and apoptotic factors, are aberrantly increased in FUS mutant motoneurons. Characterization of transcriptome changes in the cell type primarily affected by the disease contributes to the definition of the pathogenic mechanisms of FUS-linked ALS.

**INTRODUCTION**

The fatal neurodegenerative disease amyotrophic lateral sclerosis (ALS) is caused by loss of motoneurons (MNs) in the spinal cord and brain, leading to progressive muscle atrophy. Both sporadic ALS (sALS) and familial ALS (fALS) have been linked to a number of genes, including SOD1, C9ORF72, TDP-43, TAF15, and FUS/TLS (FUS) (Renton et al., 2014). The FUS gene encodes for an RNA-binding protein involved in RNA biogenesis and maturation (Lagier-Tourenne et al., 2012). Wild-type (WT) FUS is mainly localized in the nucleus, and a hallmark of FUS ALS patients is the presence of cytoplasmic inclusions containing mutated protein in the brain and spinal cord (Kwiatkowski et al., 2009; Vance et al., 2009). Many ALS-associated FUS mutations disrupt the function of the C-terminal nuclear localization signal (NLS). Therefore, defects in nuclear import, leading to aberrant cytoplasmic localization of FUS, have been proposed as the initial step in ALS pathogenesis (Bentmann et al., 2013; Dormann and Haass, 2011). Recent analyses in murine models suggest that loss of FUS function may not be sufficient to induce MN degeneration (Kino et al., 2015; Seckic-Zahirovic et al., 2016), and both loss of a nuclear function and gain of a toxic function in the cytoplasm have been proposed as contributing pathological mechanisms in FUS ALS (Ling et al., 2013).

FUS mutations are expected to exert a major change in the transcriptome. However, this has not been directly addressed in human MNs so far. Human induced pluripotent stem cells (iPSCs) represent a useful system to model ALS, as they can be differentiated into disease-relevant cell types (Sances et al., 2016). iPSCs carrying ALS mutations can be generated by reprogramming from patients (Boulting et al., 2011) or by gene-editing techniques, producing lines that differ only for the specific mutation (Kiskinis et al., 2014; Lenzi et al., 2015). Comparison of gene-edited mutant lines with their otherwise isogenic WT counterparts allows to assign any observed phenotypic difference to the ALS mutation, avoiding any bias due to different genetic backgrounds of non-isogenic mutants and controls.

Recent evidence suggests that microRNAs (miRNAs) might play a role in MN diseases, such as ALS and spinal muscular atrophy (SMA) (Kye and Gonçalves, 2014). Conditional loss of the processing enzyme Dicer in postnatal MNs causes an SMA-like phenotype in mice (Haramati et al., 2010), suggesting that, collectively, miRNAs could be required for MN survival. Global downregulation of miRNAs was observed in MNs of sporadic ALS spinal cords isolated by laser-capture microdissection (Emde et al., 2015), and a protective role for individual miRNAs, such as miR-218 and miR-375, has been shown in MNs (Amin et al., 2015; Bhinge et al., 2016). Previous work from our lab and others has shown that proteins genetically linked to ALS play a role in miRNA biogenesis. FUS stimulates miRNA processing by facilitating the recruitment of the enzyme Drosha on miRNA genes (Morlando et al., 2012), and TDP-43 regulates miRNA biogenesis at multiple levels (Buratti et al., 2010; Di Carlo et al., 2013; Kawahara and Mieda-Sato, 2012). Moreover, overexpression of SOD1, TDP-43, or FUS proteins, either WT or carrying ALS mutations, impaired miRNA processing in the cytoplasm by inducing cellular stress (Emde et al., 2015). As most of the
studies on the contribution of ALS-linked factors to miRNA biogenesis come from cell lines or non-MN cells, it is currently unknown which miRNAs could be affected by ALS mutations in MNs.

Here we took advantage of mutant FUS$^{P525L}$ and isogenic control iPSCs to generate and isolate pure human MNs, which were used for whole-transcriptome analysis by RNA sequencing (RNA-seq). We identified both long and short RNA species significantly altered in FUS mutant MNs. Changes in the transcriptome point to pathways previously associated to neurodegenerative diseases, such as those related to cell adhesion. Among differentially expressed miRNAs we focused on miR-375. Levels of miR-375 were decreased in mutant MNs, in which we observed an increase of a subset of its targets, such as p53 and the neural RNA-binding protein ELAVL4. We propose a pathological mechanism in which an impairment of miRNAs production, downstream of FUS mutations, would alter RNA metabolism and increase MN vulnerability via aberrant upregulation of pro-apoptotic targets.

**RESULTS**

**Differentiation and Isolation of iPSC-Derived MNs**

We have recently reported the generation of iPSC lines devoid of mutations in the FUS gene (hereafter FUS$^{WT}$) or carrying the severe P525L ALS-related mutation introduced by gene editing in both FUS alleles (hereafter FUS$^{P525L}$) (Lenzi et al., 2015). We stably modified these lines by inserting a Hb9:GFP reporter into the AAVS1 locus (Wainger et al., 2014) (Figures S1A and S1B). To improve time and efficiency of MN generation from iPSCs, we took advantage of an optimized differentiation protocol and the stable insertion of the Hb9:GFP reporter system have been introduced by gene editing. Around this time point we detected a peak of expression for the neural progenitor gene PAX6, followed by a raise of pan-neuronal (TUJ1) and MN (ISL1) markers at day 9. The late MN gene CHAT was expressed at day 9, with a further increase until day 12. Levels of the MN progenitor marker HB9 peaked around day 9 and decreased at later time points. The mixed population of differentiated cells at day 12 was highly enriched of ISL1/2-positive neurons (Figure S1C).

Live cell imaging and flow cytometry analysis indicated that 30%-40% of cells were GFP-positive at this time point (Figures 1C and 1D). Notably, expression of the reporter was detected only in retinoic acid and smoothened agonist (SAG)-treated cells, which induce a ventral spinal cord character, suggesting that the exogenous Hb9 promoter was correctly turned on in the MN lineage (Figure 1B).

We further validated the reporter system by gene expression analysis in distinct cell populations isolated using cell sorting (Figure S1D). Next-generation sequencing (RNA-seq) was performed on total RNA from GFP-positive and -negative cells collected after sorting. Cluster analysis based on gene expression data correctly separated the samples into GFP-positive and -negative ones (Figures S1E and S1F). GFP-positive samples had a similar expression profile, while GFP-negative ones were more dissimilar. A total of 1,631 genes were differentially expressed between GFP-positive and -negative cells, most of them having higher expression levels in GFP-negative samples (Figure S1G; Table S1). As shown in Figure 1F, levels of MN markers (in green) were higher in GFP-positive samples, with the exception of LHX1. Notably, the HB9 transcript was not significantly enriched in GFP-positive samples. This apparent inconsistency can be explained by the narrow window of time of HB9 expression: by the time point in which GFP protein reaches its maximum accumulation (day 12; Figure 1D) the endogenous HB9 mRNA is already downregulated (Figure 1B). Compared with GFP-positive cells, GFP-negative populations probably contained a fraction of MN progenitors at earlier stages, expressing high levels of HB9.

As expected, ALS-related genes SOD1, C9ORF72, FUS, and TARDBP were not enriched in GFP-positive cells. Experimental validation of selected markers by real-time qPCR confirmed the RNA-seq results (Figure 1G). Purified GFP-positive cells were re-plated on laminin-coated dishes and upon further culturing acquired a neuronal morphology (Figure S1H). Immunostaining analysis indicated that sorted GFP-positive cells were highly enriched for ISL1/2-positive MNs (Figure 1H).

Taken together, the combination of a short and efficient differentiation protocol and the stable insertion of the Hb9:GFP reporter allowed the effective isolation of a large number of purified, iPSC-derived, MNs.

**Transcriptome Profiling in FUS WT and Mutant Human MNs**

We have previously shown that ALS mutations in FUS do not impair differentiation (Lenzi et al., 2015, 2016). This observation was confirmed in MNs generated with the improved protocol described in Figure 1 from FUS$^{WT}$ and FUS$^{P525L}$ iPSCs. The fraction of GFP-positive cells was consistently comparable over six independent experiments (Figures 2A and 2B). Moreover, GFP-positive cells with a FUS$^{WT}$ or FUS$^{P525L}$ background showed comparable levels of expression of MN markers (Figure S2A). Notably, in the Hb9:GFP FUS$^{P525L}$ line both the mutation and the reporter system have been introduced by gene editing. Therefore, besides the FUS$^{P525L}$ mutation, iPSC lines used for subsequent experiments were isogenic.
We took advantage of our iPSC system to systematically analyze the consequences of the FUSP525L mutation on the transcriptome in pure populations of in-vitro-derived human MNs. GFP-positive FUSWT and FUSP525L iPSCs were sorted at day 12 of differentiation and further cultured on laminin-coated dishes for an additional 7 days (day 12 + 7). Total RNA from three independent differentiation experiments was collected and analyzed by RNA-seq. Gene expression-based cluster analysis resulted in the segregation of samples into two distinct groups based on their FUS genotype (Figures S2B and S2C). Out of 14,289 genes expressed in human MNs, 267 genes were upregulated and 244 downregulated in a consistent manner in FUSP525L compared with FUSWT (false discovery rate [FDR] < 0.05) (Table S1). The gene expression heatmap of differentially expressed genes is shown in Figure 2C. RNA-seq results were validated by real-time qPCR in at least three additional independent samples. Figure 2D shows validation of four representative genes among those downregulated (SCL17A8, NXPH2, CRIM1, and GRIN2A) and upregulated (TP53I3, CDH7, NNAT, and TSPYL5). Expression of the FUS transcript itself, as well as the mRNAs of other major
Figure 2. RNA-Seq Analysis of FUS\textsuperscript{WT} and FUS\textsuperscript{P525L} iPSC-Derived MNs
(A) Representative FACS plots of FUS\textsuperscript{WT} and FUS\textsuperscript{P525L} cells at day 12. As a negative control, FUS\textsuperscript{WT} cells were differentiated in the absence of retinoic acid and SAG (left panel).
(B) Schematic representation of flow cytometry analysis (day 12). Histogram bars represent the average of six independent experiments and error bars indicate the SD (Student’s t test; unpaired; two tails; n.s., p > 0.05).

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ALS-related genes, was not significantly altered in FUS mutant MNs (Figure S2D).

We confirmed these results in an independent FUS WT and mutant isogenic pair. A second FUS<sup>P525L</sup> iPSC line (hereafter FUS<sup>P525L</sup>#2), generated as previously described (Lenzi et al., 2015), and its parental FUS<sup>WT</sup> iPSC line (hereafter FUS<sup>WT</sup>#2) were modified by inserting the Hb9:GFP reporter into the AAVS1 locus (Figures S3A–S3D). FUS<sup>WT</sup>#2 and FUS<sup>P525L</sup>#2 were then differentiated, sorted, and collected at day 12 + 7 for RNA analysis. All genes analyzed in the original isogenic pair resulted consistently altered in MNs obtained from these new iPSC lines (Figure 2D).

A subset of these genes was also affected in MNs obtained from a Hb9:GFP-modified heterozygous FUS<sup>P525L</sup> iPSC line, hereafter FUS<sup>P525L</sup>(ht) (Lenzi et al., 2015) (Figure 2D).

**Functional Consequences of the FUS<sup>P525L</sup> Mutation in Human MNs**

We next assessed whether the change in the transcriptome detected in FUS mutant cells might be informative of pathways potentially involved in ALS MN degeneration. Gene ontology (GO) term enrichment analysis with the FIDEA tool (D’Andrea et al., 2013) highlighted relevant categories that cooperate in pathways and distinct molecular functions. In particular, we noticed a striking enrichment for categories related to cell adhesion in genes differentially expressed in FUS<sup>P525L</sup> MNs (Figure 3A). Notably, “cell adhesion” is one of the categories consistently enriched in multiple studies focusing on FUS-bound transcripts and splice targets in mouse and human neurons (Otozco and Edbauer, 2013). GO term enrichment analysis of differentially expressed genes in laser-captured MNs from postmortem sporadic ALS patients also indicated “cell adhesion” (Batra et al., 2016). Moreover, functional enrichment analysis for genome-wide association studies involving several neurological diseases (including ALS) showed significant values for the “cell-cell adhesion” GO category (Guio-Vega and Forero, 2017). This category was also enriched in the analysis of differentially expressed genes in ALS fibroblasts carrying the C9ORF72 mutation (Kotni et al., 2016). Cell adhesion-related categories were enriched also when upregulated and downregulated genes were analyzed separately (Figure S4A). Interestingly, the Kyoto Enrichment of Genes and Genomes pathway “Neuroactive ligand-receptor interaction” was specifically enriched by the downregulated genes. Previous work found this pathway enriched in RNA profiling from C9ORF72 mutant fibroblasts (Kotni et al., 2016) and linked to neurological diseases such as Parkinson’s disease (Kong et al., 2015).

Seeking for ALS signature in FUS mutant MNs, we crossed our RNA-seq data with a recently published dataset from laser-captured MNs from sALS patients (Batra et al., 2016; Kapeli et al., 2016). In these works, the authors found 2,346 upregulated and 955 downregulated genes in sALS patients. The variation of upregulated genes was more robust, since more stringent selection criteria had lower impact on their abundance. These two lists of genes were employed as two distinct gene sets in the context of a gene set enrichment analysis to evaluate whether they were over-represented in the set of genes deregulated upon FUS mutation. Notably, the set of genes downregulated in sALS patients was enriched among those downregulated in FUS<sup>P525L</sup> (FDR = 0.151), and the set of genes upregulated in sALS patients was significantly enriched among those upregulated in FUS<sup>P525L</sup> (FDR = 0.009) (Figure S4B). These results suggest a common signature in gene expression between in-vitro-derived FUS<sup>P525L</sup> MNs and sALS MNs. As suggested by FDR values, this tendency is stronger for the upregulated genes, consistent with the robustness of their deregulation in sALS patients.

We next investigated whether there is any relationship between differential gene expression and direct FUS binding in mutant MNs. The FUS interactome has never been characterized in purified human MNs; however, FUS CLIP-seq (crosslinking and immunoprecipitation, followed by high-throughput sequencing) data from human brain cortex are available (Lagier-Tourenne et al., 2012). We reanalyzed this dataset and evaluated the position of FUS binding clusters relative to the different regions of the pre-mRNAs, which are expressed in our MNs. FUS binding was enriched in the exonic regions of protein-coding genes, and in particular in the 3′ UTR, when compared with sequences located more (distal introns) or less (proximal introns) than 500 bp from the nearest exon-intron junction (Figure 3B). FUS-bound genes were classified as differentially expressed or not in FUS<sup>P525L</sup> MNs, and individual mRNA regions were analyzed separately. A significantly higher percentage of genes modulated in FUS mutant MNs was directly bound by FUS in the distal intron and 3′ UTR (Figure 3C). When the same analysis was repeated with the TDP-43-bound genes dataset (Tollervey et al., 2011), we did not observe a significant enrichment.
for the 3' UTR set (Figure S4C). Taken together, these results suggest that mRNAs bound by FUS in the 3' UTR might be more susceptible to changes in FUS activity due to ALS mutations. We validated differential expression of selected FUS-bound mRNAs in mutant MNs (TAF7, TAF15, GRIN2A, PCDH10, and CRIM1) (Figures 2D and 3D).

To assess whether the changes in gene expression downstream of FUS ALS mutations are consistent with an animal
Figure 4. miR-375 Is Downregulated in FUS Mutant MNs

(A) Venn diagram showing the relations between differentially expressed miRNAs at different time points of MN maturation, as resulting from TaqMan array cards analysis in FUSWT and FUSP525L iPSC-derived MNs at day 12 and 12 + 7, and small RNA-seq at day 12 + 7 (p < 0.05).

(B) Validation of selected miRNAs by real-time qRT-PCR in MNs at day 12 + 7. Expression levels in FUSP525L and FUSP525L#2 are shown as relative to their respective isogenic FUSWT controls, set to a value of 1. Histogram bars represent the average of at least four independent experiments and error bars indicate the SD (Student’s t test; paired; two tails; *p < 0.05; ***p < 0.001; n.s., p > 0.05).

(C) Real-time qRT-PCR analysis of the indicated miRNAs in FUSWT undifferentiated iPSCs and MNs (day 12 of differentiation, unsorted). miR-302a and miR-367 are pluripotency miRNAs; miR-218 is an MN-enriched miRNA. For each miRNA, the sample with the highest

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model exhibiting ALS features, we crossed our data with the transcriptome profiling of a recently described mouse carrying targeted deletion of the PY-NLS (FusANLS/ANLS) (Scekic-Zahirovic et al., 2016). This mutation severely impairs the nuclear localization of FUS, mimicking the human PS25L mutation and other truncations of the protein C-terminal. Among differentially expressed genes, 17 were downregulated and 8 were upregulated in both mutant mouse brain and human MNs (Figures 3E and S4D). The overlap was statistically significant both for upregulated and downregulated gene lists (p value for hypergeometric test <0.05). Interestingly, mRNA levels of TAF15, a FUS family member mutated in a subset of fALS patients (Couthouis et al., 2011), were significantly increased in both mouse (Scekic-Zahirovic et al., 2016) and human (Figure 3D). TAF15 was among the transcripts directly bound by FUS (Lagier-Tourenne et al., 2012). These findings suggest that a possible crosstalk between FUS and TAF15, hypothesized in murine models, might be conserved in human.

**FUS<sup>PS25L</sup> Mutation Impairs miR-375 Biogenesis in MNs by a Loss-of-Function Mechanism**

Previous studies in cell lines have hypothesized a link between FUS mutations and the impairment of miRNA function (Emde et al., 2015; Morlando et al., 2012), but this was never directly investigated in human MNs. We performed miRNA profiling by small RNA sequencing (small RNA-seq) in FUS<sup>WT</sup> and FUS<sup>PS25L</sup> MNs. Based on miRNA expression levels, samples could be clustered according to their FUS genotype (Figures S5A–S5C). Of the 573 miRNAs expressed in human MNs (day 12 + 7), 21 resulted downregulated and 11 upregulated in FUS<sup>PS25L</sup> mutant cells (FDR < 0.05) (Figure S5D; Table S1). We also analyzed to which extent the change in the miRNAome is maintained at different time points. TaqMan array cards and small RNA-seq identified miR-375 and miR-484 as differentially expressed miRNAs at both day 12 and day 12 + 7 (Figure 4A; Table S2). These and other selected candidates were analyzed by real-time qRT-PCR in FUS<sup>PS25L</sup>, FUS<sup>PS25L</sup>#2, and FUS<sup>PS25L</sup>(ht) MNs, and their isogenic WT controls (Figures 4B, S5E, and S5F). Among them, miR-375 was consistently downregulated in both homozygous FUS mutant lines over at least four independent experiments (Figure 4B).

miR-375 has been recently described as a protective miRNA in MNs (Bhinge et al., 2016). Consistent with previous observations (Bhinge et al., 2016), miR-375 was highly upregulated in MNs compared with undifferentiated iPSCs (Figure 4C). To gain insights into the mechanisms underlying miR-375 downregulation in FUS<sup>PS25L</sup> MNs we transfected differentiating FUS<sup>WT</sup> iPSCs with small interfering RNAs (siRNAs) targeting FUS and analyzed gene expression at day 12 of differentiation. In these conditions, FUS levels were reduced to about 60% compared with scramble siRNA-transfected cells (Figure 4D). We have previously shown that FUS localization in the nucleus in homozygous FUS<sup>PS25L</sup> cells is reduced to 50% of its normal levels, due to partial delocalization in the cytoplasm (Lenzi et al., 2015). Therefore, the decrease of FUS achieved by siRNA in WT cells is expected to reproduce possible loss of nuclear function effects of the mutation. FUS reduction did not affect differentiation, as assessed by ISL1, CHAT, and TUJ1 marker analysis (Figure 4D). Levels of miR-375 were significantly reduced upon FUS knockdown (Figure 4D). Conversely, expression of selected genes among the most upregulated (NNAT, TSPYL5, and TAF15) or downregulated (CRLM1 and SLC17A8) ones in FUS<sup>PS25L</sup> MNs was not affected by FUS knockdown (Figure 4D).

These results suggest that ALS mutations in the FUS gene might impair miR-375 production via a nuclear loss-of-function mechanism, while gain of toxic functions of the mutated protein might underlie the alteration of protein-coding genes expression.

**miR-375 Target Genes in FUS Mutant MNs**

To identify miR-375 targets in human MNs, we crossed mRNA expression data from the RNA-seq with predicted (TargetScan; Agarwal et al., 2015) and previously validated targets (miRTarBase; Chou et al., 2016). Eight miRNAs upregulated in FUS<sup>PS25L</sup> MNs were also miR-375 predicted targets (Figures 5A; Table S3), including ELAVL4 (also known as HuD), which had been previously validated as a bona fide miR-375 target (Abdelmohsen et al., 2010). The increased expression of ELAVL4 and other predicted targets (PHLDA1, EBF3, B3GAT2) in mutant MNs was validated by real-time qPCR (Figure 5B). The ELAVL4 gene encodes for a neuron-specific RNA-binding protein (Bronicki and Jasmine, 2013). In mouse MNs, the transcript of the NRN1 (CPG15) gene is among known targets of ELAVL4, which promotes NRN1 mRNA accumulation by enhancing its stability (Aketen et al., 2011). In line with this, we detected increased levels of NRN1 in FUS<sup>PS25L</sup> MNs (Figure 5B).

We then noticed that the p53 gene (TP53) was a previously described target of miR-375 (Liu et al., 2013). TP53 mRNA was unchanged or slightly upregulated,
respectively, in FUS<sup>P525L</sup> and FUS<sup>P525L</sup>#2 MNs (Figure 5B). In both lines, we detected an increase of p53 protein by western blot (Figure 5C). Among miR-375 targets, we noticed that EBF3 and PHLDA1 (also known as TDAG51) have been previously involved in the induction of apoptosis (Gomes et al., 1999; Zhao et al., 2006).

To assess whether changes in target mRNA levels could be explained by differences in miRNA levels, we transfected synthetic miR-375 mimics in FUS<sup>P525L</sup> differentiated cells. We observed a significant decrease of ELAVL4 and TP53 mRNA levels compared with control-transfected cells (mimics-C) (Figure 5D). Since levels of EBF3 and PHLDA1 were not decreased, we hypothesize that other mechanisms might contribute to their upregulation in FUS mutant cells.

Collectively, these findings suggest that dysregulation of miR-375 in FUS mutant MNs may have an impact on RNA metabolism and survival due to aberrant increase of ELAVL4 and p53, respectively.

**DISCUSSION**

In this study we report the whole-transcriptome profile of in-vitro-derived human MNs with WT or mutant (P525L) FUS background. Our analysis was performed on two pairs of isogenic lines generated by gene editing. We therefore provide significant information on the changes in the transcriptome of the neuronal subtype primarily affected by ALS and due solely to the specific, ALS-linked, FUS<sup>P525L</sup> mutation. This represents a major advance beyond previous work, in which the transcriptome of mixed populations of progenitor cells (composed for the most part of non-MN cells and derived from non-isogenic FUS control/mutant iPSCs) was profiled by exon array (Ichiyangai et al., 2016).

We found that numerous differentially expressed genes belong to functional categories related to the cell adhesion pathway. Interestingly, this correlates with transcriptome analysis of postmortem laser-capture microdissected MNs...
from ALS patients, genome-wide association studies on several neurological diseases and differential gene expression in C9ORF72 mutant cells (Aronica et al., 2015; Batra et al., 2016; Guio-Vega and Forero, 2017; Kotni et al., 2016). In the light of our findings, cell adhesion-related pathways might be considered as biomarkers and/or therapeutic targets in FUS ALS patients.

We also crossed our data with the transcriptome analysis from a recently reported murine model (Scekic-Zahirovic et al., 2016). Among genes commonly upregulated in both species we noticed TAF15, which is mutated in some ALS patients (Couthouis et al., 2011). TAF15 is a member of the FET family of RNA-binding proteins and is closely related to FUS. Similarly to FUS and TDP-43, upregulation of WT TAF15 causes neurodegeneration in Drosophila (Couthouis et al., 2011). Notably, TAF15 levels were slightly increased in FUS null mice (Kino et al., 2015; Scekic-Zahirovic et al., 2016), but not in human MNs with reduced FUS levels (Figure 4D). This discrepancy might be explained by residual FUS protein in our RNAi experiment. A shift in TAF15 solubility and its localization into FUS-positive inclusions has been observed in frontotemporal lobar degeneration, but not in ALS (Neumann et al., 2011). Therefore, it remains unclear whether TAF15 upregulation in FUS mutant MNs contributes to ALS pathogenesis.

Changes in mRNA levels may be a direct consequence of the FUS mutation. Despite the fact that specific information on FUS-bound transcripts in human MNs is still missing, FUS CLIP-seq data from human brain cortex are available (Lagier-Tourenne et al., 2012). Our results suggest a possible direct effect of FUS mutations on those transcripts that are targeted by FUS on their 3’ UTR. Interestingly, levels of several transcripts were altered in the same direction in homozygous and heterozygous FUS(P525L) MNs. The P525L mutation is inherited in an autosomal dominant way in ALS patients, and homozygous individuals have never been reported. Unexpectedly, in spinal cord tissue from heterozygous FUS ALS patients a near absence of nuclear FUS was observed in MNs, suggesting that both mutant and WT FUS can be sequestered within cytoplasmic inclusions (Vance et al., 2013). As the nuclear fraction of FUS in homozygous and heterozygous P525L cells is, respectively, 80% and 50% (Lenzi et al., 2015), the homozygous mutant could be regarded as representative of a more advanced stage of the disease. Mechanistically, toxic functions of mutant FUS might underlie the alteration of at least a subset of mRNAs, as suggested by knockdown experiments.

FUS mutations might also affect gene expression indirectly, for instance by altering miRNA levels. We found that miR-375 levels were consistently lower in MNs derived from two independent FUS(P525L) lines. Bhänge et al. (2016) recently showed that miR-375 is enriched in human spinal MNs derived from embryonic stem cells. Interestingly, miR-375 overexpression protected MNs from DNA damage-induced apoptosis. Mechanistically, miR-375 may exert this function by targeting p53 (Bhänge et al., 2016; Liu et al., 2013). We show here that p53 and other pro-apoptotic miR-375 predicted targets are upregulated in FUS mutant MNs. Notably, spinal MNs of ALS patients have been shown to display higher levels of p53 protein, which could be involved in apoptosis-mediated neuronal death (Martin, 2000; Ranganathan and Bowser, 2010).

In FUS(P525L) MNs we observed 40%–50% reduction in miR-375 levels. This is strikingly similar to the situation observed by Bhänge et al. (2016) in SMA MNs, in which miR-375 levels were reduced to 40%. Therefore, miR-375 dysregulation represents a common feature between two different MN diseases. While the molecular basis of miR-375 downregulation in SMA MNs remains currently unknown, we propose that ALS mutations causing FUS exclusion from the nucleus impair miR-375 production via a loss-of-function mechanism.

In addition to p53-induced apoptosis, miR-375 dysregulation might have an impact on other crucial pathways. Its decrease in FUS(P525L) MNs is mirrored by an increase of its target ELAVL4, an RNA-binding protein playing multiple roles in neural development, function, and degeneration (Broniccki and Jasmin, 2013). ELAVL4 regulates the stability and/or localization of several target transcripts. We propose that FUS, through the miR-375/ELAVL4 axis, is hierarchically at the top of a regulatory network with a broader role in the RNA metabolism of MNs.

In conclusion, transcriptome analysis of isogenic FUS(WT) and FUS(P525L) in-vitro-derived human MNs provided an insight on target genes and pathways altered by ALS mutations in the cell type primarily affected by the disease.

**EXPERIMENTAL PROCEDURES**

**Maintenance and Differentiation of Human iPSCs**

Generation and maintenance of iPSC lines is described in Lenzi et al. (2015). The MN differentiation protocol has been modified from Hill et al. (2016). In brief, cells were differentiated in N2B27 medium supplemented with 1 µM all-trans retinoic acid (Sigma-Aldrich) and 1 µM SAG (Merck Millipore) for 12 days in the presence of 10 µM SB431542 and 100 nM LDN-193189 (both from Milltenyi Biotec) from day 0 to 6, and 5 µM DAPT and 4 µM SU-5402 (both from Sigma-Aldrich) from day 6 to 12. Cells were sorted at day 12 using a FACSAria III (BD Biosciences) and re-plated on poly-L-ornithine-coated dishes and laminin-coated dishes (both from Sigma-Aldrich) in Neural Medium. The Hb9-GFP reporter was stably integrated in the AAV8 locus, as described previously (Wainger et al., 2014).

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RNA and Protein Analysis
Total RNA, extracted with the Quick-RNA MiniPrep (Zymo Research) and retrotranscribed with SuperScript VILO (Thermo Fisher Scientific) or miScript II RT (QIAGEN), was analyzed by real-time qRT-PCR with SYBR Green PowerUP (Thermo Fisher Scientific) or SYBR Green PCR Master Mix (QIAGEN). For miRNAs 384 array, RNA retrotranscribed using the TaqMan MicroRNA RT Kit was analyzed using the TaqMan Human MicroRNA Array A (Thermo Fisher Scientific). Primers sequences are reported in Table S4. Western blot analysis was carried out using anti-p53 (fl-393; Santa Cruz) and anti-GAPDH (sc-32233) antibodies.

RNA-Seq and Bioinformatics Analysis
Total RNA was sequenced on an Illumina HiSeq 2500 sequencing system using a TruSeq Stranded Total RNA Library Prep Kit with Ribo-Zero treatment (Illunina) or the TruSeq Small RNA Library (Illumina) at the Institute of Applied Genomics (Udine, Italy). An average of about 21 million 125 base pairs long paired-end reads or 17 million 50 base pairs long single-end reads were produced for each sample for RNA-seq or small RNA-seq, respectively. Bioinformatics analysis is described in detail in the Supplemental Experimental Procedures.

RNA-seq raw data have been deposited at the GEO (GEO: GSE94888).

Immunostaining
Immunostaining was performed with anti-Islet-1/2 (1:50, 39.4D5; DSHB) primary antibody and anti-mouse Alexa Fluor 488 (1:250, Thermo Fisher Scientific) goat secondary antibody. Images were acquired with an inverted IX73 microscope (Olympus).

FUS Knockdown and miRNA Mimics Transfection
Differentiating FUSWT iPSCs were transfected at 8 and 10 days with 40 nM anti-FUS siRNAs (SI00070518, QIAGEN) or scramble siRNAs (1027281, QIAGEN). FUSP525L iPSCs, induced to differentiate into MNs for 14 days and seeded on Matrigel-coated plates, were transfected after 48 hr with 10 nM miR/vana miRNA mimics (miR-375-3p MIMAT0000728; Negative Control No. 1 Cat. 4464058; Thermo Fisher Scientific) goat secondary antibody. Images were acquired with an inverted IX73 microscope (Olympus).

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AUTHOR CONTRIBUTIONS
A.R. and R.D.S. conceived the project. R.D.S. generated Hb9::GFP iPSC lines, set up and optimized MN differentiation and isolation, and collected and analyzed RNA and protein samples. L.S. and V.A. contributed to iPSC culture and differentiation and RNA analysis. A.C. performed the bioinformatics analysis of the RNA-seq. G.P. set up and optimized the fluorescence-activated cell sorting analysis and sorting of MNs. V.d.T. acquired and analyzed microscopy images. A.R. and I.B. coordinated the work and wrote the paper.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures, five figures, and four tables and can be found with this article online at https://doi.org/10.1016/j.stemcr.2017.09.004.
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Supplemental Information

FUS Mutant Human Motoneurons Display Altered Transcriptome and microRNA Pathways with Implications for ALS Pathogenesis

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SUPPLEMENTAL INFORMATION

FUS mutant human motoneurons display altered transcriptome and microRNA pathways with implications for ALS pathogenesis

De Santis et al.

SUPPLEMENTAL FIGURES AND TABLES
Figure S1. MN differentiation and sorting. Related to Figure 1

(A) Schematic representation of the Hb9::GFP reporter system, inserted in the AAVS1 locus by gene editing with zinc-finger nucleases (ZFN). A specific break introduced by ZFN in the AAVS1 locus (arrow) is repaired by homologous recombination, introducing a puromycin resistance gene spliced in frame with AAVS1 (SA-PUROr) and a membrane-localized GFP (M-GFP) coding sequence under the control of the Hb9 promoter. pA indicates cleavage and polyadenylation signal site. (B) End-point PCR analysis on the genomic DNA of iPSCs before (FUSWT) or after (FUSWT+Hb9::GFP; FUSP525L+Hb9::GFP) transfection with AAVS1 ZFNs and Hb9::GFP reporter system. Primers annealed to the unmodified AAVS1 locus (top), to the reporter pA and the AAVS1 locus (outside of the sequence corresponding to the homology arm region; middle) or to an unrelated genomic locus as a control, G3BP (bottom). (C) Immunostaining analysis of ISL1/2 and TUJ1 markers of cells differentiated for 12 days as in Fig. 1A, dissociated, re-plated on laminin-coated dishes and cultured for 2 additional days in Neural Medium. DAPI was used to stain nuclei. Widefield images acquired at a Zeiss Axiovert A1 microscope with a 40X objective. Scalebar: 20µm. (D) GFP-positive and -negative cells were analyzed by FACS to check the purity of the sorted samples. Plot overlay show the purity of isolated populations compared to unsorted sample. (E) MDS plot showing clustering of GFP-positive and -negative samples based on log2-transformed CPM values obtained by RNA-Seq analysis. (F) Hierarchical clustering and Pearson correlation matrix of GFP-positive and -negative samples based on log2-transformed CPM values obtained by RNA-Seq analysis. (G) Heatmap representing RNA-Seq-derived expression levels of genes that are differentially expressed between GFP-positive and -negative cells collected after sorting (3 independent experiments). Plotted values correspond to mean-centered log2-transformed RPKM values. (H) Phase contrast images of cells sorted at day 12 for GFP expression and further cultured for 1 (left panel) and 7 (right panel) days on laminin-coated dishes. Scalebar: 50µm
Figure S2. FUS\textsuperscript{WT} and FUS\textsuperscript{P525L} iPSC generate MNs with comparable efficiency. Related to Figure 2

(A) Real-time qRT-PCR analysis of the expression of the indicated genes in purified MNs (day12+7). Expression levels in FUS\textsuperscript{P525L}, FUS\textsuperscript{P525L} #2 and FUS\textsuperscript{P525L} (ht) are shown as relative to their respective isogenic FUS\textsuperscript{WT} controls, set to a value of 1.
Histogram bars represent the average of at least 3 independent experiments and error bars indicate the standard deviation (Student’s t-test; paired; two tails; *** = p<0.001; p>0.05 for all other genes). *HB9, ISL1 and CHAT* are MN markers. *TUJ1* is a pan-neuronal marker and *HPRT* is a housekeeping control. (B) MDS plot showing clustering of FUS\textsuperscript{WT} and FUS\textsuperscript{P525L} samples based on log2-transformed CPM values obtained by RNA-Seq analysis. (C) Hierarchical clustering and Pearson correlation matrix of FUS\textsuperscript{WT} and FUS\textsuperscript{P525L} samples based on log2-transformed CPM values obtained by RNA-Seq analysis. (D) Table showing the expression levels (RPKM) of ALS associated genes in RNA-Seq analysis. LogFC indicates log2 fold change in FUS\textsuperscript{WT} vs FUS\textsuperscript{P525L} comparison.
Figure S3. Characterization of FUS$^{WT\#2}$ and FUS$^{P525L\#2}$ iPSC lines. Related to Figure 2

(A) Sequencing results from FUS$^{WT\#2}$ and FUS$^{P525L\#2}$ iPSC lines. FUS$^{P525L\#2}$ iPSCs were modified by TALEN-directed HDR with the strategy described in (Lenzi et al., 2015). The arrows indicate the targeted nucleotide in codon 525 (C, wild type; T, mutant).

(B) Immunostaining showing intracellular localization of WT and mutant FUS proteins in iPSCs. Scale bar: 10 µm.

(C) End-point PCR analysis on the genomic DNA of FUS$^{WT\#2}$ and FUS$^{P525L\#2}$ iPSC lines. iPSCs before transfection with AAVS1 ZFNs and Hb9::GFP reporter system are used as control (FUS$^{WT}$). Primers annealed to the unmodified AAVS1 locus (top), to the reporter pA and the AAVS1 locus (outside of the sequence corresponding to the homology arm region; middle) or to an unrelated genomic locus as a control, G3BP (bottom).

(D) Schematic representation of flow cytometry analysis of the fraction of GFP-positive cells at day 12. Histogram bars represent the average of 4 independent experiments and error bars indicate the standard deviation (Student’s t-test; unpaired; two-tails; n.s.= p>0.05).
**Figure S4**

**A**

Downregulated genes

- Morphogenesis
- Organ development
- Cell cycle
- Cell migration
- Homophilic cell adhesion
- Single-multicellular organism process
- Multicellular organism process
- Biological adhesion
- Nervous system development
- Anatomical structure development
- System development

Upregulated genes

- Developmental process
- Cell adhesion
- Multicellular organismal development
- Single-organism process
- System process
- Nervous system development
- Biological adhesion
- Multicellular organismal process
- Single-multicellular organism process
- Cell adhesion
- Homophilic cell adhesion
- Nervous system development
- Endocrine system development

**B**

- Downregulated in sALS MNs: 942
- 7 (overlap)
- 235 (unique)
- Downregulated in FUS\(^{Q578K}\) MNs: 2315
- 26 (overlap)
- 240 (unique)

**C**

**D**

- Downregulated concordant:
  - CHODL, CRIM1, CADM2, C6orf62, RSPO2, KCNK9, C11orf87, LG13, KCND2, IL13RA1, MTURN, BAALC, NXP1, PDE1C, GRK3, OXCT1, FGF13

- Downregulated discordant:
  - ZNF267, SULF1, TMAN4

- Upregulated concordant:
  - NPTX1, ASIC4, TAF15, NHLH2, UPF3B, PSPC1, ONECUT3, FLRT2

- Upregulated discordant:
  - SHISA6, NTS, KNCNP1, RYR3, ATP1B1, PPP2R2C, HOXB3, ARPP21, OPCML, FABP3
Figure S4. Bioinformatics analysis of differentially expressed genes in FUS<sup>WT</sup> and FUS<sup>P525L</sup> MNs. Related to Figure 3
(A) Word cloud generated by FIDEA representing GO Biological Process terms enriched in the list of genes that are downregulated (left panel) or upregulated (right panel) in FUS<sup>P525L</sup> MNs. The functional categories are represented with a character size proportional to the statistical significance of their enrichment. (B) Top: Venn diagrams showing the overlap between downregulated and upregulated genes in the present work and in MNs isolated by laser capture from sALS patients (Batra et al., 2016). Bottom: table of commonly upregulated and downregulated genes. (C) Fraction of TDP-43 bound protein-coding genes (identified by Tollervey et al., 2011) which are also differentially expressed genes in FUS mutant MNs. A gene was considered to be bound if a TDP-43 iCLIP peak was found within its body; the analysis was repeated focusing only on specific pre-mRNA regions (Fisher's exact test; *= p<0.05; ***=p<0.001). (D) Table showing the identity of the genes indicated in the Venn diagram of Figure 3E (overlap between differentially expressed genes in the present work and in a FUS mutant mouse; Seekie-Zahiروفic et al., 2016).
Figure S5. Small RNA-Seq analysis. Related to Figure 4
(A) MDS plot showing clustering of FUSWT and FUSP525L samples based on log2-transformed CPM values obtained by small RNA-Seq analysis. (B) The same plot was drawn after removing samples from one differentiation experiment to appreciate the pairing of samples originated from the remaining two experiments. (C) Hierarchical clustering and Pearson correlation matrix of FUSWT and FUSP525L samples based on log2-transformed CPM values obtained by small RNA-Seq analysis. (D) Heatmap representing small RNA-Seq-derived expression levels of miRNAs that are differentially expressed between FUSWT and FUSP525L iPSC-derived MNs at day 12+7 (3 independent experiments). Plotted values correspond to mean-centered log2-transformed CPM values. (E) Analysis of the expression of selected miRNAs in MNs at day 12+7 by real time qRT-PCR. Expression levels in FUSP525L and FUSP525L #2 are shown as relative to their respective isogenic FUSWT controls, set to a value of 1. Histogram bars represent the average of at least 3 independent experiments and error bars indicate the standard deviation (Student’s t-test; paired; two tails; * = p<0.05; ** = p<0.01; n.s. = p>0.05). (F) Analysis of the expression of miR-375 in MNs at day 12+7 by real time qRT-PCR. Expression levels in FUSP525L(ht) are shown as relative to its isogenic FUSWT control. Histogram bars represent the average of at least 3 independent experiments and error bars indicate the standard deviation (Student’s t-test; paired; two tails; n.s. = p>0.05).
| Groups                                      | Total | Elements          |
|--------------------------------------------|-------|-------------------|
| day 12; day 12+7; small-seq(p-value<0,05)  | 2     | miR-375           |
|                                            |       | miR-484           |
|                                            |       |                   |
| day 12+7; small-seq(p-value<0,05)          | 3     | miR-129-3p        |
|                                            |       | miR-125b-5p       |
|                                            |       | miR-139-5p        |
|                                            |       |                   |
| day 12; small-seq(p-value<0,05)            | 6     | miR-504-5p        |
|                                            |       | miR-10b-5p        |
|                                            |       | miR-149-5p        |
|                                            |       | miR-25-3p         |
|                                            |       | miR-192-5p        |
|                                            |       | miR-185-5p        |
|                                            |       |                   |
| day 12; day 12+7                           | 47    | miR-331           |
|                                            |       | miR-106a          |
|                                            |       | miR-574-3p        |
|                                            |       | miR-93            |
|                                            |       | miR-652           |
|                                            |       | miR-124a          |
|                                            |       | miR-486           |
|                                            |       | miR-193b          |
|                                            |       | miR-20a           |
|                                            |       | miR-532           |
|                                            |       | miR-627           |
|                                            |       | miR-483-5p        |
|                                            |       | miR-200c          |
|                                            |       | miR-16            |
|                                            |       | miR-216b          |
|                                            |       | miR-886-5p        |
|                                            |       | miR-26b           |
|                                            |       | miR-660           |
|                                            |       | miR-140-3p        |
|                                            |       | miR-31            |
|                                            |       | miR-454           |
|                                            |       | miR-30b           |
|                                            |       | miR-216a          |
|                                            |       | miR-330           |
|                                            |       | miR-218           |
|                                            |       | let-7g            |
| miRNA   | miRNA     | miRNA   | miRNA   | miRNA   |
|---------|-----------|---------|---------|---------|
| miR-133a | miR-186   | miR-365 | miR-92a | miR-191 |
| miR-184 | miR-10a   | miR-26a | miR-628-5p | miR-125a-5p |
| miR-28-3p | miR-146b | miR-708 | miR-886-3p | miR-20b |
| miR-296 | miR-422a | miR-425-5p | miR-103 | miR-95 |
| miR-103 | miR-598 |

Table S2. Differentially expressed miRNAs in FUS^{WT} vs FUS^{P525L} MNs (Taqman Array Cards). Related to Figure 4
| Name           | Sequence                              |
|----------------|---------------------------------------|
| AAVS1 FW       | CAGGGCCGTTAATGTGGCT                  |
| AAVS1 HB9 FW   | CACTCTCGGACAGGGACGAGCTG              |
| AAVS1 HB9 RV   | CCAAAAGGGCACCGCTGTAGACAG             |
| AAVS1 RV       | GGGTTAGACCCAATATCAGG                 |
| ATP1B1 FW      | AAGCCCACATATCAGGGACGAGAG            |
| ATP1B1 RV      | TGCTCATGTGCTTCGTGGGATCAT             |
| ATP50 FW       | ACTCGGTTTGACCTACAGC                  |
| ATP50 RV       | GTACTGAACATCGACCT                    |
| B3GAT2 FW      | TCTTCCAGGAGATGCGAACCAC              |
| B3GAT2 RV      | ACAACTTTGCCGTTTCCACCA               |
| CDH7 FW        | AGAAGCGCTGACACCCCTGTC               |
| CDH7 RV        | GGTTCCTCAGTCAGCCATGTC               |
| ChAT FW        | TCTAGCTACAAGGCGCCTGCT               |
| ChAT RV        | ACCAGGCTGTCCTGGGTATG                |
| CRIM1 FW       | TCGAACCCTGACAGCAATCCCTT             |
| CRIM1 RV       | AGAAGACTGGACTCCACTCCAGG             |
| EBF3 FW        | TCTACGAGGCGATGGGTATG                |
| EBF3 RV        | GTCTGAGGCGTTCGTGTC                  |
| ELAVL4 FW      | CAACCCACCAGCAAGGTGTC                |
| ELAVL4 RV      | AGCCTGAACCTCTGAGCTG                 |
| G3BP1 FW       | CCAGGATTGGGAGTGGGAAGGG              |
| G3BP1 RV       | CAGACTGGGTGATCGACTCCCA              |
| GAPDH FW       | GGAAGGGTGAAGTGTCGAGT                |
| GAPDH REV      | GTACCAGGCTTGAAGGCAGGCCCC            |
| GRIN2A FW      | GTTACGCTTGAAGAAGGGGAA               |
| GRIN2A RV      | TGTACCACTCCCCGATGGTCA              |
| HB9 FW         | GAGACCAGGTTGAAAGATTG                |
| HB9 RV         | CCTCTGTTTTCTCCGCCTTCC              |
| HPRT FW        | GCCCTGGCGTCGATGATTAGT               |
| HPRT RV        | GGCCCTCCATCTCTCCTACATA             |
| ISL-1 FW       | TACAAAGTACCCAGCCACC                 |
| ISL-1 RV       | GGAAGTTGAGAGGACATTGA                |
| LRNFN2 FW      | CCGGGTGAGATGCTACAGCCT               |
| LRNFN2 RV      | TGAGTGTGCTGCGGTCTGTACAT             |
| NANOG FW       | CCAAATTCTCCTGACCACGTAC              |
| NANOG RV       | CACGTTGTTTCCAAACAAAGAAA             |
| NNNAT FW       | TGCTCATCATCGGTGTCATCA               |
| NNNAT RV       | ACACGCTGATGCGCAGTCTTCT             |
| NRN1 FW        | GGGCTTTCGACTTGTGGTCTCA              |
| NRN1 RV        | ATCTCCAGTATGCGACACG                 |
| NXPH2 FW       | TCCCCCTGCGCTTTGGTAA                |
| NXPH2 RV       | CCAATGGCTCTGAAATCTGCTG             |
| PAX FW         | ATGTGTGAGTAAATTTAGCTTGGA            |
| PAX RV         | GCTTACAAACTTCTGGAGGTCGTA            |
| Oligonucleotide         | Sequence                                      |
|-------------------------|-----------------------------------------------|
| PHLDA1 FW               | CAGAGGGCAAGGAGATCGACTT                        |
| PHLDA1 RV               | GGCCTGACGATTCTTTGACTG                        |
| SHISA6 FW               | CGAGAAACAGTACGACCCGGA                        |
| SHISA6 RV               | AGCCCTGTGGATGTTCATCTTCC                      |
| SLC17A8 FW              | ACGCCGTGGGAGATTCTTTG                        |
| SLC17A8 RV              | TGAATGTAACGCTTGGGAGAGGAGCC                  |
| TAF15 FW                | GGTCACAGGGAGGAGGTAGA                        |
| TAF15 RV                | CCATAATCCCTGGACCCACCCAA                     |
| TAF7 FW                 | GGGATCGACTGGAATCGACCC                      |
| TAF7 RV                 | TCAAGCTTCTCATAACAGGAGGCA                    |
| TP53 FW                 | TGGCCATCTCAAGCAGCTCA                       |
| TP53 RV                 | CGGATAAGATGCTGAGGAGGAGG                     |
| TP53I3 FW               | CTCACCCTCTCCAGCTTT                         |
| TP53I3 RV               | CGGCTGAGTTGGCTAGCT                         |
| TSPYL5 FW               | G0CCCAATCCCTTGAGCT                         |
| TSPYL5 RV               | TGCTTTCGACCTTGCTCT                         |
| TUJ1 FW                 | CCCGGAACCATGGACAGGT                        |
| TUJ1 RV                 | TGACCCCCCTGAGCCACGG                        |
| Hs_SNORD25_11 miScript Primer Assay | (SNORD25 small nucleolar RNA, C/D box 25) |
| Hs_miR-125b_1 miScript Primer Assay | MIMAT000423: 5'UCCUGAGACCCAUACUUGUGA         |
| Hs_miR-10b_3 miScript Primer Assay | MIMAT000254: 5'UACCCUGUAGAACGAAUUGUGA       |
| Hs_miR-184_1 miScript Primer Assay | MIMAT000454: 5'UGGACGGAGAUCUACUGGAAGAGGU   |
| Hs_miR-186_1 miScript Primer Assay | MIMAT000456: 5'CAAGAUAUCUUCCUUGGCU          |
| Hs_miR-218_1 miScript Primer Assay | MIMAT000275: 5'UUGUCUUGACUACGAGGAUGAUGA     |
| Hs_miR-302a_2 miScript Primer Assay | MIMAT000684: 5'UAAAGCGCUUACGUGAAUGUGA      |
| Hs_miR-367_2 miScript Primer Assay | MIMAT000719: 5'AAUUGCCAUUAGCAGGCGUGA       |
| Hs_miR-375_2 miScript Primer Assay | MIMAT000728: 5'UUUGUUCGUGCACGCGGCGUGA     |
| Hs_miR-484_1 miScript Primer Assay | MIMAT0002174: 5'UCAGCGCUACAGUCCCCUGCGGAU |

Table S4. Oligonucleotides and probes used in this study. Related to Experimental Procedures.
SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Maintenance and differentiation of human iPSCs

Generation and validation of iPSC lines is described in Lenzi et al. (Lenzi et al., 2015). All iPSC lines were maintained in Nutristem-XF (Biological Industries, Kibbutz Beit-Haemek, Israel) in plates coated with hESC-qualified Matrigel (Corning Inc., Corning, NY) and passaged every 4-5 days with 1 mg/ml Dispase (Thermo Fisher Scientific, Waltham, MA). MN differentiation protocol has been modified from Hill et al. (Hill et al., 2016). iPSCs were dissociated to single cells with Accutase (Thermo Fisher Scientific) and counted. 9x10^5 cells were seeded onto each well of a 6-well Matrigel-coated plate in Nutristem-XF supplemented with 10 µM ROCK-inhibitor (Y-27632, Sigma-Aldrich, St. Louis, MO) to enhance survival upon dissociation. The ROCK-inhibitor was withdrawn the next day. After two days (day 0), cells reached confluence and medium was switched to N2B27 Medium, composed of 50% DMEM/F12 (Sigma-Aldrich) and 50% Neurobasal (Thermo Fisher Scientific) supplemented with 1X N2 and 1X B27, 1X Glutamax, 1X MEM Non-Essential Aminoacids (all from Thermo Fisher Scientific) and 100 U/ml Penicillin + 100 µg/ml Streptomycin (Sigma Aldrich). 10 µM SB431542 (Miltenyi Biotec, Bergisch Gladbach, Germany) and 100 nM LDN-193189 (Miltenyi Biotec) were added to the medium from day 0 to 6, while 5 µM DAPT (Sigma-Aldrich) and 4 µM SU-5402 (Sigma Aldrich) were added from day 6 to 12. 1 µM all-trans Retinoic Acid (Sigma-Aldrich) and 1 µM SAG (Merck Millipore, Billerica, MA) were added to the medium from day 0 to 12. Medium was replaced daily during the course of differentiation.

Cells were dissociated at day 12 with the Papain Dissociation System (Worthington Biochemical Corp., Lakewood, NJ) and single cell suspensions were prepared for isolation in sorting buffer (PBS without Ca^{2+}/Mg^{2+}; 2.5% Horse Serum; 0.4% Glucose; DNaseI; all from Sigma-Aldrich) containing 1X B27 Supplement (Thermo Fisher Scientific). After sorting, cells were re-plated on poly-L-ornithine (Sigma-Aldrich) and natural mouse laminin (Sigma-Aldrich) coated dishes in Neural Medium (N2B27 Medium supplemented with 20 ng/ml BDNF, 10 ng/ml GDNF, 10ng/ml CNTF, all from Peprotech, London, UK; 200 ng/ml L-ascorbic acid, Sigma-Aldrich; and ROCK-inhibitor for the first 24 hours). Neural Medium without ROCK-inhibitor was replaced every 3 days until collection of cells. The Hb9::GFP reporter, kindly provided by Dr. Kevin Eggan, was stably integrated in the AAVS1 locus by targeted Zinc Finger Nucleases (ZFNs) as previously described (Wainger et al., 2014). Briefly, AAVS1 ZFNs and the template construct containing the reporter and a puromycin resistance gene were co-transfected in iPSCs using the Neon Transfection System (Thermo Fisher Scientific) as described (Lenzi et al., 2015). After 48 hours, transfected cells were selected with 0.5 µg/ml puromycin and then clonally expanded.

Isolation of differentiated MNs by fluorescent-activated cell sorting (FACS)

Human MNs were sorted based on GFP expression from the Hb9::GFP reporter using a FACSAriaIII (BD Biosciences, San Jose, CA) equipped with a 488nm laser and FACSDiva software (BD Biosciences version 6.1.3). Data were analyzed using the FlowJo software (Tree Star, Ashland, OR). Briefly, cells first gated based on forward and side scatter area (FSC-A and SSC-A) plot were then detected in the green fluorescence channel for GFP expression (530/50nm filter) and collected as negative (GFP- ) and positive cells (GFP+). To reduce stress for the neurons, cells were isolated in gentle conditions using a ceramic nozzle of size 100µm, a low sheath pressure of 19.84 pound-force per square inch (psi) that maintains the sample pressure at 18.96 psi and an acquisition rate of 3000 events/s. According to the different use, cells were collected in 5 and/or 15 ml polystyrene or polypropylene tubes. Following isolation, an aliquot of each tube of the sorted cells was evaluated for purity resulting in an enrichment >98-99% for each sample.

To check the course of differentiation, an aliquot of cells was acquired starting from day 6 every 2 days at the flow cytometer LSRFortessa (BD Biosciences), equipped with a 488nm laser, to evaluate GFP expression levels.

Real-time qRT-PCR and western blot analysis

Total RNA was extracted with the Quick RNA MiniPrep (Zymo Research, Irvine, CA) and retrotranscribed with SuperScript VILO (Thermo Fisher Scientific) or miScript II RT (Qiagen, Venlo, Netherlands). Real-time qRT-PCR analysis was performed with SYBR Green Power-UP (Thermo Fisher Scientific) or SYBR Green PCR Master Mix (Qiagen) in a ViiA7 Real Time PCR System (Thermo Fisher Scientific) and calculations performed with the delta delta Ct method. The internal control for mRNA analysis is the housekeeping gene ATP5O (ATP synthase, H+ transporting, mitochondrial F1 complex, O subunit). For miRNA analysis internal control is U25 (SNORD_25). Primers sequences are reported in Table S4.

Western blot analysis of p53 protein levels was carried out with NuPAGE 4-12% Bis-Tris gels (Thermo Fisher Scientific) in MOPS-SDS buffer, using anti-p53 (fl-393; Santa Cruz, Dallas, TX) and, as a loading control, anti-GAPDH (sc-32233; Santa Cruz) antibodies. Images were acquired with the Chemidoc MP (BioRad, Hercules, CA).

miRNAs 384 array
200ng total RNA from MNs was retrotranscribed using the TaqMan MicroRNA RT Kit (Thermo Fisher Scientific). The real-time detection of the miRNA levels was performed using the TaqMan® Human MicroRNA Array A (Thermo Fisher Scientific), according to manufacturer's instructions, in a ViiA7 Real Time PCR System (Thermo Fisher Scientific). The values obtained were normalized for snoRNA-U44.

**RNA sequencing and bioinformatics analysis**

Total RNA was extracted from iPSC-derived MNs (GFP-negative and positive with a FUS\(^{WT}\) genotype, day12; FUS\(^{WT}\) and FUS\(^{P525L}\), day 12+7) and sequenced on an Illumina Hiseq 2500 Sequencing system using TruSeq Stranded Total RNA Library Prep Kit with Ribo-Zero treatment (Illumina, San Diego, CA) at the Institute of Applied Genomics (IGA; Udine, Italy). An average of about 21 million 125 base pairs long paired-end reads were produced for each sample.

RNA-Seq reads were initially trimmed using the Trimmomatic software (Bolger et al., 2014) to remove adapter sequences and poor quality bases; the minimum read length after trimming was set to 18. After that, Bowtie 2 (Langmead and Salzberg, 2012) was used to align reads to human RNA sequences; reads mapping to these sequences were filtered out. In order to calculate the distribution of the inner distance between mate pairs, reads were aligned to a non-redundant set of human RNA sequences derived from Ensembl 77 gene annotation (Flicek et al., 2014) using BWA software (H. Li and Durbin, 2010); this mapper was chosen because of its ability to automatically infer the insert size. Mean and variance of inner distance distribution were estimated from aligned read pairs whose inner distance was within interval \([Q1-2(Q3-Q1),Q3+2(Q3-Q1)]\) \((Q1=first quartile, Q3=third quartile), the same as used by BWA to estimate the insert size. TopHat2 (Kim et al., 2013) was employed to align reads to GRCh38 human genome and Ensembl transcriptome using parameters \(-i\ 50\,-r\ 32\,-mate-std-dev\ 58\,-library-type\ fr\-firststrand\). Read counts for Ensembl 77 human genes were calculated using htsq-cound software (Anders et al., 2015), specifying the intersection-strict option. Number of reads and mapping statistics are reported in Table S1. edgeR software (Robinson et al., 2010) was used to find differentially expressed genes in GFP-positive vs GFP-negative and FUS\(^{WT}\) vs FUS\(^{P525L}\) comparisons. In both cases, TMM normalization was applied to read counts after filtering genes with a CPM (Count Per Million) value less than 1 in at least three (in GFP-positive vs GFP-negative comparison) or four (in FUS\(^{WT}\) vs FUS\(^{P525L}\) comparisons) samples. MDS plots and Pearson correlation-based sample correlation matrices were drawn based on log2-transformed normalized read counts. For FUS\(^{WT}\) vs FUS\(^{P525L}\) comparison, samples originating from the same differentiation experiment showed some degree of pairing and this was taken into account when performing differential expression analysis. An additive model was fitted to adjust for baseline differences between the three independent differentiation experiments: design <- model.matrix (~ experiment + condition). Model fitting and testing was performed using glmFIT and glmLRT functions. Heatmaps of differentially expressed genes were plotted based on mean-centered log2-transformed RPKM values, calculated using rpkm function.

Functional enrichment analysis of genes deregulated upon FUS mutation was performed by providing the list of differentially expressed protein-coding genes to FIDEA Web Server (D'Andrea et al., 2013), using the list of protein-coding genes that passed the CPM-based filter as background. GSEA analysis was performed using GSEA software (Hill et al., 2016; Subramanian et al., 2005), with the permutation type parameter set to gene set.

Cufflinks software (Trapnell et al., 2010) was employed to evaluate transcript-level expression in FUS\(^{WT}\) and FUS\(^{P525L}\) conditions, using parameters --compatible-hits-norm --max-frag-multihits 1. Transcripts with FPKM > 0.1 and belonging to genes with FPKM > 0.5 in at least three samples were flagged as expressed.

**CLIP-Seq data reanalysis**

Raw reads from FUS HITS-CLIP experiment conducted by Lagier-Tourenne and coworkers (Lagier-Tourenne et al., 2012) on human brain cortex were downloaded from GEO. First, adapter and quality trimming of reads was performed using Trimmomatic; Cutadapt was then used to remove all the adapter sequences that were not trimmed in the first phase. Trimmed reads were aligned to GRCh38 using Bowtie (Langmead et al., 2009) with parameters -a -m 1 --best --strata. Duplicate reads, which could represent PCR artifacts, were removed using MarkDuplicates from Picard (picard.sourceforge.net/command-line-overview.shtml). Tools from the Pyicoteo suite (Althammer et al., 2011) were used to call CLIP-Seq peaks. First, all reads were extended to a length of 36 nucleotides using the pyicos extend tool. Then, CLIP-Seq peak calling was performed using the picoclip tool. Ensembl 77 GTF file was supplied to generate exploratory regions, using the option --region-magic genebody. Only peaks with p-value < 0.001 were retained. Colocalization of FUS CLIP-Seq peaks with different regions of expressed protein-coding transcripts was evaluated as follows: each protein-coding locus was divided into coding sequence (CDS), 5' UTR, 3'UTR, proximal and distal intron regions (the latter being defined as the intronic regions which are more than 500 bp far from the nearest exon-intron boundary), giving priority to CDS over the other regions; then each peak was assigned a region based on the position of the peak summit using the BEDTools software suite (http://bedtools.readthedocs.io/). Enrichment of peaks in each region relative to region size was computed as described (Kapeli et al., 2016). For the analysis of differential FUS binding between deregulated and non-deregulated genes, bound genes were defined as those hosting at least one FUS CLIP-Seq peak, either within the entire gene body or in a specific pre-mRNA region.
TDP-43 binding sites obtained by iCLIP in human brain (Tollervey et al., 2011) were downloaded from starBase v2.0 Web Server (J.-H. Li et al., 2014). hg19 coordinates were converted to GRCh38 coordinates using the liftOver tool (The UCSC Genome Browser Database: update 2006). The center of each iCLIP cluster was used to assign it to a pre-mRNA region.

Small RNA-Seq
Total RNA was extracted from iPSC-derived MNs (FUS\textsuperscript{WT} and FUS\textsuperscript{P525L}, day 12+7) and sequenced on a Illumina Hiseq 2500 Sequencing system using TruSeq Small RNA Library (Illumina) at the Institute of Applied Genomics (IGA; Udine, Italy). An average of about 17 million 50 base pairs long single-end reads were produced for each sample. mirPRo software (Shi et al., 2015; Subramanian et al., 2005) was used to remove adapter sequences, map reads to human pre-miRNA sequences downloaded from mirBase v21 (Kozomara and Griffiths-Jones, 2014) using NovoAlign (H. Li and Homer, 2010), and calculate read counts for mature miRNAs. Number of reads and mapping statistics are reported in Table S1. Starting from the raw counts, sample clustering and differential expression of mature miRNAs were performed using the same procedure adopted for FUS\textsuperscript{WT} vs FUS\textsuperscript{P525L} comparison. Samples originating from the same differentiation batch were mildly correlated, so we opted for a paired design when performing differential expression analysis. The heatmap of deregulated mature miRNAs was plotted based on mean-centered log2-transformed CPM values.

Immunostaining
MN were re-plated on matrigel-coated dishes and cultured for additional 3 days (day12+3), then were fixed in 2% paraformaldehyde for 15 minutes at room temperature and washed twice with PBS. Fixed cells were then permeabilized with PBS containing 1% BSA and 0.2% Triton X-100 and incubated 1h with primary antibody anti-Islet-1/2 (1:50, 39.4D5 DSHB, Iowa City, Iowa) and TUJ1 (1:200; T2200; Sigma-Aldrich) at room temperature. The secondary antibodies are goat anti-mouse Alexa Fluor 488 (1:250, Thermo Fisher Scientific) and goat anti-rabbit Cy3 (1:250, Jackson ImmunoResearch, Suffolk, UK). DAPI (Sigma-Aldrich) was used to stain nuclei. Fluorescent images were acquired using a 20X LWD objective mounted on an inverted ix73 microscope (both Olympus, Tokyo, Japan) with Lumencor Spectra X LED illumination, a CoolSNAP MYO CCD camera (Photometrics, Tucson, AZ), and MetaMorph software (Molecular Devices, Sunnyvale, CA).

iPSCs were fixed in 4% paraformaldehyde for 15 minutes at room temperature and washed twice with PBS. Fixed cells were then permeabilized with PBS containing 1% BSA and 0.2% Triton X-100 and incubated with the primary antibody anti-FUS/TLS (4H11) (1:100; SC-47711; Santa Cruz Biotechnologies) for 1 hour at room temperature. The secondary antibody is goat anti-mouse Alexa Fluor 488 (1:250, Thermo Fisher Scientific). DAPI (Sigma-Aldrich) was used to stain nuclei. Confocal images were acquired at the Olympus iX83 FluoView1200 laser scanning confocal microscope using a UPLSAPO60xO, NA 1.35 oil objective. The standard setting for DAPI and Alexa Fluor 488 emission and 405nm and 473nm laser for excitation were used. Images were acquired at 800x800 pixel, 4x zoom, line scan sequential mode.

FUS knockdown
For FUS knockdown experiments, FUS\textsuperscript{WT} iPSCs were induced to differentiate and transfected at 8 and 10 days with 40 nM anti-FUS siRNAs (Hs_FUS_4 FlexiTube siRNA, SI00070518, Qiagen) or scramble control siRNAs (AllStars Neg. Control 414 siRNA, 1027281, Qiagen) using Lipofectamine RNAi-Max (Thermo Fisher Scientific), according to manufacturer’s instructions.

miRNA mimics transfection
FUS\textsuperscript{P525L} iPSCs were induced to differentiate into MNs for 14 days. After dissociation with Accutase (Thermo Fisher Scientific), cells were seeded on Matrigel-coated plates and then transfected after 48 hours with 10nM mirVana miRNA mimics (Thermo Fisher Scientific) (microRNA-375-3p MIMAT0000728; mirRNA Mimic Negative Control#1 Cat. 4464058) using Lipofectamine 2000 (Thermo Fisher Scientific), according to manufacturer’s instructions. RNA was collected for real-time qRT-PCR analysis after 48 hours.
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