The SYNGAP1 3′UTR Variant in ALS Patients Causes Aberrant SYNGAP1 Splicing and Dendritic Spine Loss by Recruiting HNRNPK

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Fused in sarcoma (FUS) is a pathogenic RNA-binding protein in amyotrophic lateral sclerosis (ALS). We previously reported that FUS stabilizes Synaptic Ras-GTPase activating protein 1 (Syngap1) mRNA at its 3′ untranslated region (UTR) and maintains spine maturation. To elucidate the pathologic roles of this mechanism in ALS patients, we identified the SYNGAP1 3′UTR variant rs149438267 in seven (four males and three females) out of 807 ALS patients at the FUS binding site from a multicenter cohort in Japan. Human-induced pluripotent stem cell (hiPSC)-derived motor neurons with the SYNGAP1 variant showed aberrant splicing, increased isoform α1 levels, and decreased isoform γ levels, which caused dendritic spine loss. Moreover, the SYNGAP1 variant excessively recruited FUS and heterogeneous nuclear ribonucleoprotein K (HNRNPK), and antisense oligonucleotides (ASOs) blocking HNRNPK altered aberrant splicing and ameliorated dendritic spine loss. These data suggest that excessive recruitment of RNA-binding proteins, especially HNRNPK, as well as changes in SYNGAP1 isoforms, are crucial for spine formation in motor neurons.

Key words: amyotrophic lateral sclerosis; antisense oligonucleotides; dendritic spine; hnRNPK; iPSC-derived motor neuron; SYNGAP1
Significance Statement

It is not yet known which RNAs cause the pathogenesis of amyotrophic lateral sclerosis (ALS). We previously reported that Fused in sarcoma (FUS), a pathogenic RNA-binding protein in ALS, stabilizes synaptic Ras-GTPase activating protein 1 (Syngap1) mRNA at its 3′ untranslated region (UTR) and maintains dendritic spine maturation. To elucidate whether this mechanism is crucial for ALS, we identified the SYNGAP1 3′UTR variant rs149438267 at the FUS binding site. Human-induced pluripotent stem cell (hiPSC)-derived motor neurons with the SYNGAP1 variant showed aberrant splicing, which caused dendritic spine loss along with excessive recruitment of FUS and heterogeneous nuclear ribonucleoprotein K (HNRNPK). Our findings that dendritic spine loss is because of excess recruitment of RNA-binding proteins provide a basis for the future exploration of ALS-related RNA-binding proteins.

Introduction

Fused in sarcoma (FUS) is one of the major causative genes for amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD; Kwiatkowski et al., 2009; Neumann et al., 2009). The key pathologic findings of ALS-FUS or ALS/FTLD are the cytosolic mislocalization and aggregate formations of FUS (Neumann et al., 2009; Mackenzie et al., 2011), suggesting either the gain-of-toxic function (Sharma et al., 2016; López-Erauskin et al., 2018) or loss-of-function (Ishigaki et al., 2018; Humphrey et al., 2020) of FUS. Abnormal liquid–liquid phase separation of FUS is also reported to be involved in the potential pathogenesis of FUS aggregation (Hofweber et al., 2018; Niaki et al., 2020).

FUS is an RNA-binding protein that is involved in various RNA metabolisms, including alternative splicing (Ishigaki et al., 2012; Lagier-Tourenne et al., 2012), mRNA stability (Kapeli et al., 2016), transcription (Tan and Manley, 2010; Masuda et al., 2015), mRNA transport (Fujii et al., 2005), and translation (Yasuda et al., 2013; Kamelgarn et al., 2018). FUS binds to the 3′ untranslated region (UTR) of its target mRNAs (Ishigaki et al., 2012; Lagier-Tourenne et al., 2012; Nakaya et al., 2013) and post-transcriptionally regulates mRNA expression (Udagawa et al., 2015; Yokoi et al., 2017; Akiyama et al., 2019; Garone et al., 2020). While aberrant RNA metabolism of FUS has been suggested to correlate with the pathogenesis of ALS/FTLD, it is still not clear which specific RNA pathway might directly cause ALS/FTLD.

Synaptic dysfunction is considered an initial pathologic change in ALS and various neurodegenerative diseases (Herms and Dorostkar, 2016; Henstridge et al., 2018). Importantly, FUS is known to be involved in synaptic function (Fujii et al., 2005; Qiu et al., 2014; Deshpande et al., 2019). We have investigated the relationship between FUS and synaptic function and found that FUS regulates the mRNA stability of Glutamate ionotropic receptor AMPA type subunit 1 (Gria1; Udagawa et al., 2015) and Synaptic Ras-GTPase activating protein 1 (Syngap1; Yokoi et al., 2017) at their 3′UTR, thus maintaining spine morphology and cognitive function. SYNGAP1 is a pathogenic gene for intellectual disability including mental retardation, epilepsy, and autism spectrum disorders (Mignot et al., 2016), and SYNGAP1 is a major protein located at the postsynaptic density and negatively regulates the Ras/Rap pathway (Kim et al., 1998; Jeyabalan and Clement, 2016; Walkup et al., 2016). Syngap1 knock-out mice exhibit an increased number of mature spines (Kim et al., 2003; Clement et al., 2012). SYNGAP1 has four isoforms that exert different synaptic functions and are defined by their C terminals: α1, α2, β, and γ (McMahon et al., 2012; Araki et al., 2020). For instance, the isoform α1 decreases synaptic strength, while α2 increases synaptic strength (McMahon et al., 2012). We previously reported that FUS specifically binds to the long 3′UTR of Syngap1, which is mainly connected to the Syngap1 isoform α2 mRNA involved in strengthening synaptic function (Yokoi et al., 2017). Although these findings indicate that FUS-mediated SYNGAP1 mRNA regulation is important for spine maturation in mice, its involvement in the pathogenesis of ALS/FTLD has never been described.

Therefore, we explored the whole exome sequencing data of the Japanese Consortium for Amyotrophic Lateral Sclerosis Research (JaCALS; a multicenter ALS cohort in Japan; Hayashi et al., 2020; Nakamura et al., 2021). We searched for a causative variant in the SYNGAP1 3′UTR binding site of FUS that might induce the aberrant binding properties of FUS, which cause aberrant SYNGAP1 mRNA metabolism and synaptic dysfunction. As the SYNGAP1 3′UTR binding site of FUS in humans is different from that in mice (Nakaya et al., 2013), analyses using human-derived samples are mandatory for investigating the pathogenesis of the variant. Thus, the isogenic model of human-induced pluripotent stem cell (iPSC)-derived motor neurons were used to validate the pathogenic mechanism of the novel SYNGAP1 variant in human ALS patients.

Materials and Methods

Study approval

The experiments using information and material related to patients with the SYNGAP1 variant were approved by the ethics committee of Nagoya University Graduate School of Medicine (2004-0281). All the experimental procedures for the production and use of iPSCs were approved by the ethics committee of the Aichi Medical University School of Medicine (approval number 14-004, 2020-213).

Genetic analysis

We performed whole exome sequencing in 807 patients with sporadic ALS and in 191 normal controls who had previously participated in the Japanese Consortium for Amyotrophic Lateral Sclerosis Research (JaCALS), which consists of 32 neurologic facilities. The details of JaCALS have been described elsewhere (Hayashi et al., 2020).

Genomic DNA was extracted from peripheral blood leukocytes. The exomes of patients with sporadic ALS were captured with the SureSelect Human All Exon V5+UTR or V6+UTR (Agilent Technologies). The libraries were indexed, pooled, and sequenced on an Illumina HiSeq 2000 Sequencer (paired-end, 100 base reads). The reads were aligned to a human reference genome (UCSC hg19) using BWA 0.6.2. The Picard tools 1.73 software was used to remove duplicate reads. Variants and insertions/deletions were identified with GATK 1.6–13 and filtered to the coordinates with variant quality score recalibration. NM_006772 (RefSeq) was referred to identify the 3′UTR region of the SYNGAP1 gene, and variants within the FUS binding sites of the SYNGAP1 3′UTR were then selected. According to the CLIP-seq data of FUS in the human brain (Nakaya et al., 2013), the FUS binding sites were defined as those identified in multiple patients. The SYNGAP1 3′UTR variants (rs149438267) found in four males and three females were validated.
using Sanger sequencing. The sequence variants were validated by sequencing in both directions. There were no known pathogenic mutations for ALS in patients with the SYNGAP1 3'UTR variant. The sequencing details have been previously described (Nakamura et al., 2021).

**hiPSC culture and differentiation in vitro**

The experiments were performed as described previously (Shimojo et al., 2015; Onodera et al., 2020; Okada et al., 2021). 20B7 cells (a gift from Shinya Yamanaka) were maintained on mitomycin-C-treated murin fibroblast STO cell line transformed with neomycin resistance and murine LIF genes (SNL) feeder cells in 0.1% gelatin-coated tissue culture dishes in human embryonic stem cell (hESC) medium, and were used to induce motor neurons. For differentiation, the SNL feeder cells were first removed. Then, hiPSC colonies were detached using a dissociation solution containing 0.25% trypsin, 100 μg/ml Collagenase IV, 1 μM CaCl₂, and 20% knock-out serum replacement (KSR) medium, and were cultured in suspension in bacteriologic dishes in standard hESC medium.

On day 1, the medium was changed to human embryoid body (hEB) medium containing DMEM/F-12, 5% KSR, 2 mM L-glutamine, 1% non-essential amino acids solution (NEAA) and 0.1% 2-mercaptoethanol with 300 mM LDN-193189, 3 μM SB 431542, and 3 μM CHIR 99021. On day 2, the medium was changed to fresh hEB medium containing 300 mM LDN-193189, 3 μM SB431542, 3 μM CHIR99021, and 1 μM retinoic acid. On days 4–14, hEBs were cultured in hEB medium containing 1 μM retinoic acid and 1 μM purmorphamine; the medium was changed every 2–3 d. On day 14, hEBs were dissociated into single cells using TrypLE Select. The dissociated cells were plated on dishes coated with growth factor reduced Matrigel at a density of 1 × 10⁵ cells/cm², and were cultured in motor neuron medium consisting of KMB Neural Stem Cell medium supplemented with 2% B27 supplement, 1% CultureOne supplement, 1% NEAA, 50 mM retinoic acid, 500 mM purmorphamine, 10 μM cyclic AMP, 10 ng/ml recombinant brain-derived neurotrophic factor (BDNF), 10 ng/ml recombinant glial cell line derived neurotrophic factor (GDNF), 10 ng/ml recombinant human insulin-like growth factor-1 (IGF-1), and 200 ng/ml L-ascorbic acid for up to four weeks. Half of the medium was changed every 2–3 d.

**Scarless genome editing in iPSCs**

The donor plasmid (Yoshimatsu et al., 2019) was generated for scarless genome editing. The TTA site in SYNGAP1 3’UTR was selected as the center of the 5’ and 3’ arm. The primers for cloning the SYNGAP1 3’UTR sequence are listed in Table 1. The plasmids pENTR-L3-PBL-R1 (for the 3’ arm) and pENTR-R2-PBB-L4 (for the 3’ arm) were linearized by HpaI and ligated with the cloned sequences by Gibson’s assembly. To construct the donor plasmid, Multisite Gateway LR cloning was performed with the generated vectors with the arms as follows: pENTR-L1-PGK-PuroTK-L2 and pUC-DEST-R3R4. To generate single-guide RNAs (sgRNAs) targeting the SYNGAP1 variant, the Guide-it sgRNA In Vitro Transcription kit (Takara) was used according to the manufacturer’s instructions. We generated sgRNA targeting AAGAGAGGGCAAGACCCCATG to generate the homozygous mutation and CCCCTTTTTCTTCCCCATGCC to generate the heterozygous mutation. Y-27632 (10 μM) and SB431542, 3 μM CHIR99021, and 1 μM recombinant human LIF genes (SNL) feeder cells in 0.1% gelatin-coated tissue culture dishes in human embryonic stem cell (hESC) medium, and were used to induce motor neurons. For differentiation, the SNL feeder cells were first removed. Then, hiPSC colonies were detached using a dissociation solution containing 0.25% trypsin, 100 μg/ml Collagenase IV, 1 μM CaCl₂, and 20% knock-out serum replacement (KSR) medium, and were cultured in suspension in bacteriologic dishes in standard hESC medium. On day 1, the medium was changed to human embryoid body (hEB) medium containing DMEM/F-12, 5% KSR, 2 mM L-glutamine, 1% non-essential amino acids solution (NEAA) and 0.1% 2-mercaptoethanol with 300 mM LDN-193189, 3 μM SB 431542, and 3 μM CHIR 99021. On day 2, the medium was changed to fresh hEB medium containing 300 mM LDN-193189, 3 μM SB431542, 3 μM CHIR99021, and 1 μM retinoic acid. On days 4–14, hEBs were cultured in hEB medium containing 1 μM retinoic acid and 1 μM purmorphamine; the medium was changed every 2–3 d. On day 14, hEBs were dissociated into single cells using TrypLE Select. The dissociated cells were plated on dishes coated with growth factor reduced Matrigel at a density of 1 × 10⁵ cells/cm², and were cultured in motor neuron medium consisting of KMB Neural Stem Cell medium supplemented with 2% B27 supplement, 1% CultureOne supplement, 1% NEAA, 50 mM retinoic acid, 500 mM purmorphamine, 10 μM cyclic AMP, 10 ng/ml recombinant brain-derived neurotrophic factor (BDNF), 10 ng/ml recombinant glial cell line derived neurotrophic factor (GDNF), 10 ng/ml recombinant human insulin-like growth factor-1 (IGF-1), and 200 ng/ml L-ascorbic acid for up to four weeks. Half of the medium was changed every 2–3 d.
Quantitative analysis of immunofluorescence data

The immunofluorescent signal intensity was measured by ZEN software (Zeiss). The quantification of spine number was performed as described previously (Yokoi et al., 2017). Regions of dendrites located 30–100 μm from the cell soma that did not intersect with other dendrites were selected. The numbers of spines, labeled with Alexa Fluor 488-conjugated phalloidin, were counted over a region of 20 μm in length. The number of spines was counted independently, without sample information. Synaptophysin-positive F-actin particles were detected using Zen software. The differentiation efficacy of iPSC-derived motor neurons...
was calculated by ImageJ. The intensities were calculated, and cells with intensities above the same threshold were considered positive.

**3′ RACE and TA-cloning**

To identify the SYNAP1 3′UTR variants, we used the SMARTer RACE 5′/3′ kit (Takara) according to the manufacturer’s instructions. The primer used in the assay was 5′-CTGTCCCCAGGAAAGAACCACGCAA-3′.

The PCR products were inserted into the pRACE vector within the kit, and the sequences were analyzed.

**Immunocytochemistry and F-actin staining of neuronal cultures**

Cultured motor neurons were immunostained as described previously (Yokoi et al., 2017), with some modifications. Samples were fixed with 4% paraformaldehyde at 37°C for 10 min, washed with PBS twice, and...
Table 2. The allele frequency of the SYNAP1 variant rs149438267

<table>
<thead>
<tr>
<th>Population</th>
<th>Allele count</th>
<th>Frequency of G/T allele</th>
</tr>
</thead>
<tbody>
<tr>
<td>JaCALS patient group</td>
<td>1614</td>
<td>0.00434*</td>
</tr>
<tr>
<td>JaCALS control group</td>
<td>382</td>
<td>0</td>
</tr>
<tr>
<td>ToMMo8.3KJPN</td>
<td>16,760</td>
<td>0.0017</td>
</tr>
<tr>
<td>GEM-JWGA</td>
<td>14,452</td>
<td>0.0017</td>
</tr>
<tr>
<td>ToPMeD (phase 3)</td>
<td>125,568</td>
<td>0.00014</td>
</tr>
<tr>
<td>gnomAD (v2.1.1)</td>
<td>31,136</td>
<td>0.000032</td>
</tr>
<tr>
<td>European (non-Finnish)</td>
<td>15,280</td>
<td>0.000065</td>
</tr>
<tr>
<td>African</td>
<td>8634</td>
<td>0</td>
</tr>
<tr>
<td>Latino</td>
<td>846</td>
<td>0</td>
</tr>
<tr>
<td>Ashkenazi Jewish</td>
<td>290</td>
<td></td>
</tr>
<tr>
<td>East Asian</td>
<td>1552</td>
<td></td>
</tr>
<tr>
<td>European (Finnish)</td>
<td>3458</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>1076</td>
<td></td>
</tr>
</tbody>
</table>

* p < 0.05 (p = 0.037, compared with ToMMo 8.3KJPN; p = 0.036, compared with GEM-J WGA, Fisher’s exact test).
** All alleles are heterozygotes.
\* The filter status of GEM-J WGA is not high confident region.

Results

The SYNAP1 3’UTR variant at the FUS binding site was identified in the Japanese ALS cohort

First, we referred to the CLIP-seq data of FUS in the human brain (Nakaya et al., 2013) for the FUS binding sites of human SYNAP1 3’UTR. We found that most of the FUS CLIP tags were located in relative close proximity to exon 19, the 5’ side of 3’UTR (Extended Data Fig. 1A-1A), unlike the FUS CLIP tags in mice that are mostly located at the distant side to the exons at the 3’ side of 3’UTR (Nakaya et al., 2013; Yokoi et al., 2017). This indicates that FUS regulation of SYNAP1 mRNA might differ between humans and mice. Based on these findings, we examined the SYNAP1 3’UTR in the whole exome sequencing data of 807 Japanese patients with sporadic ALS and 191 normal controls registered in JaCALS database. We found eight variants in the SYNAP1 3’UTR and the heterozygous SYNAP1 3’UTR variant rs149438267 (G to T) in seven out of 807 ALS patients (1614 alleles), while it was absent in all 191 controls (382 alleles). The allele frequency of this variant was 0.00434, which was significantly higher than those in the nation-wide Japanese database ToMMo8.3KJPN (0.0017, p = 0.037, Fisher’s exact test) and GEMJ-WGA (0.0017, p = 0.036, Fisher’s exact test; Tadaka et al., 2018; Table 2). Interestingly, the frequency of the variant was higher in the Japanese database compared with TOPMED and gnomAD (Karczewski et al., 2020), suggesting different incidence rates among different populations. In ALS patients with this variant, upper limb weakness or dysarthria were frequently observed as initial symptoms, whereas cognitive functions were well preserved (Table 3). The clinical courses were relatively rapid, especially in patients with dystarthis during the initial stage of the disease.
The SYNGAP1 homozygous mutation induced a loss of dendritic spines

To reproduce the relationship between RNA-binding proteins and SYNGAP1 3’UTR by a single-nucleotide polymorphism (SNP) of noncoding sequence, we performed scarless genome editing by CRISPR-Cas9 and inserted the SYNGAP1 3’UTR variant rs149438267 into iPSCs in isogenic condition (Fig. 1B). We chose 201B7 because they have been well characterized (Takahashi et al., 2007) and because they are one of the most commonly and widely used high quality iPSCs (Imamura et al., 2017; Fujimori et al., 2018; Akiyama et al., 2019). First, we generated iPSCs carrying the rs149438267 variant in homozygosis to analyze the full genetic effect expressed by this polymorphism (Fig. 1C; Extended Data Fig. 1-1B). These screened iPSCs were then differentiated into motor neurons (Extended Data Fig. 1-1C; Shimojo et al., 2015; Onodera et al., 2020; Okada et al., 2021), and cell differentiation efficiencies were evaluated using the following procedure: after one week by immunocytochemistry of Islet-1 (ISL-1), homeobox protein HB9 (HB9), and β-III-tubulin (Extended Data Fig. 1-1D,E), and after four weeks by choline acetyltransferase (ChAT) and microtubule-associated protein 2 (MAP2; Fig. 1D,E). These data suggested that the differentiation efficiencies were similar in the wild-type (WT) and the homozygous mutant iPSC-derived motor neurons. Next, we evaluated spines in the motor neurons using F-actin immunolabeling (Extended Data Fig. 1-1F,G), and the number of F-actin particles was significantly decreased in the rs149438267 homozygous motor neurons (Fig. 1F,G). These results indicate that the SYNGAP1 3’UTR variant rs149438267 from patients with ALS is involved in the spine formation of motor neurons.

The SYNGAP1 homozygous mutant increased isoform 71 levels without altering total expression of SYNGAP1

SYNGAP1 has four isoforms (α1, α2, β, and γ), based on its spliced variant between exon 17, 18, and 19 (Fig. 2A; Jeyabalan and Clement, 2016; Kilinc et al., 2018). Each splice variant results in frameshifts and coded specific C-terminal amino acid sequences. Moreover, 3’UTR connected to each open reading frame (ORF) sequence is also known to have spliced variant in mice (Yokoi et al., 2017). First, we analyzed the spliced variants of SYNGAP1 3’UTR using rapid amplification of cDNA ends (RACE) of the RNA sequences from the WT iPSC-derived motor neurons. We found that short 3’UTR was present but to a lesser extent than full-length 3’UTR (Extended Data Fig. 2-1A). Cloning of RACE fragments revealed that the major ORF isoform of human SYNGAP1 was α2, coinciding with that of mice (Yokoi et al., 2017), and that the ORF isoform ratio between full-length 3’UTR and spliced short UTR was similar (Extended Data Fig. 2-1B). Unexpectedly, short UTR has many variations of potential spliced isoforms (Extended Data Fig. 2-1C). According to these data, we performed RT-PCR from SYNGAP1-ORF to the end of 3’UTR, and found no differences in the proportion of each isoform between the WT and the rs149438267 homozygous motor neurons (Fig. 2B). Although the SYNGAP1 variant rs149438267 was located at the 3’UTR, the results suggest that this variant did not affect the splicing pattern of SYNGAP1 3’UTR.

Next, we focused on ORF expression of SYNGAP1. qRT-PCR of SYNGAP1-ORF revealed that ORF expression levels remained unchanged in the rs149438267 homozygous motor neurons (Fig. 2C). Western blotting showed an increase in SYNGAP1 isoform α1, while PAN-SYNGAP1 and SYNGAP1 α2 remained unchanged (Fig. 2D,E), suggesting that the increase in SYNGAP1 isoform α1 might be because of changes in alternative splicing. Note that the only commercially available antibodies are against PAN-SYNGAP1 and α1 and α2 isoforms. We also generated iPSCs with the heterozygous mutation (Extended Data Fig. 2-1D) but could not detect the same alteration in SYNGAP1 isoform expression (Extended Data Fig. 2-1E,F). This suggests that the heterozygous mutation might exert less effect on the SYNGAP1 isoform under the current culture conditions.

SYNGAP1 isoform α1 is reported to decrease synaptic strength (Rumbaugh et al., 2006; McMahon et al., 2012), and we also reported that SYNGAP1 α1 overexpression decreases the number of mature spines in mouse primary hippocampal neurons (Yokoi et al., 2017). To confirm the effect of SYNGAP1 α1 on synaptic function, we overexpressed SYNGAP1 α1 in the WT motor neurons (Fig. 2F) and confirmed that SYNGAP1 α1 decreased the number of spines (Fig. 2G,H). These results suggest that an increase in SYNGAP1 α1 expression causes dendritic spine loss in the rs149438267 homozygous motor neurons.

The SYNGAP1 variant decreased SYNGAP1 isoform γ, which exerted a positive effect on spine formation

As mentioned above, SYNGAP1 has four isoforms (α1, α2, β, and γ) defined according to the alternative splicing between exons 17, 18, and 19. Because of the complexity of the isoform patterns, no study to date has evaluated all the isoforms simultaneously (Araki et al., 2020). To analyze how SYNGAP1 variants change the alternative splicing of ORF, we performed fragment analysis of SYNGAP1-ORF between exon 17, 18, and 19 (Fig. 3A). Unexpectedly, we found a significant depletion of the isoform γ in the rs149438267 homozygous motor neurons, which could not be evaluated by Western blotting because of the lack of

Table 3. The clinical features of ALS patients with SYNGAP1 variant rs149438267

<table>
<thead>
<tr>
<th>Sex</th>
<th>Age at onset</th>
<th>Clinical diagnosis based on the revised El Escorial criteria</th>
<th>Initial symptoms</th>
<th>Duration from onset to death (months)</th>
<th>Upper motor symptom</th>
<th>Lower motor symptom</th>
<th>Active denervation in EMG</th>
<th>Chronic denervation in EMG</th>
<th>Cognitive tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>64</td>
<td>Probable laboratory supported</td>
<td>Upper limb weakness</td>
<td>30</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Not examined</td>
</tr>
<tr>
<td>F</td>
<td>57</td>
<td>Probable laboratory supported</td>
<td>Upper limb weakness</td>
<td>64</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>MMSE 23, FAB 7</td>
</tr>
<tr>
<td>M</td>
<td>66</td>
<td>Definite supported</td>
<td>Dysarthria</td>
<td>29</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Not examined</td>
</tr>
<tr>
<td>F</td>
<td>66</td>
<td>Probable supported</td>
<td>Dysarthria, dysphagia, dyspnea</td>
<td>7</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>MMSE 26, FAB 13</td>
</tr>
<tr>
<td>M</td>
<td>75</td>
<td>Probable supported</td>
<td>Dysarthria</td>
<td>18</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Not examined</td>
</tr>
<tr>
<td>F</td>
<td>77</td>
<td>Probable supported</td>
<td>Dysarthria</td>
<td>38</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>MMSE 20, FAB 14</td>
</tr>
<tr>
<td>M</td>
<td>75</td>
<td>Probable supported</td>
<td>Dysarthria</td>
<td>11</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Not examined</td>
</tr>
</tbody>
</table>
a specific antibody (Fig. 3B; Extended Data Fig. 3-1A,B). While a significant increase in isoform α1 RNA levels in homozygous neurons was observed at the protein level (Fig. 2E), isoform α1 RNA levels did not significantly increase in some lines (Fig. 3B; Extended Data Fig. 3-1A,B). The isoform γ has also been found in mouse brain (Araki et al., 2020); however, its function in spine formation has not been elucidated (Kilinc et al., 2018). Interestingly, the overexpression of the SYNGAP1 isoform γ
rescued a loss of dendritic spines in rs149438267 homozygous motor neurons (Fig. 3C–F), indicating that the SYNGAP1 isoform γ is important for spine formation in motor neurons and can ameliorate spine abnormality in rs149438267 homozygous motor neurons, even in the presence of excess isoform α1. These results suggest that the aberrant splicing, an increase in isoform α1 and a decrease in isoform γ, cause spine loss in the rs149438267 homozygous motor neurons.

The SYNGAPI 3’UTR mutation excessively recruited FUS and HNRNPK
To clarify how the SYNGAPI 3’UTR variant rs149438267 affects the aberrant splicing of SYNGAPI isoforms α1 and γ, we next evaluated the binding efficacy of FUS and collaborating RNA-binding proteins. We generated biotinylated RNA probes with 240-nt sequences for the 5’ side of SYNGAPI 3’UTR with or without the mutation (Fig. 4A), and performed RNA pull-down
assay to analyze the binding efficacy of RNA-binding proteins to each probe by Western blotting. Interestingly, the binding efficacy of FUS to the probe with the mutation was increased compared with the probe without the mutation (Fig. 4B, C), suggesting that the SYNGAP1 3’UTR variant rs149438267 recruited FUS to 3’UTR. In addition to FUS, gel digestion and LC/MS analysis of the specific bands in each sample were performed to examine the proteins that bind to the SYNGAP1 3’UTR (Extended Data Fig. 4-1A; Extended Data Table 4-1). We found that the binding efficacy of heterogeneous nuclear ribonucleoprotein K (HNRNPK) also increased with the mutated probe (Fig. 4B, C). HNRNPK is a DNA/RNA-binding protein that plays important roles in transcription (Michelotti et al., 1996; Wang et al., 2020), paraspeckle formation (Fox et al., 2018), splicing (Liu et al., 2018; Thompson et al., 2018), and post-transcriptional regulation at 3’UTR (Yano et al., 2005; Liepelt et al., 2014; Shin et al., 2017). The relationships between HNRNPK and diseases such as cancer (Barboro et al., 2014), Kabuki-like syndrome (Dentici et al., 2018), and Au-Kline syndrome (Au et al., 2018) have been described. The anti-HNRNPK antibody recognizes two splice variants (Barboro et al., 2009). We also analyzed other candidates found in the LC/MS analysis, including ELAVL2 and PUF60, which remained unchanged (Extended Data Fig. 4-1B, C). Importantly, HNRNPK is also known to interact with FUS (Groen et al., 2013). The expression level of HNRNPK remained unchanged between the WT and the rs149438267 homozygous motor neurons (Extended Data Fig. 4-1D, E). To validate whether FUS and HNRNPK bind to SYNGAP1 mRNA, we performed RNA-IP of the motor neuron cultures using the antibodies for each RNA-binding protein. The binding efficacy of SYNGAP1 mRNA to FUS and HNRNPK tended to be higher in the rs149438267 homozygous motor neurons than in the WT motor neurons (Extended Data Fig. 4-1F–I). Moreover, co-immunoprecipitation assay (Co-IP) by the anti-FUS antibody with the RNase A treatment revealed that FUS and HNRNPK bound with each other via protein-protein interactions (Fig. 4D). Co-IP by anti-HNRNPK antibody showed that the level of FUS bound to HNRNPK was low and reduced by RNase A treatment. This suggests that HNRNPK is not always bound to FUS, and that some of the FUS proteins bind to HNRNPK via protein–protein interactions and others bind indirectly to HNRNPK via RNA. These results suggest that HNRNPK is a crucial collaborator of FUS, and that both proteins are excessively recruited by the SYNGAP1 3’UTR variant.

HNRNPK, rather than FUS, altered the expression pattern of SYNGAP1 isoforms

Next, we performed knock-down experiments to elucidate how FUS and HNRNPK regulate SYNGAP1 isoforms. It should be noted that after HNRNPK knock-down, motor neurons could not survive for four weeks, and thus we collected samples on day 14. Western blot analysis (Fig. 5A–D) showed that FUS knock-
Figure 5. HNRNPK, rather than FUS, alters SYNGAP1 isoforms similar to those observed in motor neurons with the rs149438267 homozygous mutation. **A, B**, The lysates from wild-type motor neurons infected with shCtrl, shFUS1, and shFUS2 (A) or shCtrl, shHNRNPK1, and shHNRNPK2 (B; n = 3) were subjected to Western blotting with the indicated antibodies. **C, D**, Quantification of the band intensities of the indicated proteins in **A, B**. Data are presented as the mean ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001, one-way ANOVA, Tukey’s post hoc test. **E, F**, Total RNA was extracted from wild-type motor neurons infected with shCtrl, shFUS1, and shFUS2 (E) or shCtrl, shHNRNPK1, and shHNRNPK2 (F; n = 3 neuron cultures each), and the mRNA expression levels of SYNGAP1, FUS, and HNRNPK were analyzed with qRT-PCR. Data are presented as the mean ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001, one-way ANOVA, Tukey’s post hoc test. **G, H**, Total RNA from wild-type motor neurons infected with shCtrl, shFUS1, and shFUS2 (G; n = 4) or shCtrl, shHNRNPK1, and shHNRNPK2 (H; n = 3) was analyzed with...
down did not result in significant changes in SYNGAP1 expression levels (Fig. 5C). More importantly, HNRNPK knock-down decreased PAN-SYNGAP1 as well as isoforms α1 and α2 (Fig. 5D). qRT-PCR revealed that HNRNPK knock-down, but not FUS knock-down, decreased the total expression of SYNGAP1-ORF (Fig. 5E,F), consistent with the results of Western blotting (Fig. 5C,D). Fragment analysis also revealed that HNRNPK knock-down decreased isoform α1 and increased isoform γ levels, while FUS knock-down slightly decreased isoform γ levels (Fig. 5G,H). HNRNPK knock-down also induced another undescribed alternative splicing variant, which could be translated into isoform γ but had longer intron sequences than the conventional γ sequence (Extended Data Fig. 5-1A). These results indicate HNRNPK-controlled alternative splicing of SYNGAP1 mRNA, especially isoform γ. In addition, the changes in SYNGAP1 expression or isoform alteration in the double-knock-down of FUS and HNRNPK were similar to those in the HNRNPK knock-down model (Extended Data Fig. 5-1B–E), which suggests that HNRNPK plays a major role in controlling SYNGAP1 expression. FUS had no significant effect on the transcript levels of SYNGAP1 variants, but it affected SYNGAP1 α1 protein expression, which was decreased in the HNRNPK knock-down but not in the double-knock-down. Given that the SYNGAP1 variant rs149438267 caused aberrant splicing, thus increasing isoform α1 and decreasing isoform γ, these results indicate that HNRNPK, rather than FUS, might contribute to the altered splicing of SYNGAP1 isoforms through the variant. However, the knock-down experiments could not directly explain the relationship between the excessive recruitment of RNA-binding proteins, aberrant splicing, and dendritic spine loss.

The antisense oligonucleotides toward the HNRNPK binding sites ameliorated spine abnormalities

To further elucidate how the excessive recruitment of HNRNPK contributes to the aberrant splicing and dendritic spine loss in the rs149438267 homozygous motor neurons, we considered blocking the recruitment of RNA-binding proteins with antisense oligonucleotides (ASOs), which may represent a therapeutic reagent for ALS (Kole et al., 2012; Bennett et al., 2019). According to the eCLIP of K562 cells from the ENCODE datasets (Davis et al., 2018), the SYNGAP1 3’UTR variant rs149438267 was located between the putative FUS and HNRNPK binding sites (Extended Data Fig. 6-1A). Moreover, the RegRNA database showed that the exonic splicing enhancer was present at the 3’ side of the variant (Chang et al., 2013), and the mCrossBase database showed that the HNRNPK binding motifs were distributed around the exonic splicing enhancer (Feng et al., 2019; Fig. 6A).

Referring to these data, we designed three ASOs toward SYNGAP1 3’UTR (E1, E2, and E3) and treated the rs149438267 homozygous motor neurons with 10 or 50 nM of these ASOs, followed by evaluation after 14 d (Extended Data Fig. 6-1B). We then selected E2-ASO as SYNGAP1-ASO, which exhibited an increase of SYNGAP1 isoform γ at 50 nM, as observed using fragment analysis in a single experiment (Extended Data Fig. 6-1C,D). Pull-down assay confirmed that SYNGAP1-ASO could block the excessive binding of HNRNPK to the probe with the SYNGAP1 3’ UTR mutation (Fig. 6B,C). Unexpectedly, SYNGAP1-ASO significantly increased the binding efficacy of FUS to the mutation probe (Extended Data Fig. 6-1E,F). Moreover, pull-down assay of HNRNPK knock-down showed that the depletion of HNRNPK did not increase FUS binding to the mutation probe (Extended Data Fig. 6-1G,H), which suggests that HNRNPK and FUS interact indirectly at SYNGAP1 3’UTR, and that ASO independently blocked HNRNPK and increased FUS binding. Next, we analyzed the effects of SYNGAP1-ASO on the expression pattern of the SYNGAP1 isoforms. Fragment analysis revealed that the treatment of SYNGAP1-ASO for 14 d in the rs149438267 homozygous motor neurons increased the expression ratio of isoform γ, decreased the expression ratio of isoform α2, and did not alter the expression ratio of isoform α1 (Fig. 6D). qRT-PCR revealed that the expression level of total SYNGAP1 was not affected by SYNGAP1-ASO (Fig. 6E), suggesting that SYNGAP1-ASO could solely affect the alternative splicing of SYNGAP1 isoforms. In addition, SYNGAP1-ASO did not alter the SYNGAP1 isoforms in WT neurons 14 d after ASO treatment (Extended Data Fig. 6-1G,H), suggesting that SYNGAP1-ASO could specifically correct the excessive recruitment of HNRNPK induced by the variant. Western blotting showed that the expression of SYNGAP1 α2 was decreased in the rs149438267 homozygous motor neurons 14 d after SYNGAP1-ASO treatment, while SYNGAP1 α1 and PAN-SYNGAP1 remained unchanged (Fig. 6F,G), consistent with the results of fragment analysis (Fig. 6D).

Finally, SYNGAP1-ASO treatment for 14 d could ameliorate a loss of dendritic spines in the rs149438267 homozygous motor neurons (Fig. 6H). This suggests that the excessive recruitment of HNRNPK was the major pathogenic mechanism for dendritic spine loss in the rs149438267 homozygous motor neurons. Together, these results clarify that the SYNGAP1 3’UTR variant rs149438267 identified in the Japanese ALS cohort excessively recruits RNA-binding proteins, especially HNRNPK, and causes a loss of dendritic spines in iPSC-derived motor neurons (Fig. 7).

Discussion

In this study, we screened ALS patients in a combined Japanese cohort at the FUS binding site in the SYNGAP1 3’UTR. We found the variant rs149438267 was associated with a loss of dendritic spines in iPSC-derived motor neurons. While previous studies have demonstrated that SYNGAP1 is a pathogenic candidate for autism spectrum disorder (Mignot et al., 2016), no report to date has demonstrated a relationship between SYNGAP1 and ALS. Interestingly, SYNGAP1 mutations in individuals with autism spectrum disorder represent loss-of-function mutations (Mignot et al., 2016), and Synap1 hetero knock-out mice show an increase in the proportion of mature spines (Clement et al., 2012). Our findings of spine loss by SYNGAP1 3’UTR variant rs149438267 differ from the function of SYNGAP1 observed in previous research. Synapse loss has been reported as an early pathologic feature of neurodegenerative diseases, including ALS (Sasaki and Maruyama, 1994; Hermz and Dorostkar, 2016; Henstridge et al., 2018). Also, synaptic dysfunction has been reported to contribute to early motor and cognitive dysfunctions before neuronal death in a mouse model of ALS/FTLD (Sunico et al., 2011; Yokoi et al., 2017). In this study, we confirm the presence of dendritic spine loss by the SYNGAP1 3’ UTR rs149438267 in iPSC-derived homozygous motor neurons, which could help to elucidate the pathogenesis of early-stage ALS.
Figure 6. Antisense oligonucleotides blocked the HNRNPK binding site and ameliorated a loss of dendritic spines. 

A. A schematic overview of antisense oligonucleotides toward SYNGAP1 3′ UTR. The scheme shows the sequences representing the binding motifs of HNRNPK according to the mCrossBase database (underlined in blue) and the exonic splicing enhancer according to the RegRNA database (orange frame). 

B. RNA pull-down assay was performed with the lysates from the wild-type motor neurons and biotinylated RNA probe with rs149438267. Pull-down samples were analyzed by Western blotting using the indicated antibodies. 

C. Quantification of the band intensities in B. Data are presented as the mean ± SEM \( n = 3 \) each; * \( p < 0.05 \), unpaired t test. 

D. The isoform ratio. 

E. RNA expression of SYNGAP1-ORF. Data are presented as the mean ± SEM \( n = 4 \) each; * \( p < 0.05 \), * \( p < 0.01 \), n.s., not significant, unpaired t test. 

F. RNA samples (same as in D) were analyzed with RT-PCR and the primer set for SYNGAP1-ORF. Data are presented as the mean ± SEM \( n = 4 \) each; unpaired t test. 

G. Motor neurons with the homozygous mutation were treated with Ctrl-ASO or SYNGAP1-ASO for 14 d and were then subjected to Western blotting with the indicated antibodies. 

H. The number of spines. 

I. Quantification of the band intensities of the proteins in F. Data are presented as the mean ± SEM \( n = 3 \); * \( p < 0.05 \); n.s., not significant,
We found that the SYNGAP1 3’UTR rs149438267 variant excessively recruited FUS and HNRNPK. Interestingly, the cooperative protein with FUS is quite different from that in mice (Yokoi et al., 2017). Moreover, we found that ASO, which blocks the excessive recruitment of HNRNPK to SYNGAP1 3’UTR, could alter the SYNGAP1 splicing, and ameliorate dendritic spine loss in the rs149432867 homozygous motor neurons. This result emphasizes that excess HNRNPK recruitment is the crucial mechanism underlying dendritic spin formation in motor neurons. SYNGAP1 3’UTR contains exonic splicing enhancers close to the HNRNPK binding site, and HNRNPK is known to affect the association between RNA-binding proteins and exonic splicing enhancers or silencers (Marchand et al., 2011). The variants at the 3’UTR have been reported to affect the alternative splicing of ORF (Zhao et al., 2019). Because of such splicing machinery, the excessive binding of HNRNPK itself or HNRNPK and other collaborating RNA-binding proteins could have introduced the aberrant splicing in the rs149432867 homozygous motor neurons.

We also found that HNRNPK affected the SYNGAP1 isoform γ. SYNGAP1 has four isoforms, of which isoform α is the most validated isoform that has a PDZ domain that interacts with proteins at the postsynaptic density and regulates synaptic plasticity (Kim et al., 1998; Araki et al., 2013). There have been few reports on the function of SYNGAP1 isoform γ (Araki et al., 2020). Our findings indicate that the SYNGAP1 isoform γ plays an important role in spine formation in human models. While the SYNGAP1 isoform γ was increased, α remained unchanged, and α2 was decreased by ASO treatment. Together with the results of double-knock-down of FUS and HNRNPK, this indicates that FUS affects HNRNPK-dependent alternation of the SYNGAP1 α level. Although the decrease in the SYNGAP1 isoform α2 might have suppressed synaptic strength (McMahon et al., 2012), the decrease in α1 and increase in γ improved spine abnormality, possibly by overcoming the effects of α2. Also, ASO could ameliorate spine loss without sequestering FUS from SYNGAP1 3’UTR by ASO. These data indicate that HNRNPK regulation of the SYNGAP1 isoform γ, which is independent of FUS and SYNGAP1 isoform α1, is a crucial mechanism underlying spine abnormality caused by the SYNGAP1 3’UTR rs149438267 variant. Further evaluations in motor neurons with FUS mutation are needed to elucidate whether FUS has the pathogenic correlation with SYNGAP1.

In this study, there were some discrepancies between the Western blot and fragment analysis results in the evaluation of SYNGAP1 α isoforms. These could be a result of translational control of SYNGAP1 mRNA (Popovitchenko et al., 2020) or isoform-specific protein stability control (Vuong et al., 2022). Since there was a difference of only one base pair between isoform α1 and α2, it is difficult to distinguish each mRNA to analyze translational control. Moreover, given that SYNGAP1 isoforms are defined within a long region of alternative splicing between exons 17, 18, and 19, it is difficult to evaluate all isoforms simultaneously, even with next-generation sequencing (Araki et al., 2020). These discrepancies might be also because of the insufficient detection capability of fragment analysis. In addition, protein expression of isoforms β and γ could not be analyzed because of the absence of specific antibodies. Further developments in methods to elucidate the regulation of SYNGAP1 isoforms are expected.

We could not confirm aberrant SYNGAP1 alterations in the rs149432867 heterozygous motor neurons. This might be because of the experimental settings, e.g., the duration of motor neuron culturing or the composition of the motor neuron medium (Bardy et al., 2015; Odawara et al., 2016). In addition, we could not generate iPSCs from patients with the SYNGAP1 variants rs149432867 because of ethical limitations. We could not obtain informed consent from patients with SYNGAP1 variant rs149432867 to generate iPSCs from their samples. It should also be noted that the heterozygous variant might have milder pathologies than the homozygous variant. Thus, a more detailed analysis of heterozygous motor neurons is needed to fully demonstrate the relevance of this variant in the pathogenesis of ALS.

In summary, we identified the SYNGAP1 3’UTR variant rs149432867 from the Japanese sporadic ALS cohort as a major

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unpaired t test. *M*, Motor neurons were immunostained for F-actin (green) and MAP2 (red). Scale bars: 10 μm. I, Quantification of the number of spines per 20 μm. Data are presented as the mean ± SEM n = 15 each; ***p < 0.001, n.s. not significant, Kruskal–Wallis test, Bonferroni post hoc test. The fundamental data of SYNGAP1-ASO toward the binding site of HNRNPK are displayed in Extended Data Figure 6-1.
candidate involved in a loss of dendritic spines in iPSC-derived motor neurons. In addition, we demonstrated that the SYNGAP1 3’ UTR variant altered SYNGAP1 isoforms via the excess recruitment of FUS and HNRNPK; in particular, HNRNPK played an important role in SYNGAP1 splicing and spine formation. Our findings provide a basis for the future exploration of ALS-related RNA-binding proteins.

References


