Intranuclear Aggregation of Mutant FUS/TLS as a Molecular Pathomechanism of Amyotrophic Lateral Sclerosis*

Received for publication, September 8, 2013, and in revised form, November 18, 2013. Published, JBC Papers in Press, November 26, 2013, DOI 10.1074/jbc.M113.516492

Takao Nomura, Shoji Watanabe, Kumi Kaneko, Koji Yamanaka, Nobuyuki Nukina, and Yoshiaki Furukawa

From the Department of Chemistry, Laboratory for Mechanistic Chemistry of Biomolecules, Keio University, Yokohama, Kanagawa 223-8522, the Graduate School of Brain Science, Doshisha University, Kyoto 619-0225, the Laboratory for Motor Neuron Disease, RIKEN Brain Science Institute, Wako, Saitama 351-0198, the Department of Neuroscience and Pathobiology, Research Institute of Environmental Medicine, Nagoya University, Nagoya, Aichi 464-8601, and the Department of Neuroscience for Neurodegenerative Disorders, Juntendo University Graduate School of Medicine, Tokyo 113-8421, Japan

Significance: A loss of functional TLS/FUS in the nucleus will lead to neurodegeneration.

Background: Abnormal accumulation of mutant FUS/TLS is a pathological change in patients with amyotrophic lateral sclerosis (ALS).

Results: A pathogenic mutation, G156E, increases propensities of FUS/TLS for aggregation in vitro and in vivo.

Conclusion: Intranuclear aggregation of mutant FUS/TLS is a molecular pathomechanism of ALS.

Dominant mutations in FUS/TLS cause a familial form of amyotrophic lateral sclerosis (fALS), where abnormal accumulation of mutant FUS proteins in cytoplasm has been observed as a major pathological change. Many of pathogenic mutations have been shown to deteriorate the nuclear localization signal in FUS and thereby facilitate cytoplasmic mislocalization of mutant proteins. Several other mutations, however, exhibit no effects on the nuclear localization of FUS in cultured cells, and their roles in the pathomechanism of ALS remain obscure. Here, we show that a pathogenic mutation, G156E, significantly increases the propensities for aggregation of FUS in vitro and in vivo. Spontaneous in vitro formation of amyloid-like fibrillar aggregates was observed in mutant but not wild-type FUS, and notably, those fibrils functioned as efficient seeds to trigger the aggregation of wild-type protein. In addition, the G156E mutation did not disturb the nuclear localization of FUS but facilitated the formation of intranuclear inclusions in rat hippocampal neurons with significant cytotoxicity. We thus propose that intranuclear aggregation of FUS triggered by a subset of pathogenic mutations is an alternative pathomechanism of FUS-related fALS diseases.

Fused in sarcoma (FUS), also called as TLS (1), is a DNA/RNA-binding protein involved in physiological processes related to RNA metabolism in particular (2–4). Recently, dominant mutations have been identified in the FUS gene to cause a familial form of amyotrophic lateral sclerosis (fALS) (5, 6). Wild-type FUS protein is localized mostly in the nucleus of a motor neuron, but a subset of pathogenic mutations in FUS was found to facilitate its cytoplasmic mislocalization (5, 6). Upon mutations, FUS would hence lose its physiological functions performed in the nucleus, possibly contributing to the reduced viability of cells.

As represented in Fig. 1, FUS is composed of several distinct domains: a SYGQ-rich region (SYGQ), RNA recognition motif (RRM), three RGG-rich regions (RGG1, RGG2, and RGG3), and a zinc finger motif (ZnF) (7). FUS is also characterized by its extreme C-terminal sequence motif, R/H/KX2–5PY, which functions as a nuclear localization signal called PY-NLS (8). PY-NLS binds to nuclear import receptor, karyopherin β2 (Kapβ2), by which FUS usually localizes at the nucleus (8). Notably, the C-terminal region of FUS is a hot spot for pathogenic mutations (Fig. 1), and those C-terminal mutations have been shown to weaken the affinity of FUS with Kapβ2, thereby resulting in the cytoplasmic mislocalization of mutant FUS (9).

Despite this, many fALS-causing mutations have been reported also in the N-terminal SYGQ region as well as RGG1 region of FUS (Fig. 1) and did not affect the nuclear localization of FUS at least in cultured cells (10). In addition, to our knowledge, neuropathological and biochemical analysis of spinal cords of fALS patients with those mutations have not been available so far; therefore, it remains unknown if FUS with a mutation in its SYGQ and RGG1 regions undergoes cytoplasmic mislocalization under pathological conditions.

FUS is an intrinsically aggregation-prone protein even without any mutations (11). Given the sequence analysis predicting the high aggregation propensities at the N-terminal SYGQ region of FUS (12), we suspect that pathogenic mutations at the SYGQ region modulate the aggregation kinetics of FUS. In this study, therefore, we have examined the effects of pathogenic mutations at SYGQ and RGG1 regions on the aggregation propensities of FUS proteins. Among the mutations tested (G156E,
G225V, M254V, and P525L), introduction of the G156E mutation at the SYGQ region was found to render FUS highly prone to aggregation in vivo as well as in vitro. Such insoluble aggregates exhibited fibrillar morphologies and were capable to triggering the aggregation of wild-type FUS through a seeding reaction. Based upon these results, an alternative pathomechanism of FUS-related fALS has been discussed in which mutations increase the aggregation propensities of FUS proteins.

EXPERIMENTAL PROCEDURES

Preparation of Recombinant FUS Proteins—For preparation of GST-FUS proteins, cDNA of human FUS was cloned in a multiple cloning site (BamHI and SalI) of a vector, pGEX6P-2 (GE Healthcare). A plasmid for expression of GST-FUS-His was prepared by seamlessly inserting six consecutive CAT codons at the C-terminal end of the human FUS coding sequence in the above pGEX6P-2 plasmid containing the GST-FUS gene. Mutations were introduced by the In-Fusion PCR method, and all constructs examined in this study were confirmed by DNA sequencing.

After transfection of a plasmid in Escherichia coli (Rosetta™ (DE3)), expression of GST-FUS and GST-FUS-His proteins was induced by addition of 0.1 mM isopropyl β-D-thiogalactoside, and the cultures were shaken at 20 °C for 46 h. Cells were lysed in PBS containing 5 mM MgCl2, 2% Triton X-100, 6.7 mg/liter of DNase I, 0.12 g/liter of lysozyme and a protease inhibitor mixture, cOmplete EDTA-free (Roche Applied Science), and centrifuged to collect soluble supernatant, which was mixed with glutathione-Sepharose 4B resins (GE Healthcare). After being washed with a TN-trehalose buffer (50 mM Tris, 100 mM NaCl, and 200 mM trehalose) at pH 8.0, the resins were treated with a TN-trehalose buffer containing 20 mM reduced glutathione to elute the bound GST-FUS/GST-FUS-His proteins. The eluted GST-FUS-His proteins were then loaded to HisTrap HP column (GE Healthcare) fitted to BioLogic LP (Bio-Rad) and washed with a buffer containing 50 mM Tris, 100 mM NaCl, and 10 mM imidazole, pH 8.0. GST-FUS-His proteins were then eluted from the column by a TN-trehalose buffer with 250 mM imidazole, pH 6.8 (or 8.0, see text), concentrated by using Vivaspin 15 (MWCO: 10 kDa, Sartorius) and checked by SDS-PAGE. The protein concentration was estimated by using BioRad Protein Assay, in which bovine serum albumin was used as the standard.

Electrophoresis—A sample solution containing 10 µg of total proteins was mixed with SDS-PAGE sample buffer containing β-mercaptoethanol and loaded on a 10% polyacrylamide gel after boiling for 5 min. Insoluble pellets were re-dissolved in buffer containing 2% SDS and then treated with β-mercaptoethanol before loading on a gel. Following electrophoresis, the gel was stained with Coomassie Brilliant Blue, by which the protein bands were visualized. For Western blotting, the gel was further electroblotted on a 0.2-µm PVDF membrane (Bio-Rad), where the protein was detected using a mouse monoclonal anti-GST antibody (1:2,000 dilution, Wako) as a primary antibody and a stabilized goat anti-mouse IgG(H+L) peroxidase-conjugated antibody (1:5,000 dilution, Thermo) as a secondary antibody. Blots were developed with ImmunoStar LD (Wako), and images were obtained using Limited-STAGE (AMZ System Science).

Aggregation of Recombinant FUS Proteins—To examine aggregation of FUS, 150 µM of 5 µM soluble GST-FUS-His proteins in a TN-trehalose buffer with 250 mM imidazole, pH 6.8, was set in a 96-well plate and left at room temperature. Turbidity was monitored at every 5 min in a plate reader (Epoch, BioTek) by measuring absorbance at 350 nm. After the aggregation reaction for 90 min, the sample was ultracentrifuged at 110,000 g for 15 min to prepare soluble supernatant (150 µl) and insoluble pellets. Pellets were then re-dissolved in 150 µl of an SDS-PAGE sample buffer, which contains 0.1% SDS and β-mercaptoethanol, and soluble supernatant was also mixed with 5× SDS-PAGE sample buffer. Both soluble and insoluble fractions were then boiled and loaded on a 10% SDS-PAGE gel.

For diagnosis of amyloid-like FUS aggregates, 250 µM thioflavin T was added to the reaction mixture, which was prepared by incubating 5 µM GST-FUS-His in a TN-trehalose buffer with
Mutation-induced Aggregation of FUS/TLS in ALS

250 mM imidazole, pH 6.8, for 2 h at room temperature. Fluorescence spectra (460–600 nm) were then recorded by using F-4500 (HITACHI) with excitation at 442 nm.

Electron microscopic examination of in vitro FUS aggregates was also performed. GST-FUS-His aggregates collected with ultracentrifugation at 110,000 × g for 15 min were first adsorbed on STEM100C grids coated by elastic carbon (Oken-shoji), washed with pure water, and then negatively stained with 2% phosphotungstic acid. Images were obtained using an electron microscope (Tecnai Spirit, FEI).

For a seeding reaction, 200 μl of 5 μM GST-FUS1156E-His in TN-trehalose buffer with 250 mM imidazole, pH 6.8, was incubated at room temperature for 2 h, and the resultant aggregates were collected with ultracentrifugation at 110,000 × g for 15 min and then resuspended in 20 μl of TN-trehalose buffer, pH 6.8. After being sheared with ultrasonication, 1.5 μl of the aggregates was added to 150 μl of 5 μM GST-FUS-His in TN-trehalose buffer with 250 mM imidazole, pH 6.8, and incubated at room temperature without any agitation. Turbidity of the reaction mixture was monitored at every 5 min in a plate reader (Epoch, BioTek) by measuring absorbance at 350 nm.

DNA Constructs, Cell Culture, and Transfection—A vector, pCMV-HA or pCMV-myc (Clontech), was used for the construction of plasmids expressing HA-tagged or myc-tagged human FUS, respectively, by utilizing its multiple cloning site (SalI/NotI). Mutations were introduced by QuikChange mutagensis (Stratagene).

Human neuroblastoma SH-SY5Y cells were maintained in DMEM/F-12 (Invitrogen) with 10% fetal bovine serum (FBS) in 5% (v/v) CO2 at 37 °C. For immunostaining experiments, cells were seeded on a 4-well chamber slide (Lab-Tek II Chamber Slide with a cover CC2 glass slide, Nalge Nunc international). Cells were transfected with plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. After transfection for 4 h, culture media were replaced with Neurobasal medium (Invitrogen) supplemented with B-27 supplement (Invitrogen), 500 μM l-glutamine, and 5 mM N6,2’-O-dibutyryl cAMP (Nacalai Tesque) for differentiating SH-SY5Y cells. Following further incubation for 15 h, cells were fixed and immunostained as mentioned below.

Pure neuronal cells were prepared from embryonic rat hippocampus (E18) with a previous method (13) using a poly-l-lysine-coated coverslip. Primary cultured neurons were then grown and maintained in serum-free neuronal maintenance medium containing minimal essential medium (Nacalai tesque), 1 mM sodium pyruvate (Invitrogen), 0.6% glucose (Nacalai tesque), N2 supplement, and 1% B27 supplement (Life Technologies) (14). Neurons were transiently transfected with plasmids by using Lipofectamine 2000 (Invitrogen) at 7 DIV. Transfected neurons were further incubated for 2 days at 37 °C with 5% CO2 in neuronal maintenance medium.

Immunocytochemistry—For immunostaining of HA-FUS proteins, cultured SH-SY5Y cells were fixed with 4% paraformaldehyde containing 0.12 M sucrose in PBS for 10 min, permeabilized with 0.5% Triton X-100 in PBS for 5 min, and blocked with 0.1% BSA in PBS for 30 min. Cells were then incubated with anti-HA-fluorescein, High Affinity (3F10) (1:100 dilution, Roche Applied Science) in PBS containing 0.1% BSA for 1 h, washed once with 0.1% Triton X-100 in PBS and twice with 0.1% BSA in PBS. Nuclei were counterstained with DAPI (1:3000 dilution, Molecular Probes).

Transfected neurons at 9 DIV were fixed with 4% paraformaldehyde containing 4% sucrose in Mg2+- and Ca2+-free Dulbecco’s phosphate-buffered saline for 15 min at room temperature, and then blocked with 4% skim milk in TBS containing 0.1% Triton X-100 for 1 h. These neurons were probed with rabbit anti-HA tag polyclonal antibody (MBL), mouse anti-Myc tag monoclonal antibody (clone My3, MBL), and mouse anti-MAP2 monoclonal antibody (clone AP-20, Sigma) for 2 h at room temperature and then further incubated with the corresponding secondary antibodies conjugated with Alexa Fluor 594 or Alexa Fluor 488 (Invitrogen) for 1 h at room temperature. Nuclei were counterstained with bisbenzimide H33342, Nacalai tesque). Confocal images with a slice thickness of ~1 μm were obtained by a laser-scanning microscope of the LSM5 Exciter system (Carl Zeiss, Germany) using a ×40 objective lens for SH-SY5Y cells and by Axiovert 200M microscope (ZEISS) with a ×40 and ×63 objective lenses for primary neurons.

RESULTS

Purification of Recombinant Full-length FUS Proteins in a Dual Tag System—In a previous studies (e.g. Ref. 15), recombinant FUS was overexpressed in E. coli as a GST-fused protein, and we thus first attempted to purify GST-fused FUS proteins (GST-FUS) by affinity chromatography using glutathione (GSH)-Sepharose resins. Although most of GST-FUS proteins were detected in insoluble pellets after lysis of E. coli cells, a soluble fraction of the lysates was treated with GSH-Sepharose resins, by which a soluble form of GST-FUS was obtained (Fig. 2A). The purified GST-FUS samples, however, exhibited the absorption peak centered at 260 nm (Fig. 2B), suggesting the contamination of nucleic acids. Indeed, washing the resins with a buffer containing 1 M NaCl can remove the species with the absorption at 260 nm (Fig. 2B and C), which were digested with RNase but not DNase (Fig. 2D). Accordingly, GST-FUS tightly binds endogenous RNAs in E. coli, consistent with a physiological role of FUS as an RNA-binding protein.

Even after removal of bound RNAs, it should also be noted that the purified GST-FUS samples are not homogenous but contain impurities with smaller molecular weight (Fig. 2A). Assuming that these smaller proteinaceous species were bound to GSH-Sepharose, the purified GST-FUS samples were considered to contain significant amounts of the proteins truncated in the middle of FUS. To prepare the recombinant protein containing a full-length sequence of FUS, therefore, we have introduced a polystyristide (His6) tag at the C-terminus of FUS by using GST-FUS, GST-FUS-His was mostly detected in insoluble pellets after cell lysis (Fig. 3A), and incubation of soluble supernatant with GSH-Sepharose resin was further performed Ni2+-affinity chromatography and removed the truncated proteins that lack a His6 tag. A single major band corresponding to the molecular mass of full-length GST-FUS-
His (about 81 kDa) was confirmed with SDS-PAGE (Fig. 3A) and Western blotting analysis (Fig. 3B). Furthermore, purification of GST-FUS-His using Ni²⁺-affinity chromatography was found to remove contaminants of nucleic acids (Fig. 3C). These results have, therefore, indicated that a full-length FUS protein without bound nucleic acids was successfully prepared with the two-step purification procedure utilizing N-terminal GST and C-terminal His₆ tags.

A Pathogenic G156E Mutation Increases the Aggregation Propensities of FUS—After being eluted from Ni²⁺-affinity chromatography resins with an imidazole-containing buffer at pH 8.0, purified GST-FUS-His proteins were concentrated by a centrifugal concentrator to prepare 5 μM solution. The sample solution was, however, found to gradually become turbid when left at 25 °C (Fig. 3D), and almost all GST-FUS-His proteins after incubation for 1 h were detected as insoluble pellets after ultracentrifugation (Fig. 3D, inset). Just after being eluted from the resins, GST-FUS-His proteins started to aggregate, but several procedures including determination of the protein concentration were required for the aggregation assay. It is, therefore, difficult to characterize the aggregation reaction with reproducible kinetic parameters. This is also the case with GST-FUS-His proteins with ALS-causing mutations. In contrast, when the pH of an elution buffer was decreased from 8.0 to 6.8, we serendipitously found no change in the solution turbidity of purified GST-FUS-His even after incubation for 1 h, and GST-FUS-His proteins remained in soluble supernatant after ultracentrifugation (Fig. 3D). Although a detailed mechanism of FUS aggregation still remains obscure, a solution pH appears to be a critical factor affecting the aggregation propensities of our FUS proteins.

We have then tested effects of pathogenic mutations (G156E (16), G225V (17), and M254V (18)) on the aggregation propensity of GST-FUS-His, pH 6.8. As shown in Fig. 4A, no change in the solution turbidity of GST-FUS-His was observed with G225V and M254V mutations (FUSG225V, FUSM254V), but the G156E mutation in GST-FUS-His (FUSG156E) increased solution turbidity, suggesting the formation of aggregates. GST-FUS-His with a pathogenic mutation at the C-terminal region, P525L (5), did not form aggregates and remained soluble at pH 6.8 within the incubation time examined here (Fig. 4, A and B), consistent with limited roles of C-terminal mutations in the aggregation propensities of FUS (15). It is notable that the solution turbidity of FUSG156E increased just after the sample was set in a 96-well plate; therefore, we have speculated that aggregation of FUSG156E already starts when eluted from the resins. In our experimental protocol, it usually required approximately 40 min for starting the turbidity measurement after elution of FUS proteins from the Ni²⁺-affinity chromatography resins. Indeed, the sample solution of FUSG156E was sometimes already turbid when the sample was set for turbidity measurements; therefore, the initial time point of the FUSG156E aggregation remains obscure. Despite this, FUSG156E was found as insoluble pellets after ultracentrifugation, whereas FUSG225V and FUSM254V remained in soluble supernatant (Fig. 4B). These data have thus clearly shown a distinct role of G156E mutation in increasing the aggregation propensities of a FUS protein at least in the physiological range of solution pH. Notably, furthermore, G144E, G154E, and G156D, which have not been reported as pathogenic mutations, also increased the aggregation propensities of FUS albeit to a lesser extent compared with G156E (Fig. 4B). It is thus likely that the introduction of a neg-
ative charge to the SYGQ domain contributes to the increase in aggregation propensities of FUS proteins.

**FUS Aggregates Exhibit Amyloid-like Properties**—Abnormal accumulation of FUS-positive cytoplasmic inclusions have been characterized as a major pathological change in FUS-related FALS cases (5, 6), albeit no report in FALS cases with G156E-mutant FUS. Ultrastructural analysis on those pathological inclusions has further revealed the presence of fibrillar FUS aggregates (19, 20). Consistent with such molecular pathologies, insoluble FUSG156E aggregates in this study (Fig. 4B) was found to possess fibrillar morphologies with approximately 10 nm of the width (Fig. 4C). These structures were not observed in wild-type GST-FUS and GST-FUS-His are indicated by arrows. A GST-FUS sample was found to contain several forms of FUS-GST in which FUS was truncated, whereas most of such truncated proteins were successfully removed in a GST-FUS-His sample. C, UV-visible spectrometric analysis of 10 μg GST-FUS-His quantified by a Bradford assay was performed after purification with GSH-Sepharose resins (dotted curve) and then further with Ni²⁺-affinity chromatography (solid curve). D, 5 μM GST-FUS-His in a TN-trehalose buffer with 250 mM imidazole at pH 8.0 (filled circles) or 6.8 (open circles) was incubated at room temperature, and the solution turbidity was monitored by measuring the absorption at 350 nm. Inset, after incubation for 2 h, sample solutions (T) were fractionated into soluble supernatant (S) and insoluble pellets (I) by ultracentrifugation and analyzed by SDS-PAGE using 10% polyacrylamide gel. GST-FUS-His was found to form insoluble aggregates at pH 8.0 but not at pH 6.8.

**FUS Aggregates Exhibit Amyloid-like Properties**—Abnormal accumulation of FUS-positive cytoplasmic inclusions have been characterized as a major pathological change in FUS-related FALS cases (5, 6), albeit no report in FALS cases with G156E-mutant FUS. Ultrastructural analysis on those pathological inclusions has further revealed the presence of fibrillar FUS aggregates (19, 20). Consistent with such molecular pathologies, insoluble FUSG156E aggregates in this study (Fig. 4B) were found to possess fibrillar morphologies with approximately 10 nm of the width (Fig. 4C). These structures were not observed in wild-type GST-FUS-His proteins (FUSWT), which remained soluble at pH 6.8 (Fig. 4B). We have also found that the aggregates of FUSG156E increase the fluorescence intensity of thioflavin T, a diagnostic dye of amyloid-like protein aggregates (Fig. 4D). Again, FUSWT did not increase the thioflavin T fluorescence after incubation for 1 h. Pathological inclusions containing FUS in patients with frontotemporal lobar degeneration were not stained with thioflavin dyes (21), but there have been no detailed studies examining reactivity of thioflavin dyes to inclusions in patients with fALS-causing mutations in FUS.

Taken together, the G156E mutation is considered to trigger the formation of fibrillar amyloid-like aggregates of FUS proteins in vitro.

**G156E Mutation Facilitates Formation of Intranuclear FUS-positive Foci in Cells**—We next tested if the G156E mutation leads to aggregation of FUS in the intracellular environment. Although FUS appears to shuttle between the nucleus and cytoplasm (4), immunocytochemistry has revealed intracellular localization of wild-type FUS predominantly to the nucleus (10, 22, 23). In differentiated human neuroblastoma SH-SY5Y cells, we have confirmed the nuclear localization of endogenous FUS proteins (data not shown) as well as the transiently transfected human wild-type FUS with an N-terminal HA tag (HA-FUSWT); furthermore, intranuclear inclusions/foci were not observed (Fig. 5A). As reported previously, cytoplasmic staining (including diffuse and foci) of FUS was evident in almost all cells overexpressing HA-FUS with R522G and P525L mutations (Fig. 5, D and E), which is consistent with roles of the C-terminal region as a nuclear localization signal (10, 22, 23). In contrast, HA-FUS with the G156E mutation (HA-FUSG156E) remained nuclear but notably formed intranuclear foci in transfected cells (approximately 40% of
total cells expressing HA-FUS\textsuperscript{G156E}, white arrows in Fig. 5B). The G225V mutation appears to have minimal effects on both intracellular localization and foci formation of FUS (Fig. 5C). Given increased propensities of FUS aggregation in vitro with G156E but not the G225V mutation, these observations using cultured cells support our idea that the G156E mutation facilitates aggregation of FUS even in the intranuclear environment.
We have also examined effects of pathogenic mutations on FUS proteins in primary neurons. Rat hippocampal primary neurons (DIV 7) were transfected with plasmids for overexpression of HA-FUS proteins and immunostained with anti-HA antibodies after incubation for 2 days at 37 °C. Cells were fixed, stained with anti-HA (red) and anti-MAP2 (green) antibodies, and observed using a confocal microscope. Nuclei were counterstained with Hoechst 33342. In each panel, the image immunostained with anti-HA antibody (magnification: ×63) and the merged images (magnification: ×40) are shown at the left and right, respectively. F, mean intensity of immunofluorescence observed by staining with anti-MAP2 antibody was measured in a cell body with Image J software and found to significantly decrease when HA-FUS with pathogenic mutations were expressed in cells. Measurements were performed in 15, 12, 18, and 6 cells for NT (non-transfected cells), and cells expressing HA-FUSWT, HA-FUSG156E, and HA-FUSP525L, respectively.

![Image](A) HA-FUSWT
![Image](B) HA-FUSG156E
![Image](C) HA-FUSG156E
![Image](D) HA-FUSG156E
![Image](E) HA-FUSP525L

**FIGURE 6.** FUSG156E forms intranuclear inclusions in rat hippocampal primary neurons. A–E, rat hippocampal primary neurons at DIV 7 were transfected with a plasmid for expression of HA-FUSWT (A), HA-FUSG156E (B–D), or HA-FUSP525L (E) and incubated for 2 days at 37 °C. Cells were fixed, stained with anti-HA (red) and anti-MAP2 (green) antibodies, and observed using a confocal microscope. Nuclei were counterstained with Hoechst 33342. In each panel, the image immunostained with anti-HA antibody (magnification: ×63) and the merged images (magnification: ×40) are shown at the left and right, respectively. F, mean intensity of immunofluorescence observed by staining with anti-MAP2 antibody was measured in a cell body with Image J software and found to significantly decrease when HA-FUS with pathogenic mutations were expressed in cells. Measurements were performed in 15, 12, 18, and 6 cells for NT (non-transfected cells), and cells expressing HA-FUSWT, HA-FUSG156E, and HA-FUSP525L, respectively.

Staining for MAP2 by transfecting cells to overexpress HA-FUSG156E and HA-FUSP525L but not HA-FUSWT (Fig. 6F). Loss of MAP2 has been considered as an early marker for neuronal damage following cerebral ischemia (25), spinal cord injury (26), and traumatic brain injury (27); therefore, potential toxicities of FUSG156E and FUSP525L to neurons would be reflected by reduced intensity of MAP2 immunostaining in primary neurons. In addition, primary neurons expressing HA-FUSG156E and HA-FUSP525L often exhibited abnormal cell shapes (e.g. Fig. 6D) that were characterized by less numbers of dendrites than those of non-transfected cells as well as cells expressing HA-FUSWT, albeit difficult to be quantified. These observations are considered to support toxic roles of FUSG156E and FUSP525L in neurons.

**Seeded Aggregation of Wild-type FUS with G156E Mutant FUS in Vitro and in Vivo**—It remains obscure how the G156E mutation in FUS exerts toxicity toward neurons, but physiological functions of FUS such as translational and splicing regulations are well expected to be retarded upon formation of aggregates in the nucleus. Notably, neurodegenerative diseases including FUS-related ALS are characterized by their rapid progression after the disease onset, which appears to be regulated by a “seeding reaction” at a molecular level (28). A seeding reaction is an important feature of amyloid-like fibrillar aggregates, in which a piece of protein fibrils can function as a structural template (called as a seed) for facilitating the fibrillation of as-yet-unaggregated protein molecules (29). Once mutant FUS proteins form aggregates in the nucleus, therefore, a seeding reaction could effectively transform the soluble functional FUS into insoluble aggregates, resulting in the progressive dysfunc-
tion of FUS with cytotoxicity. Indeed, a seeded fibrillation of proteins has been increasingly noticed as a molecular patho-
mechanism that describes progression of several neurodegen-
erative diseases (28).

To test the seeding ability of our FUSG156E aggregates 
in vitro, we have added well sheared FUSG156E aggregates with 
ultrasonication to the solution containing aggregation-resis-
tant GST-FUSWT-His at pH 6.8. As shown in Fig. 7A, no increase in the turbidity of FUSWT-containing solution was 
confirmed in the absence of any seeds, but addition of small 
amounts (10%) of sheared FUSG156E aggregates immediately 
made the solution turbid, suggesting the seeded aggregation of 
FUSWT with FUSG156E aggregates. Ultracentrifugal fraction-
atation has further confirmed that almost all FUSWT molecules 
are detected in insoluble pellets by addition of FUSG156E aggre-
gates (Fig. 7B). Based upon these results, the G156E mutation 
will increase the propensities of FUS proteins for fibrillar aggre-
gation, which would further facilitate the aggregation of wild-
type FUS via a seeding mechanism.

We further examined our seeding mechanism by co-trans-
fection of rat hippocampal primary neurons with plasmids 
expressing Myc-tagged FUSWT (myc-FUSWT) and HA-FUS. 
As shown in Fig. 7C, co-expression of myc-FUSWT and HA-
FUSWT did not result in the formation of any inclusions, and 
both wild-type FUS proteins remained diffused in the nucleus. In contrast, when myc-FUSWT was expressed 
together with HA-FUSG156E in neurons, HA-FUSG156E 
formed intranuclear structures of ring-like inclusions (Fig. 
7D) and dot-like foci (Fig. 7E), and importantly, myc-FUSWT 
was also detected in those abnormal structures (Fig. 7, D and 
E). Based upon these results, intranuclear aggregates of 
FUSG156E can recruit wild-type FUS proteins into their own 
structures, consistent with a seeding ability of FUSG156E 
aggregates in vitro. A seeded aggregation would, therefore, 
accelerate the functional impairment of intranuclear FUS 
proteins.

**DISCUSSION**

As described in the original paper reporting the G156E muta-
tion in FUS (16), Gly156 located in the SYGQ domain is evolu-
tionarily conserved among vertebrates, implying an important 
role in physiological functions of FUS. A low complexity region 
such as the SYGQ domain of FUS has been predicted to exhibit a 
prion-forming propensity by using several computer simula-
tions (12). Particularly, Gly156 locates at the middle of a 
sequence composed exclusively of Gln, Asn, and Tyr residues 
(Fig. 8). Several precedents including yeast prion protein, 
Sup35, have shown high fibrillation propensities of polypep-
tides rich in Gln, Asn, and Tyr. Although it is still unclear why a
Gly to Glu mutation at position 156 specifically increases the aggregation propensities of FUS, an N-terminal low-complexity (LC) region of FUS (amino acids 2–214), which covers the SYGQ domain and almost an N-terminal half of RGG1, has been recently reported to form amyloid-like fibrils in vitro with significant reversibility (32, 33). Those in vitro reversible fibrils formed by an LC region of FUS have been further shown to act as seeds, from which LC regions of FUS and the other RNA-binding proteins such as heterogeneous nuclear ribonucleoprotein A1 are polymerized (33). It should also be noted that the SYGQ domain alone is resistant to aggregation in yeast cells, and certain interactions between SYGQ and RGG2 would play roles in the formation of aggregates (15). Taken together, these results support important roles of the SYGQ domain in the aggregation of FUS in vitro and in vivo. Given that the replacement of Gly to Glu adds a negative charge on FUS, the G156E mutation might modulate interactions between SYGQ and RGG2 and thereby facilitate the aggregation. An increase of the solution pH will also add negative charges on FUS, which are consistent with our observation that GST-FUS-His becomes prone to aggregation at increased pH (Fig. 3D). Charge distribution of FUS might thus be important in the description of its aggregation propensities. Once the aggregation of mutant FUS starts in the nucleus, furthermore, the aggregates will function as seeds to trigger aggregation of the wild-type protein. FUS deficiency in a mouse has been previously shown to lead to perinatal mortality (34), and loss of physiological functions of FUS appears to be detrimental. Such recruitment of wild-type as well as mutant FUS into the aggregate structures is, therefore, considered to disturb physiological processes regulated by FUS, resulting in the deterioration of cellular activities, in particular, RNA metabolisms including transcription, alternative splicing, and mRNA transport (35). Recently, it has been reported that alternative splicing of certain neurological disease-associated genes such as Mapt (encoding Tau protein) is regulated by FUS (36–38). Given that abnormalities in the splicing events of the Tau gene are linked to frontotemporal lobar degeneration (39), intracellular amounts of alternatively spliced Tau could also be altered by aggregation of mutant FUS, which might then contribute to the appearance of neurodegenerative phenotypes.

It is clinically notable that a patient with the G156E mutation in FUS developed dementia concurrently with the presence of both upper and lower motor neuron signs (16). Although many patients with ALS have been reported to show cognitive impairment (40), cognitive dysfunction is considered to be absent or rare in ALS patients with other types of mutations in FUS (6, 16, 41–47). There is a caveat that no autopsy has been reported in G156E cases and also that no animal models of G156E-FUS are available as of now. More numbers of patients with FUS mutations, especially the G156E mutation, should hence be described before conclusions, but it is tempting to speculate that increased propensities of G156E-mutant FUS for aggregation in the nucleus are responsible for its distinct clinical features.

In summary, we have successfully purified a protein containing full-length FUS that is free from truncated proteins and E. coli endogenous nucleotides. Among several pathogenic mutations, G156E did not affect functional PY-NLS at the C terminus of FUS but increased propensities of FUS for aggregation in vitro and in vivo. Furthermore, a seeding activity of FUS\textsuperscript{G156E} aggregates was found to trigger the transformation of soluble, functional FUS\textsuperscript{WT} into aggregated structures, which would contribute to a significant loss of physiological functions of FUS in cells (Fig. 8). Even with intact PY-NLS, aggregation of intranuclear FUS proteins caused by amino acid mutations (e.g. G156E) and/or environmental changes is an alternative pathomechanism of FUS-related ALS diseases.

Acknowledgment—We thank Prof. Hiroaki Misonou for helping with experiments on rat primary culture.
REFERENCES
1. Crozat, A., Aman, P., Mandahl, N., and Ron, D. (1993) Fusion of CHOP to a novel RNA-binding protein in human myoid liposarcoma. Nature 363, 640–644
2. Urunashi, H., Tetsuka, T., Yamashita, M., Asamitsu, K., Shimizu, M., Itobu, M., and Okamoto, T. (2001) Involvement of the pro-oncoprotein TLS (translocated in liposarcoma) in nuclear factor-κB p65-mediated transcription as a coactivator. J. Biol. Chem. 276, 13395–13401
3. Yang, L., Embree, L. J., Tsai, S., and Hickstein, D. D. (1998) Oncoprotein TLS interacts with serine-arginine proteins involved in RNA splicing. J. Biol. Chem. 273, 27761–27764
4. Zinszner, H., Sok, J., Immanuel, D., Yin, Y., and Ron, D. (1997) TLS (FUS) binds RNA in vivo and engages in nu-cleo-cytoplasmic shuttling. J. Cell Sci. 110, 1741–1750
5. Kwiatkowski, T. J., Jr., Bosco, D. A., Leclerc, A. L., Tamrazian, E., Vanderburg, C. R., Russ, C., Davis, A., Gilchrist, J., Kasarskis, E. J., Munsat, T., Valdimars, P., Routley, G. A., Hosler, B. A., Cortelli, P., de Jong, P. J., Yoshinaga, Y., Haines, J. L., Pericak-Vance, M. A., Yan, J., Yoon, N., Siddique, T., Mckenna-Yasek, D., Sapp, P. C., Horvitz, H. R., Landers, J. E., and Brown, R. H., Jr. (2009) Mutations in the FUS/TLS gene on chromosome 16 cause familial amyotrophic lateral sclerosis. Science 323, 1205–1208
6. Vance, C., Rogeli, B., Hörtobagyi, T., De Vos, K. J., Nishiura, A. L., Sreedharan, J., Kasarskis, E. J., Munsat, T., Willams, K. L., Tripathi, V., Al-Sarraj, S., Al-Chalabi, A., Leigh, P. N., Blair, I. P., Nicholson, G., de Bellerocche, J., Gallo, J. M., Miller, C. C., and Shaw, C. E. (2009) Mutations in FUS, an RNA processing protein, cause familial amyotrophic lateral sclerosis type 6. Science 323, 1208–1211
7. Iko, Y., Kodama, T. S., Kasai, N., Oyama, T., Morita, E. H., Muto, T., Okumura, M., Fujii, R., Takumi, T., Tate, S., and Morikawa, K. (2004) Domain architectures and characterization of an RNA-binding protein. TLS. J. Biol. Chem. 279, 44834–44840
8. Lee, B. J., Cansizoglu, A. E., Sael, K. E., Louis, T. H., Zhang, Z., and Chook, Y. M. (2006) Rules for nuclear localization sequence recognition by karyopherin β2. Cell 126, 543–558
9. Zhang, Z. C., and Chook, Y. M. (2012) Structural and energetic basis of ALS-causing mutations in the atypical proline-tyrosine nuclear localization signal of the Fused in Sarcoma protein (FUS). Proc. Natl. Acad. Sci. U.S.A. 109, 12017–12021
10. Dormann, D., Rodde, R., Edbauer, D., Bentmann, E., Fischer, I., Hrusch, I., Hatta, R., Hata, R., Ueda, H., Handa, N., Fukunaga, R., Isaka, Y., and Kimura, K. (1989) Microtubule-associated protein 2 as a sensitive marker for cerebral ischemic damage. Immunohistochemical investigation of dendritic damage. Neuroscience 31, 401–411
11. Springer, J. E., Azbill, R. D., Kennedy, S. E., George, J., and Geddes, J. W. (1997) Rapid calpain I activation and cytoskeletal protein degradation following traumatic spinal cord injury. Attenuation with riluzole pretreatment. J. Neurochem. 69, 1592–1600
12. Taft, W. C., Yang, K., Dixon, C. E., and Hayes, R. L. (1992) Microtubule-associated protein 2 levels decrease in hippocampus following traumatic brain injury. J. Neurotrauma 9, 281–290
13. Prasad, D. D., Ouchi, M., Lee, L., Rao, V. N., and Reddy, E. S. (1994) TLS/FUS fusion domain of TLS/FUS-erg chimERIC protein resulting from the t(16;21) chromosomal translocation in human myeloid leukemia functions as a transcriptional activation domain. Oncogene 9, 3717–3729
14. Han, T. W., Kato, M., Xie, S., Wu, L. C., Mirzaei, H., Pei, J., Chen, M., Xie, Y., Allen, J., Xiao, G., and McKnight, S. L. (2012) Cell-free formation of RNA granules. Bound RNAs identify features and components of cellular assemblies. Cell 149, 768–779
15. Hicks, G. G., Singh, N., Nasabhi, A., Mai, S., Bozek, G., Kowalski, R., Arapovic, D., White, E. K., Koury, M. J., Otley, E. M., Van Kaer, L., and Ruley, H. E. (2000) Fus deficiency in mice results in defective B-lymphocyte development and activation, high levels of chromosomal instability and peri-
natal death. Nat. Genet. 24, 175–179
35. Lagier-Tourenne, C., Polymenidou, M., and Cleveland, D. W. (2010) TDP-43 and FUS/TLS. Emerging roles in RNA processing and neurodegeneration. Hum. Mol. Genet. 19, R46-R64
36. Ishigaki, S., Masuda, A., Fujioka, Y., Iguchi, Y., Katsuno, M., Shibata, A., Urano, F., Sobue, G., and Ohno, K. (2012) Position-dependent FUS-RNA interactions regulate alternative splicing events and transcriptions. Scientific Reports 2, 529
37. Fujioka, Y., Ishigaki, S., Masuda, A., Iguchi, Y., Udagawa, T., Watanabe, H., Katsuno, M., Ohno, K., and Sobue, G. (2013) FUS-regulated region- and cell-type-specific transcriptome is associated with cell selectivity in ALS/FTLD. Scientific Reports 3, 2388
38. Orozco, D., Tahirovic, S., Rentzsch, K., Schwenk, B. M., Haass, C., and Edbauer, D. (2012) Loss of fused in sarcoma (FUS) promotes pathological Tau splicing. EMBO Rep. 13, 759–764
39. Lee, V. M., Goedert, M., and Trojanowski, J. Q. (2001) Neurodegenerative tauopathies. Annu. Rev. Neurosci. 24, 1121–1159
40. Phukan, J., Pender, N. P., and Hardiman, O. (2007) Cognitive impairment in amyotrophic lateral sclerosis. Lancet Neurol. 6, 994–1003
41. DeJesus-Hernandez, M., Kocerha, J., Finch, N., Crook, R., Baker, M., Desaro, P., Johnston, A., Rutherford, N., Wojtas, A., Kennelly, K., Wszolek, Z. K., Graff-Radford, N., Boylan, K., and Rademakers, R. (2010) De novo truncating FUS gene mutation as a cause of sporadic amyotrophic lateral sclerosis. Hum. Mutat. 31, E1377–E1389
42. Groen, E. J., van Es, M. A., van Vught, P. W., Spliet, W. G., van Engelen-Lee, J., de Visser, M., Wokke, J. H., Schelhaas, H. J., Ophoff, R. A., Fumoto, K., Pasterkamp, R. J., Dooijes, D., Cuppen, E., Veldink, J. H., and van den Berg, I. H. (2010) FUS mutations in familial amyotrophic lateral sclerosis in the Netherlands. Arch. Neurol. 67, 224–230
43. Hewitt, C., Kirby, J., Highley, J. R., Hartley, J. A., Hibberd, R., Hollinger, H. C., Williams, T. L., Ince, P. G., McDermott, C. J., and Shaw, P. J. (2010) Novel FUS/TLS mutations and pathology in familial and sporadic amyotrophic lateral sclerosis. Arch. Neurol. 67, 455–461
44. Robertson, J., Bilbao, J., Zinman, L., Hazrati, L. N., Tokuhiro, S., Sato, C., Moreno, D., Strome, R., Mackenzie, I. R., and Rogaeva, E. (2011) A novel double mutation in FUS gene causing sporadic ALS. Neurobiol. Aging 32, 553.e27–553.e30
45. Suzuki, N., Aoki, M., Warita, H., Kato, M., Mizuno, H., Shimakura, N., Akiyama, T., Furuya, H., Hokonohara, T., Iwaki, A., Togashi, S., Konno, H., and Itoyama, Y. (2010) FALS with FUS mutation in Japan, with early onset, rapid progress and basophilic inclusion. J. Hum. Genet. 55, 252–254
46. Waibel, S., Neumann, M., Rabe, M., Meyer, T., and Ludolph, A. C. (2010) Novel missense and truncating mutations in FUS/TLS in familial ALS. Neurology 75, 815–817
47. Yan, J., Deng, H. X., Siddique, N., Fecto, F., Chen, W., Yang, Y., Liu, E., Donkervoort, S., Zheng, J. G., Shi, Y., Ahmeti, K. B., Brooks, B., Engel, W. K., and Siddique, T. (2010) Frameshift and novel mutations in FUS in familial amyotrophic lateral sclerosis and ALS/dementia. Neurology 75, 807–814