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Aggregation of ALS-linked FUS mutant sequesters RNA binding proteins and impairs RNA granules formation

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ABSTRACT

Protein aggregate/inclusion is one of hallmarks for neurodegenerative disorders including amyotrophic lateral sclerosis (ALS). *FUS/TLS*, one of causative genes for familial ALS, encodes a multifunctional DNA/RNA binding protein predominantly localized in the nucleus. C-terminal mutations in *FUS/TLS* cause the retention and the inclusion of FUS/TLS mutants in the cytoplasm. In the present study, we examined the effects of ALS-linked FUS mutants on ALS-associated RNA binding proteins and RNA granules. FUS C-terminal mutants were diffusely mislocalized in the cytoplasm as small granules in transiently transfected SH-SY5Y cells, whereas large aggregates were spontaneously formed in ~10% of those cells. hnRNP A1, hnRNP A2, and SMN1 as well as FUS wild type were assembled into stress granules under stress conditions, and these were also recruited to FUS mutant-derived spontaneous aggregates in the cytoplasm. These aggregates stalled poly(A) mRNAs and sequestered SMN1 in the detergent insoluble fraction, which also reduced the number of nuclear oligo(dT)-positive foci (speckles) in FISH (fluorescence *in situ* hybridization) assay. In addition, the number of P-bodies was decreased in cells harboring cytoplasmic granules of FUS P525L. These findings raise the possibility that ALS-linked C-terminal FUS mutants could sequester a variety of RNA binding proteins and mRNAs in the cytoplasmic aggregates, which could disrupt various aspects of RNA equilibrium and biogenesis.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is the most frequent form of adult-onset motor neuron diseases and ~10% of all ALS cases are hereditary [1]. Recently a variety of genes encoding RNA binding proteins have been reported as causative genes for familial ALS, including TAR DNA binding protein-43 (*TDP-43*), *FUS/TLS* (fused in sarcoma/translocated in liposarcoma), *TAF15*, *ESWR1*, *hnRNP A1*, and *hnRNP A2/B1* [2–7].

FUS/TLS contains an N-terminal QGSY (Gln-Gly-Ser-Tyr)-rich region, a G(Gly)-rich region, an RRM (RNA recognition motif), two RGG (Arg-Gly-Gly) repeats divided by a ZnF (zinc finger) motif, and a highly conserved C-terminus that encodes a non-classic NLS (nuclear localization signal) recognized by transportin. FUS/TLS, continuously nucleo-cytoplasmic shuttling, predominantly exists in the nucleus. The vast majority of ALS-linked mutations are clustered in the C terminal NLS, most of which result in the retention and the inclusion of FUS/TLS mutants in the cytoplasm [1,8].

FUS/TLS could collaborate with SMN1 (Survival motor neuron protein1) in RNP (Ribonucleoprotein) biogenesis and RNA splicing [9,10]. TDP-43 interacts with hnRNP A1 and hnRNP A2 possibly involved in pre-mRNA processing and transport of poly(A) mRNA [11,12]. Postmortem analysis in ALS patients showed abnormal cytoplasmic aggregations of these RNA binding proteins in affected neurons or muscles [2–4,7]. FET/TET family, DNA/RNA-binding proteins including FUS/TLS, TAF15 and ESWR1, were all co-localized in pathological inclusions of FTLD (frontotemporal lober degeneration)-FUS patients, suggesting the implication of FET proteins in neurodegeneration [13]. These results indicate that "gain of toxic function" and/or "loss of nuclear function" of various RNA binding proteins might be implicated in ALS pathogenesis [14,15].

RNA granules are composed of RNAs and a variety of RNA binding proteins, including stress granules(SGs), processing-bodies (PBs)/GW bodies, germ cell (or polar) granules, and neuronal transporting granules [16]. SGs are dense RNP-containing cytoplasmic bodies that arise during cell stress and could serve as a component of an adaptive mechanism sequestering and protecting cytoplasmic mRNAs. The core constituents of SGs are translationally silent 48S pre-initiation complex, early initiation factors (eIF3, eIF4), and RNA-binding proteins (TIA-1, TIAR, PABP1) [17,18]. Various RNA

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binding and RNA metabolism-related proteins, including TDP43, FUS/TLS, hnRNP A1, hnRNP A2, and SMN1 are recruited to SGs under stress conditions. In addition, SGs marker proteins were found to be additional components of the mutant TDP-43- or FUS-positive cytoplasmic inclusion in ALS patients, suggesting the mutant protein-derived SGs might play a pivotal role in the pathogenesis of ALS [14,15,19]. The translationally repressed mRNAs, with the mRNA decay machinery and translation repressors, accumulate in cytoplasmic foci referred to as PBs [20]. SGs and PBs, sharing certain proteins, are assembled and disassembled in response to stress and reagents that promote or inhibit polysome disassembly, and can contain the same species of mRNA. However, they differ in that SGs contain components of the translation initiation machinery, whereas PBs contain components of the mRNA decay machinery [16].

In the present study, we examined the effect of C-terminal FUS mutant over-expression on ALS-related RNA binding proteins and RNA granules in cell-based culture system.

2. Materials and methods

2.1. Cell culture

HEK293 and neuronal SH-SY5Y cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C in a 5% CO_2 atmosphere. Transient transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

2.2. Constructs and mutagenesis

Human cDNAs for *FUS/TLS* was generated by PCR using HEK293 cDNA as template. The resulting PCR products were subcloned into pAcGFP C1 (Clontech) vector, pDsRed C1 plasmid (Clontech) or FPC1-HA (HA-tagged protein expressing vector). Human *DCP1* cDNA was obtained from RIKEN BRC (Tsukuba, Japan) and subcloned into pAcGFP C1 plasmid (Clontech).

2.3. Western blot analysis, immunocytochemistry

Western blot analysis and immunohistochemistry were performed as described previously [21]. The antibodies used in the present study are listed in Supplementary Table 1.

2.4. Extraction of detergent-insoluble protein

To isolate detergent-insoluble fraction, cells were processed as previously described [21,22].

2.5. Quantification for western blot bands

Western blot images were analyzed by ImageJ software (National Institutes of Health), which evaluates the relative amount of protein staining with normalization to the corresponding controls.

2.6. Fluorescence in situ hybridization (FISH)

FISH was performed as described previously with modifications [23].

2.7. Statistical analysis

Quantitative data were analyzed by the Student's *t*-test, with *p*-values less than 0.05 considered as statistically significant. Data were expressed as mean ± S.D. or S.E. as indicated in figure legend.

3. Results

3.1. FUS C-terminal mutant spontaneously forms cytoplasmic aggregate sequestering FUS wild type

Since FUS-linked familial ALS cases are mostly inherited as an autosomal dominant trait, it prompted us to investigate whether the cytoplasmic inclusion of FUS C-terminal mutants could interfere with RNA equilibrium and RNA metabolism. One of the possible mechanisms is FUS mutants could sequester various RNA binding proteins in the cytoplasmic aggregate/inclusion. For this purpose, we constructed the plasmids encoding GFP or HA-tagged FUS C-terminal mutants, including FUS P525L and FUS dC (Fig. 1A). FUS P525L is one of the most severe types of familial ALS, while FUS dC lacks last 12 amino acids at C-terminal NLS similar to the severe type FUS R495X [3,4].

Neuronal SH-SY5Y cells were transfected with construct encoding GFP (Cont), GFP-FUS wild type (wt), -FUS P525L, and -FUS dC, respectively. FUS wt, a nucleo-cytoplasmic shuttling protein, predominantly localized in the nucleus, while FUS P525L and FUS dC were remarkably mislocalized in the cytoplasm as diffusely distributed granules (Fig. 1B). Most of cells transfected with GFP-FUS P525L or FUS dC plasmid formed only cytoplasmic small "granules" ($\leqslant$3 μm) (Fig. 1C, left panels), whereas approximately ~10% of transfected cells spontaneously generated large irregular "aggregate/inclusion" (>5 μm) at 48 h after transfection (Fig. 1C, right panels).

To characterize these FUS mutant-derived aggregate morphologically, we then performed immunocytochemistry using antibodies against γtubulin ("aggresome" marker), eIF3 ("SGs" marker), and Edc4 ("PBs" marker) in SH-SY5Y cells. As a result, FUS mutant-derived aggregate was mostly co-localized with eIF3 ("SGs" marker) (Supplementary Fig. 1A). In addition, endogenous FUS was recruited to SGs and co-localized with eIF3, when exposed to sodium arsenite, a representative SGs inducer (Supplementary Fig. 1B).

We then examined whether FUS wild type is recruited to FUS mutant-derived aggregate. SH-SY5Y cells were co-transfected with plasmid expressing GFP-FUS wt and HA-tagged FUS mutant (P525L or dC). At 48 h after transfection, GFP-FUS wt was recruited to the FUS P525L and FUS dC-derived cytoplasmic aggregate, respectively (Fig. 1D).

3.2. hnRNP A1 and A2 were recruited to SGs under stress conditions and sequestered in FUS P525L-derived aggregate

hnRNP A1 and A2, nucleocytoplasmic shuttling proteins, belong to A/B subfamily of ubiquitously expressed heterogeneous nuclear ribonucleoproteins (hnRNPs), implicated in pre-mRNA processing and transport of poly(A) mRNA [11,12]. We investigated whether these hnRNPs are assembled into SGs under stress conditions. hnRNPs and FUS predominantly localized in the nucleus in SH-SY5Y cells in normal condition (Supplementary Fig. 2). When exposed to sodium arsenite (0.5 mM, 30 min) or osmotic stressor sorbitol (0.4 M, 60 min), endogenous hnRNP A1 and A2 were co-localized with endogenous FUS in the cytoplasmic SGs (Fig. 2A, B). We then examined whether hnRNP A1 and A2 are sequestered in FUS P525L-derived aggregate. SH-SY5Y cells were transfected with GFP-FUS P525L plasmid and then fixed at 48 h after transfection. hnRNP A1 and A2 were recruited to FUS P525L-derived cytoplasmic aggregate, respectively (Fig. 2C).

3.3. SMN1 is sequestered in the cytoplasmic insoluble fraction by FUS P525L over-expression

SMN1, a causative gene for infantile muscular atrophy, encodes a nucleo-cytoplasmic shuttling SMN protein essential in assembly

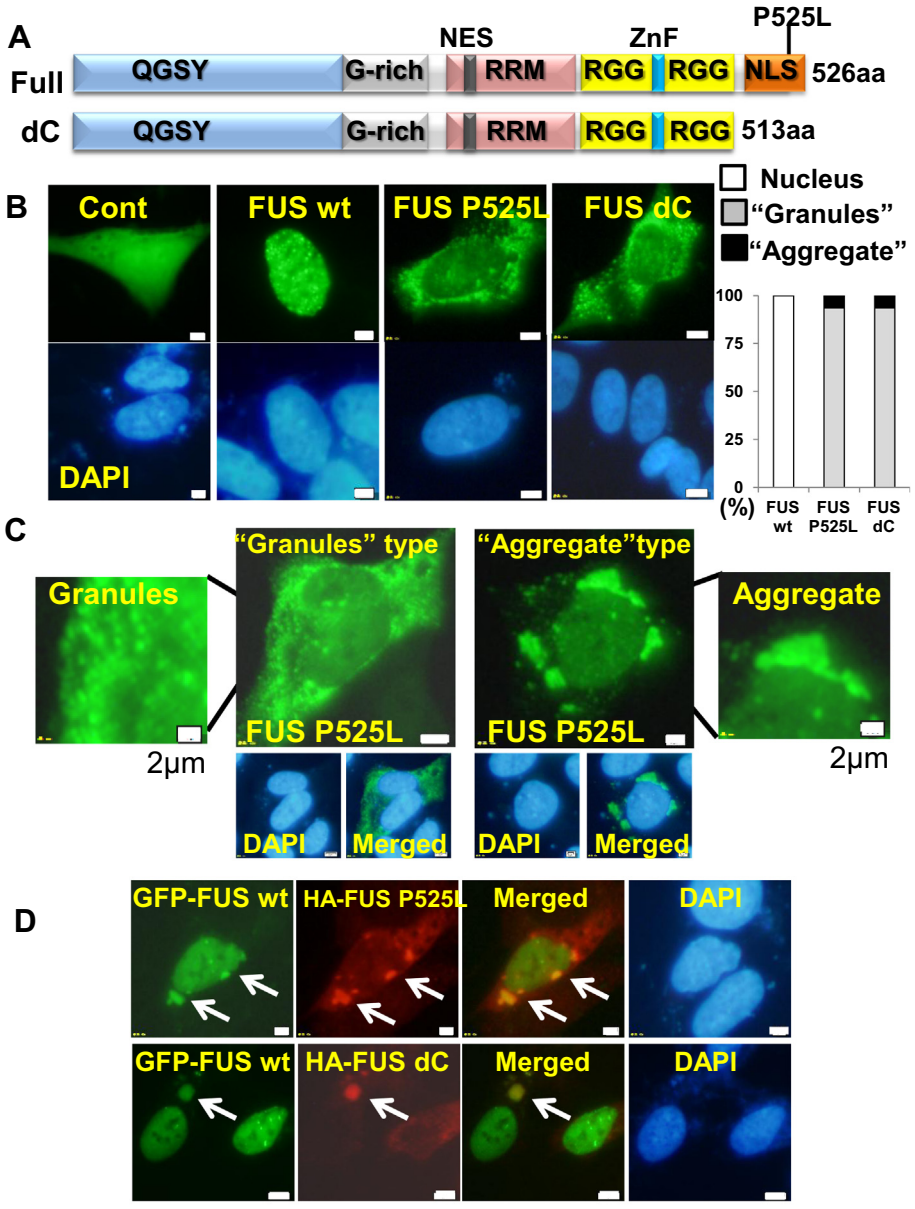

Fig. 1. FUS C-terminal mutants spontaneously form cytoplasmic aggregate that sequesters FUS wild type. (A) Diagrams representing FUS full length (Full) with missense mutation (P525L) and FUS C-terminal NLS deletion mutant (dC). QGSY, Gln-Gly-Ser-Tyr-rich region; G-rich, Gly-rich region; RRM, RNA recognition motif; NES, nuclear export signal; RGG, Arg-Gly-Gly repeats; ZnF, zinc finger domain; NLS, nuclear localization signal. (B) SH-SY5Y cells were transiently transfected with plasmid encoding GFP (Cont), GFP-fused FUS wild type (FUS wt), FUS P525L, and FUS dC, respectively. Samples were fixed at 48 h after transfection and then stained with DAPI (diamidino-2-phenylindole). Images were obtained using fluorescence microscopy. Bars, 5 μm. Bar graph on right side shows the ratio (%) of localization type ("Nucleus", "Granules", and "Aggregate" type) per GFP-positive cells at 48 h after transfection. (C) High magnification pictures for "Granules" type (left panels) and "Aggregate" type (right panels). Bars, 2 μm (as indicated) or 5 μm (otherwise). (D) SH-SY5Y cells were co-transfected with plasmid expressing GFP-FUS wt and HA-FUS mutant (P525L or dC), respectively. Samples were fixed at 48 h after transfection, stained with anti-HA antibody, and then incubated with DAPI (diamidino-2-phenylindole). Arrows indicate the co-localization. Bars, 5 μm.

of small nuclear ribonucleoprotein (snRNP) complexes. Since Spinal Muscular Atrophy (SMA) results from the absence of or mutations in the *SMN1* gene, we next addressed whether FUS P525L over-expression affects the subcellular localization and solubility of SMN1 protein. Under normal condition, SMN1 localized diffusely in the cytoplasm with nuclear foci called Gems in SH-SY5Y cells (Fig. 3A). When cells were treated with sodium arsenite (0.5 mM, 30 min), SMN1 co-localized with FUS in the cytoplasmic foci (Fig. 3B), which were stained with anti-eIF3 antibody as previously described (Supplementary Fig. 1B). Then to examine whether FUS mutant derived-aggregate sequester SMN1, SH-SY5Y cells were transfected with HA-tagged FUS P525L or dC plasmid and then fixed at 48 h after transfection. SMN1 was recruited to FUS mutant (P525L or dC)-derived spontaneous aggregate (Fig. 3C).

The insoluble protein aggregation in neuron is one of hallmarks for the neurodegenerative disorders including ALS, Parkinson's, and Alzheimer's disease. To address whether overproduced FUS P525L sequesters SMN1 protein in the detergent insoluble fraction, we performed soluble–insoluble fraction assay. HEK293 cells were transfected with plasmid expressing GFP-FUS wt or P525L, and soluble/insoluble fractions were separated on SDS–PAGE at 48 h after transfection. FUS P525L over-expression significantly increased the amount of SMN1 in the insoluble fraction and instead slightly reduced that in the soluble fraction (Fig. 3D).

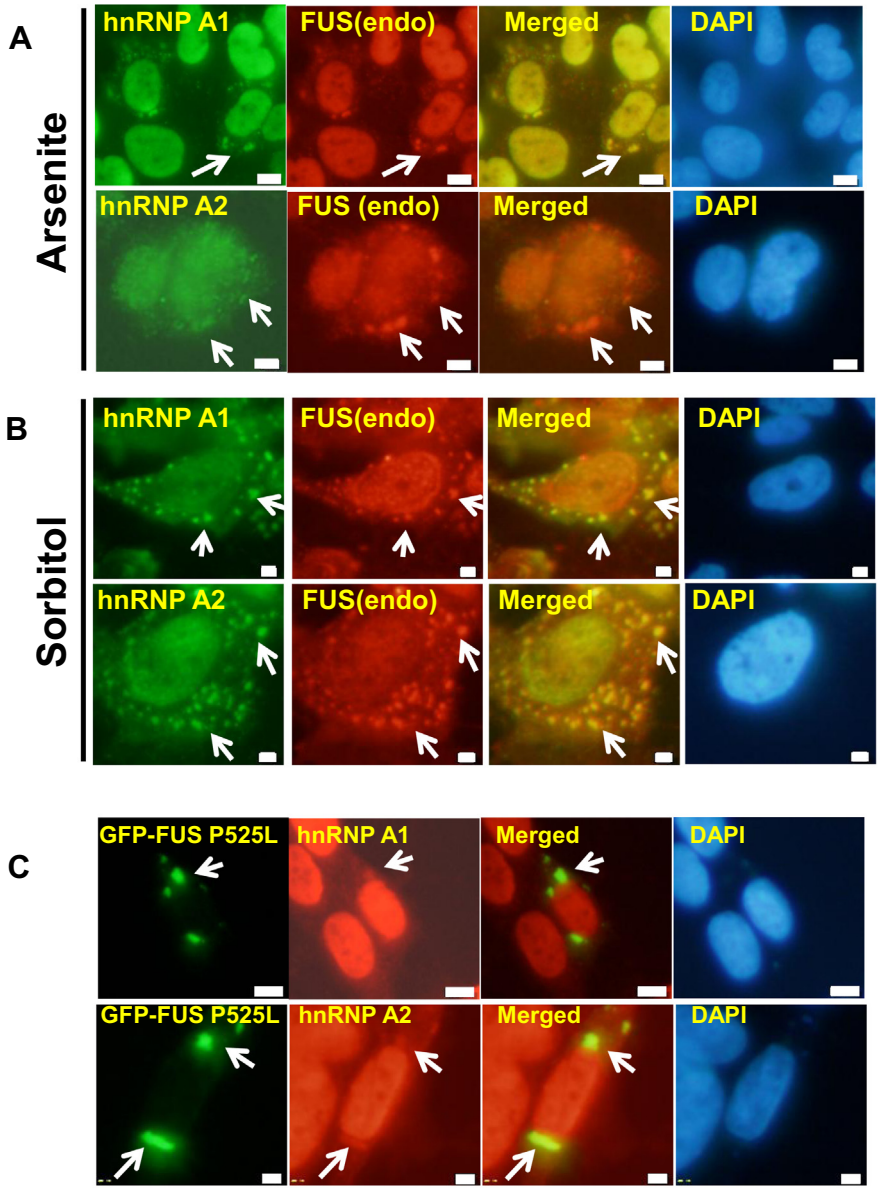

Fig. 2. hnRNP A1 and A2 are assembled to SGs under stress conditions and sequestered in FUS P525L-derived aggregate. (A, B) SH-SY5Y cells were treated with sodium arsenite (0.5 mM, 30 min) and sorbitol (0.4 M, 1 h), respectively. Cells were fixed, co-stained with anti-FUS and anti-hnRNP A1 or hnRNP A2 antibody respectively, and followed by the incubation with fluorescence conjugated secondary antibody. (C) SH-SY5Y cells were transfected with GFP-FUS P525L plasmid, and then fixed at 48 h after transfection. Cells were stained with anti-hnRNP A1 or hnRNP A2 antibody, respectively. Samples were then stained with DAPI. Images were obtained using fluorescence microscopy. Arrows indicate the co-localization. Bars, 5 μm.

3.4. FUS P525L-derived aggregate sequestered poly(A) mRNAs and reduced nuclear foci

SGs are dense RNP-containing cytoplasmic bodies serving as a component of an adaptive mechanism sequestering and protecting cytoplasmic mRNAs. We then investigated whether FUS P525L-derived aggregates enfold mRNAs in FISH (fluorescence *in situ* hybridization) assay using Cy3-labeled oligo(dT) probe against poly(A) tail. SH-SY5Y cells, transfected with plasmid encoding GFP-control or GFP-FUS P525L, were fixed at 48 h after transfection. Cy3-labeled oligo(dT) probe-positive foci in the cytoplasm were co-localized with FUS P525L-derived "aggregate" (Fig. 4A, bottom panels). Oligo(dT) probe-positive nuclear foci, which were the sites of splicing (speckles) [24], appeared to be decreased in cells harboring GFP-P525L-derived "aggregate" compared to those only with "granules" (Fig. 4A, middle and bottom panels). Then we attempted to quantify this by counting the number of oligo(dT) probe-positive foci in the nucleus. The number of oligo(dT) probe-positive nuclear foci was reduced in cells with "aggregate" (average ~ 7 foci/nucleus) compared to those only with "granules" (average ~ 13 foci/nucleus) (Fig. 4B).

Finally, we addressed whether the mislocalization of FUS P525L affects the formation of cytoplasmic RNA granules. The formation of PBs, one of RNA granules, is dependent on RNA [16]. SH-SY5Y cells were co-transfected with GFP-tagged DCP1, a representative marker of PBs, and DsRed-FUS wt or P525L plasmid respectively. The number of visible PBs was reduced in cells transfected with FUS P525L plasmid, in which aggregate was not formed, compared to those with FUS wt plasmid at 24 and 48 h after transfection (Fig. 4C, D).

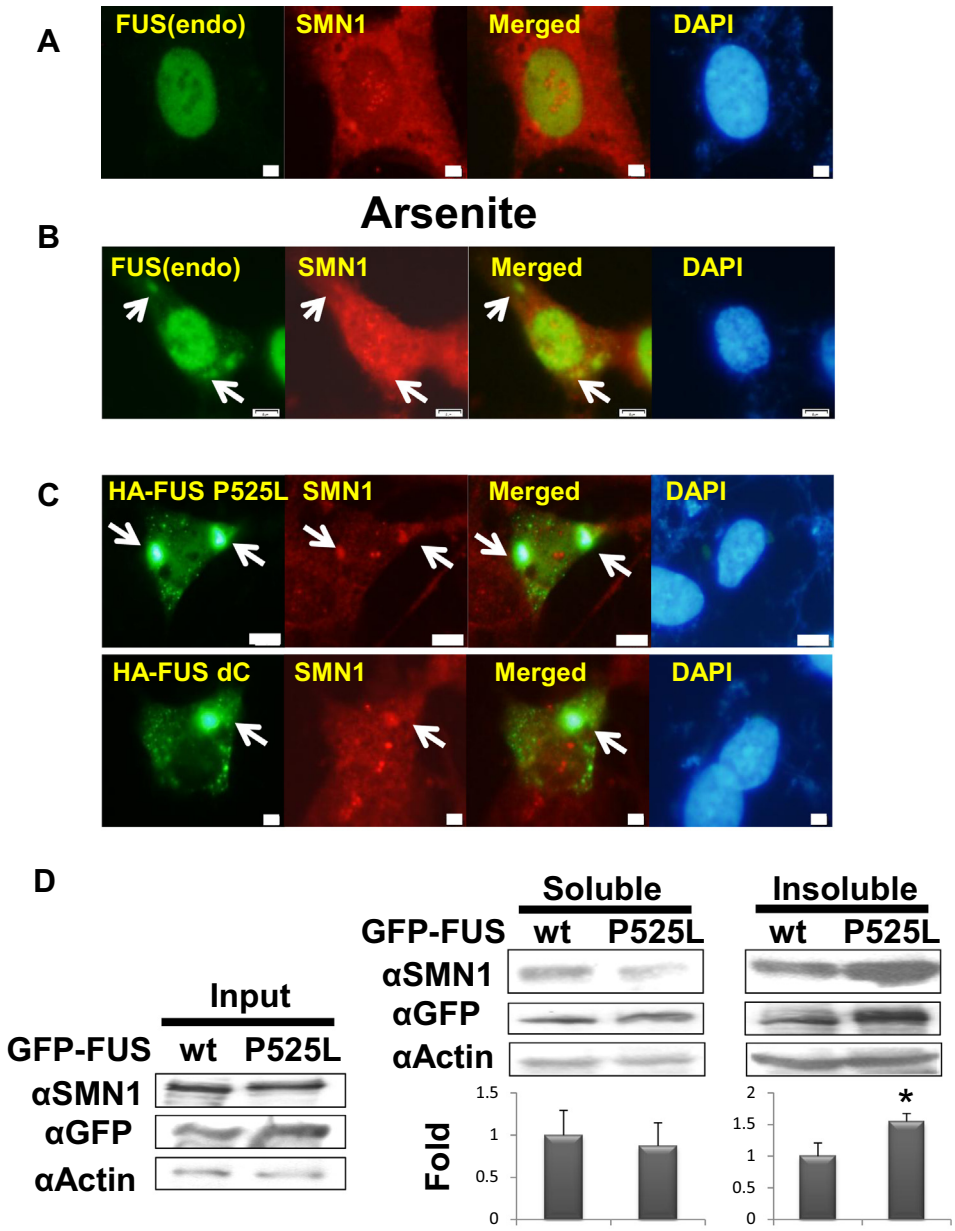

Fig. 3. SMN1 is sequestered in the insoluble fraction by FUS P525L over-expression. (A, B) SH-SY5Y cells were treated with or without 0.5 mM sodium arsenite for 30 min and fixed. Cells were co-stained with anti-SMN1 and FUS antibody. (C) SH-SY5Y cells were transfected with HA-FUS P525L or dC plasmid respectively, and fixed at 48 h after transfection. Cells were co-stained with anti-HA and anti-SMN1 antibody. Samples were then stained with DAPI. Images were obtained using fluorescence microscopy. Arrows indicate the co-localization. Bars, 5 μm. (D) HEK293 cells, transfected with GFP-FUS wt or GFP-P525L plasmid, were processed for cell fractionation assays, and subjected to the immunoblots with indicated antibodies. Immunoreactivity was normalized to the amount of actin. Relative intensities of bands are expressed as mean ± S.E. of 3 separate experiments. $^{*}p < 0.05$ vs. wt. As to input, an aliquot (20 μg) of each extract was analyzed by 10% SDS–PAGE and subjected to the immunoblots with indicated antibodies.

4. Discussion

In the present study, FUS C-terminal mutants (P525L and dC) mislocalized diffusely in the cytoplasm as granules in transiently transfected cells, whereas ~10% of those cells spontaneously generated large cytoplasmic aggregate/inclusion, which colocalized with SGs marker in SH-SY5Y cells. Since endogenous FUS is recruited into SGs under stress conditions, these findings suggest the aggregate was originally derived from spontaneous FUS mutant-associated SGs, which could eventually trigger irreversible aggregate formation of 'pathological' SGs [14,15]. ALS-linked FUS mutants are assembled into SGs in proportion to the cytoplasmic expression levels and that prolonged stress could trigger aggregate formation [14,15], indicating the possible triggers might be cytoplasmic expression levels and various stressors to induce SGs. In fact, FUS N-terminal prion-like domain was found to polymerize into uniform, amyloid-like fibers when incubated at high concentrations [25,26], and the degree of cytoplasmic mislocalization correlates with the age of onset negatively and with disease severity [8,14]. The aggregation of FUS mutant also induces the activation of eIF2α that is required for SGs assembly [27], therefore the aggregate formation could accelerate its own aggregation. Furthermore, RNA-dependent seeding of FUS assembly was observed [28], indicating RNA could accelerate FUS aggregate formation.

N-terminus containing QGSY domain of FUS is prion-like or low-complexity (LC) domain. Although morphologically similar

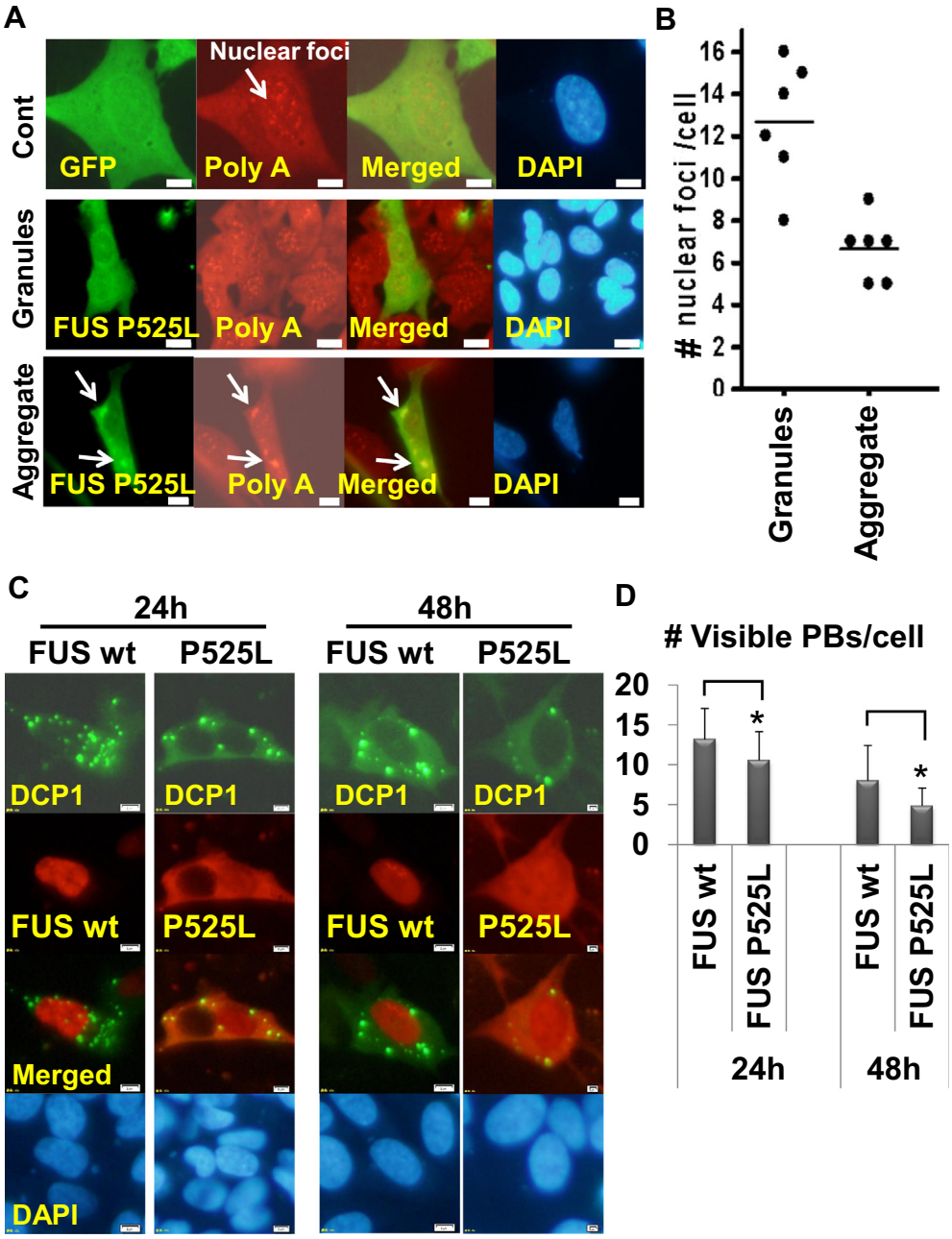

Fig. 4. FUS P525L-derived aggregate sequester poly (A) mRNAs and reduced nuclear foci. (A) SH-SY5Y cells were transfected with GFP (Cont) or GFP-FUS P525L plasmid, fixed at 48 h after transfection and processed for FISH assay using Cy3-labeled oligo(dT) probe. "Granules" indicates cells with only granules, and "Aggregate" indicates cells with aggregate of GFP-FUS P525L. Arrows indicate the co-localization. (B) Dot plot indicates the number of Cy3-labeled oligo(dT) positive foci in the nucleus in SH-SY5Y cell with granules and aggregate, respectively. (C) SH-SY5Y cells were co-transfected with GFP-DCP1 and DsRed-FUS wt or P525L plasmid, respectively. The cells were fixed at 24 h and 48 h after transfection. Samples were then stained with DAPI. Bars, 5 μm. (D) Bar graph indicates the number of visible PBs (GFP-DCP1 foci) in SH-SY5Y cells co-transfected with GFP-DCP1 and DsRed-FUS wt or P525L, respectively. The experiments were performed in duplicate at least 40 cells counted per experiment at 24 h and 48 h after transfection. $^{*}p < 0.05$ vs. wt.

to pathogenic amyloid aggregates, amyloid-like fibers derived from the FUS LC domain can reversibly transform from solubility to a polymeric, amyloid-like state [25,26]. The amyloid-like fibers derived from the FUS LC domain could retain proteins with LC sequence of FUS homotypically or that of other RNA-binding proteins heterotypically [26]. Thus extreme cytoplasmic accumulation of FUS protein, once it reaches a threshold, could transform to amyloid-like state trapping other RNA binding proteins dependently or independently of RNA [27]. In accordance with this, we found that various motor neuron disease-linked proteins (FUS wt, hnRNP As, SMN1) were assembled into SGs under stress conditions and also recruited to FUS mutant-derived spontaneous cytoplasmic aggregate.

The overproduced FUS C-terminal mutant sequestered SMN1 protein in detergent insoluble fraction and reduced the number of oligo(dT) positive nuclear foci, sites of splicing (speckles), and the formation of PBs. SMN1 protein is essential in assembly of small nuclear ribonucleoprotein (snRNP) complexes, and the previous study showed that mislocalized FUS mutants also stall spliceosomal snRNPs in the cytoplasm [29]. Therefore, Cy3-labeled

oligo(dT) positive nuclear foci, sites of splicing (speckles), could be reduced by the stalled SMN1 and snRNP complexes in the cytoplasm possibly impairing mRNA splicing. FUS as well as TDP-43 regulates the splicing of mRNAs transcribed from genes with exceptionally long introns and these products are essential for neuronal integrity [30–32], which might be one of the reasons why neuron is vulnerable to FUS mutations.hnRNP A1 and A2, single-stranded RNA binding proteins with two RRMs, are involved in many aspects of mRNA biogenesis such as transcription, splicing, stability, export through nuclear pores and translation of cellular and viral transcripts. Their functions are not limited to mRNA biogenesis, but extend to the processing of microRNAs, telomere maintenance and the regulation of transcription factor activity [33], raising the possibility that FUS mutant could disrupt these functions by trapping hnRNP A1/A2 in the cytoplasmic aggregate.

Since PBs are the sites of mRNA decay and translation repression including RNA interference and nonsense-mediated decay (NMD), the reduction of PBs by FUS C-terminal mutant could disrupt the RNA equilibrium and the quality control of mRNA. What is the mechanism in which overproduced FUS C-terminal mutant reduces the PBs formation? PBs and polysomes exist in a dynamic equilibrium. PBs increase in size and number upon inhibition of protein synthesis by polysomes-disrupting puromycin, whereas PBs shrink or even disappear when polysomes are stabilized by cycloheximide [16,17]. Since PBs formation is dependent on mRNAs released from polysomes, the cytoplasmic mislocalized FUS P525L without aggregate formation could trap mRNAs and eventually inhibit the PBs formation.

In summary, we here showed that FUS mutant-derived aggregate sequesters various motor neuron disease-linked RNA binding proteins and impair RNA granules formation. Although it remains obscure whether "loss of nuclear function" and/or "gain of toxic function" contribute to the pathogenesis, FUS mutant-derived cytoplasmic aggregate could disrupt various aspects of RNA metabolism in neuron leading to the devastating neurodegenerative disorder.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.08.115.

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