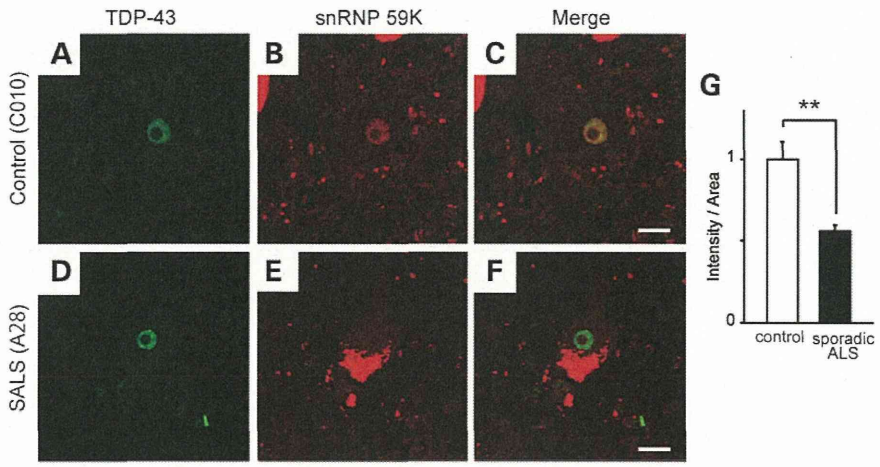

Figure 7. Decreased amount of snRNP 59-K ribonucleoprotein associated with the minor spliceosome in spinal motor neurons of ALS patients. The immunostaining of spinal motor neurons from control tissue (**A**–**C**) and sporadic ALS case (**D**–**F**) was performed using anti-TDP-43 (green) and anti-PDCD antibodies (red) and observed with confocal microscopy. We measured the intensity of snRNP 59 K in the nucleus of each motor neuron in which TDP-43 still remained. (G) The intensity of snRNP 59 K in each spinal motor neuron from controls ($n = 14$) and ALS patients ($n = 13$). Data represent the mean with standard error. Scale bar: 20 μm (A–F). The statistical comparisons were performed using the Mann–Whitney U test. $^{**}P < 0.01$.

splice sites (21,23). U11 snRNA recognizes splicing donor sites, and U12 snRNA recognizes branch sites (21,23). A reduction in the levels of these U snRNAs may hinder recognition of the donor and acceptor sites and induce delayed or altered splicing of pre-mRNAs, consequently affecting the quality and quantity of the products of not only introns of a the minor spliceosome but also introns of a the major spliceosome (23,40). Although the population of genes whose splicing is regulated by the minor spliceosome is small, the perturbation of pre-mRNA regulation by the minor spliceosome causes cell dysfunction and death. Indeed, decreased levels of U11 and U12 snRNA contribute to global alteration of pre-mRNA splicing and result in motor neuron death in SMA (31–33). Moreover, mutations in the minor spliceosome *U4atac* snRNA gene cause brain and bone malformations and postnatal death in humans (44,45). Supporting this hypothesis, we have shown that RNA with spliced U2- and U12-type introns decreases in motor cortex affected by ALS. Global alterations of pre-mRNA splicing have been documented in TDP-43-depleted mice and tissues affected by ALS (12,20). Assuming that the molecular pathogenesis for ALS is caused by reduction in the minor spliceosome, we find the hypothesis that pre-mRNA lariats likely act as decoys to sequester TDP-43, preventing it from interfering with essential cellular RNAs and RNA-binding proteins, to be interesting (46). It has been reported that the absence of Dbr1 enzymatic activity, which debranches a pre-mRNA lariat, suppresses TDP-43-mediated cytotoxicity by accumulating pre-mRNA lariats in the cytoplasm. In contrast, decreasing U11/U12 snRNA levels might decrease the number of pre-mRNA lariats and increase TDP-43-mediated toxicity. These perturbations of pre-mRNA metabolism might underlie the molecular pathogenesis in ALS. Future investigations of these perturbations in affected motor neurons or surrounding glial cells are warranted.

We propose two possibilities to explain the link between a decrease in the number of GEMs and depletion of TDP-43: (1) TDP-43 affects levels of the component proteins of GEMs or (2) TDP-43 facilitates the formation of GEMs. To test the former hypothesis, we investigated whether TDP-43 is a component protein of GEMs. However, GEM component proteins were not co-immunopreciptated with TDP-43. This finding is consistent with a recent report that used global proteomic analysis (14), which indicated that TDP-43 is not a component protein of GEMs. However, we found a decrease in the SMN protein and all SMN splicing variants in TDP-43-depleted cultured cells. The mechanism for this phenomenon is unclear, and we did not observe a decrease in the amounts of *SMN* mRNA in tissue affected by ALS. Therefore, depletion of SMN cannot fully explain the decreased number of GEMs in spinal motor neurons with ALS.

Regarding the latter hypothesis, GEMs are formed in a stochastic self-organizing manner in which the component proteins and snRNAs accumulate at the transcription site (47,48). If TDP-43 facilitates an accumulation of these components or increases the stability of a subset of GEMs, TDP-43 may associate with GEMs. Indeed, we found that a subset of TDP-43 foci contact GEMs in human spinal motor neurons. Furthermore, the volume of GEMs was similar between ALS and control neurons, suggesting that once GEMs have formed, their maturation process is not affected by ALS. In addition, the number of Cajal bodies, which also are in contact with TDP-43 foci, was decreased in spinal motor neurons from tissue affected by ALS, suggesting that TDP-43 may facilitate formation of a subset of nuclear bodies. Taken together, these results suggest that TDP-43 is associated with initiation of the formation of a subset of GEMs. However, further study is required to prove this hypothesis.

We considered the possibility that the number of GEMs decreased in cells in which nuclear TDP-43 disappeared. However, we observed that the number of GEMs decreased even in neurons with nuclear TDP-43. This result indicates that dysfunction of TDP-43 has already occurred in spinal motor neurons with nuclear TDP-43 immunostaining. It has been argued whether the disappearance of TDP-43 from the nucleus is a cause or result of TDP-43 dysfunction. Our

finding supports the hypothesis that an alteration of TDP-43 function occurs in the nucleus, resulting in dislocation of TDP-43 from the nucleus to the cytoplasm, followed by the formation of cytoplasmic inclusions. Indeed, several reports have suggested that dislocation of TDP-43 from the nucleus precedes the formation of cytoplasmic inclusions (49,50). Moreover, during preparation of our report, Yamazaki *et al.* reported reduction in the number of GEMs without TDP-43 aggregation in fibroblasts obtained from familial ALS tissue carrying p.Gly298Ser or Met337Val mutation in *TDP-43* (51). This report further supports our conclusion.

In this study, we could not determine why the repertories of U snRNAs differed between SMN- and TDP-43-depleted cells or why both SMN- and TDP-43-depleted cells demonstrated altered U snRNA repertoires in a cell type-specific manner (32). These findings suggest that U snRNAs are regulated in a complex manner by each nuclear body in each type of cell. Further study is needed to clarify these issues. A disadvantage of this study is that we analyzed RNA samples that included both glial cells and neurons. Therefore, it is unclear whether U12 snRNA decreased in motor neurons. The failure to show inhibition of U12-type intron splicing in tissues affected by ALS might have resulted from the analysis of these samples. However, we have demonstrated a decrease in U11/U12 snRNPs in spinal motor neurons from tissue affected by ALS. Moreover, it has been demonstrated that TDP-43 pathology is frequently observed in glial cells (1,5,38,52). We demonstrated that the U12 snRNA level was significantly decreased in TDP-43-depleted U87-MG cells, which are glial-derived cells. We therefore speculate that decreased U12 snRNA levels may also exist in glial cells and motor neurons in ALS patients.

Our results suggest that TDP-43 contributes to GEM formation and the biogenesis of U snRNAs, particularly U12 snRNA, in tissues affected by ALS. Although TDP-43 dysfunction directly alters pre-mRNA splicing (12,13,15–18), decreased U12 snRNA levels might also result in alterations of pre-mRNA splicing in tissues affected by ALS (20). Decreased U11 and U12 snRNA levels have been reported to result in alterations in global pre-mRNA splicing in SMA (32,33). These results demonstrate that motor neurons may be vulnerable to alterations in pre-mRNA metabolism regulated by the minor spliceosome. The observation that two causative proteins for motor neuron disease play an important role in the biogenesis of U snRNAs for the minor spliceosome provides a novel perspective on the pathogenesis of motor neuron diseases.

MATERIALS AND METHODS

Plasmid constructs

Full-length human *TDP-43* and *SMN* complementary DNAs (cDNAs) were isolated from a human whole-brain cDNA library (Clontech, Palo Alto, CA, USA). Full-length human *TDP-43* cDNA was subcloned into a pcDNA DEST-40 (Invitrogen, Carlsbad, CA, USA) or pmCherry-C1 (Clontech) vector. Full-length human *SMN* cDNA was inserted into the green fluorescent protein (GFP) fusion vector pEGFP-C3 (Clontech).

Cell culture

Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37°C in 5% CO_2. The plasmid DNA was transfected into these cells using Lipofectamine 2000 (Invitrogen). Control (ID: D-001810-10, Dharmacon, Lafayette, CO, USA), *TDP-43*-targeted (ID: L-012394-00, Dharmacon), four different *TDP-43* 3′ UTR-targeted (ID: KB-BBS3784-1_1542, 2190, 2234 and 2319; Hokkaido System Science Co., Hokkaido, Japan) or *SMN*-targeted siRNAs (ID: L-011108-00, Dharmacon) were transfected into the cultured cells using Lipofectamine RNAi MAX (Invitrogen).

Immunofluorescent analysis and quantification for spinal motor neurons

HeLa cells were immunostained with an antibody directed against SMN (1:1000; ab5831, Abcam, Cambridge, MA, USA) and myc-tag (1:400; Cell Signaling Technology, Beverly, MA, USA). Paraffin-embedded sections of the human spinal cord were fixed in 10% formaldehyde and processed for double immunofluorescence with anti-TDP-43 (1:2000; 10782-1-AP, ProteinTech, Chicago, IL, USA), anti-SMN antibodies (1:100; 610646, BD Transduction Laboratories, San Diego, CA, USA) and anti-snRNP 59 K (1:50; ab131258, Abcam). Alexa Fluor 488 rabbit anti-mouse IgG and Alexa Fluor 594 goat anti-rabbit IgG (1:1000, Molecular Probes, Eugene, OR, USA) were used as secondary antibodies. The images were acquired using a Zeiss LSM 510 Meta confocal microscope with 1-μm Z sectioning (Carl Zeiss, Inc., Oberkochen, Germany). The reconstruction of 3D images was performed with Imaris software (BitPlane, Saint Paul, MN, USA) using 6–10 serial images in which the nucleolus was observed, followed by measurement of the number and volume of GEMs. For snRNP 59 K immunolabeling, regions of interest were selected over individual cell nuclei in which TDP-43 remained, and mean fluorescence intensity per area was measured. In each individual, 16–42 motor neurons were evaluated for GEMs, and 3–4 motor neurons were evaluated for snRNP 59 K.

For evaluation of Cajal bodies, we prepared 4 serial, 6-μm-thick, formalin-fixed and paraffin-embedded sections from the lumbar spinal cord of ALS patients. From control lumbar spinal cord tissue, we prepared one section. These sections were immunostained with anti-TDP-43 rabbit polyclonal antibody (1:2000; 10782-2-AP, ProteinTech) and anti-coilin mouse monoclonal antibody (1:50; P delta; Sigma, Saint Louis, MI). Alexa Fluor 568 goat anti-rabbit IgG (1:1000; Molecular Probe) and Alexa Flour 488 goat anti-mouse IgG (1:1000; Molecular Probe) were used as secondary antibodies. Tissue sections were examined with a confocal laser-scanning microscope (LSM510META, Carl Zeiss, Oberkochen, Germany). Original imaging data were viewed with the LSM Image Browser (Carl Zeiss). The number of Cajal bodies was counted in serial sections in which nuclei were presented.

Immunoblot analysis

Cells were lysed in RIPA buffer (25 mM Tris–HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1%

sodium dodecyl sulfate) with a protease inhibitor cocktail (1:200; Sigma) at 4°C. Lysates were centrifuged for 10 min at 10 000*g*, and the resulting supernatants were collected. Protein concentration was determined with a BCA protein assay kit (Pierce, Rockford, IL, USA), and equal amounts of protein from cell lysates were analyzed. Lysates were subjected to sodium dodecyl sulfate–polyacrylamide gel (Wako, Japan) electrophoresis and transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). Immunoblotting was performed using the antibodies listed in Supplementary Material, Table S1.

Real-time quantitative RT-PCR analysis

Minimum information for publication of quantitative real-time PCR experiments (MIQE) guidelines for real-time qRT-PCR experiments were followed (53). Total RNA was extracted from cultured cell pellets or frozen human tissues using the mirVana™ miRNA isolation kit (Ambion, Austin, TX, USA). RNA quality was assessed based on the RNA integrity number, as determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Detailed information for each sample is listed in Supplementary Material, Tables S2–S4.

First-strand cDNA synthesis was performed using the SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen). Real-time qRT-PCR was conducted using SYBR Premix Ex Taq (Takara, Shiga, Japan) in a Thermal Cycler Dice Real-time System (Takara). To select the normalization factors, we calculated the relative stability of the expression of 16 housekeeping genes in each cultured cell or tissue sample using the analysis program geNorm (http://medgen.ugent.be/~jvdesomp/genorm/). Two genes were selected as stable expressing endogenous controls. The normalization factor was calculated as the square root of the product of CT values of these two genes. Each sample was measured in triplicate. The $\Delta\Delta C_T$ method was used for quantification. Statistical analyses were performed using the REST2009 program (http://www.gene-quantification.de/rest-2009.html). The primers used for U snRNAs have been previously published (32). Primer specificity was evaluated using the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi), dissociation temperature measurements and agarose gel electrophoresis analysis. The information on primer sequences is listed in Supplementary Material, Table S5.

Informed consent was obtained from relatives prior to the study, and ethical approval was obtained from research ethics committees of Niigata University.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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Symposium: Amyotrophic Lateral Sclerosis. Discovery of TDP-43 and Subsequent Development

Minor splicing pathway is not minor any more: Implications for the pathogenesis of motor neuron diseases

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To explore the molecular pathogenesis of amyotrophic lateral sclerosis (ALS), the nuclear function of TAR-DNA binding protein 43 kDa (TDP-43) must be elucidated. TDP-43 is a nuclear protein that colocalizes with Cajal body or Gem in cultured cells. Several recent studies have reported that the decreasing number of Gems accompanied the depletion of the causative genes for ALS, *TDP-43* and *FUS*. Gems play an important role in the pathogenesis of spinal muscular atrophy. Gems are the sites of the maturation of spliceosomes, which are composed of uridylate-rich (U) snRNAs (small nuclear RNAs) and protein complex, small nuclear ribonuclearprotein (snRNP). Spliceosomes regulate the splicing of pre-mRNA and are classified into the major or minor classes, according to the consensus sequence of acceptor and donor sites of pre-mRNA splicing. Although the major class of spliceosomes regulates most pre-mRNA splicing, minor spliceosomes also play an important role in regulating the splicing or global speed of pre-mRNA processing. A mouse model of spinal muscular atrophy, in which the number of Gems is decreased, shows fewer subsets U snRNAs. Interestingly, in the central nervous system, U snRNAs belonging to the minor spliceosomes are markedly reduced. In ALS, the U12 snRNA is decreased only in the tissue affected by ALS and not in other tissues. Although the molecular mechanisms underlying the decreased U12 snRNA resulting in cell dysfunction and cell death in motor neuron diseases remain unclear, these findings suggest that the disturbance of nuclear bodies and minor splicing may underlie the common molecular pathogenesis of motor neuron diseases.

Key words: amyotrophic lateral sclerosis, gemini of coiled bodies, RNA splicing, spliceosomes, U12 small nuclear RNA.

INTRODUCTION

Motor neuron system selectivity is a major mystery of motor neuron diseases. Although research has shown that the pathology is not restricted to motor neurons but also extends into other neurons as well as glial cells, the selective vulnerability of motor neurons is a characteristic feature of amyotrophic lateral sclerosis (ALS). However, the molecular mechanism underlying the vulnerability of the motor neuron system has not been fully explained. To clarify this issue, researchers must clarify what distinguishes the motor neuron.

Researchers have identified several molecular markers and physiological characters that distinguish motor neurons from others.[1] However, the morphology and location of the cell have been used as the most significant signature for identifying motor neurons in tissues. The cells of the CNS are diverse and complex, and they are mostly defined by their shape, size and location in the tissues. The complexity of the cells reflects the complexity of the cells' RNAs. The diversity of RNAs results in part from the methylation of DNA, but studies have shown that other mechanisms also control cell-specific RNA diversity.

A higher structure of the nucleus, chromatin, and nuclear bodies, is another mechanism that regulates the cell-specific RNA diversity. Recent findings have revealed that chromatin has a unique structure and location in the nucleus in each type of cell. The chromatin structure is strongly associated with the diversity of RNA.[2] Moreover,

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the other intranuclear structures also play an important role in maintaining cell function and cell survival. Thus, the intracellular location or character of nuclear bodies may also differ in each cell type.

In 1906, Ramón y Cajal won the Nobel Prize for his description of the intranuclear fine structure.[3] In the nucleus, he identified several distinct structures, including the Cajal body. It has taken a long time to understand the functions of these intranuclear structures. However, little research has been conducted to clarify the differences of nuclear bodies in each cell type or in healthy versus pathogenic conditions. To clarify the molecular mechanisms underlying the systemic pathology of neurodegenerative disorders, we must investigate the nucleus structure and related functions, which might help us to determine the unique characteristics of motor neurons. In this review, we first focus on the alteration of nuclear bodies in ALS and then discuss the association between a disturbance of uridylate-rich (U) small nuclear (sn)RNA and motor neuron diseases.

ALS IS A TDP-43 PROTEINOPATHY

Disease-specific intra- and extracellular inclusions serve as the diagnostic signature for each neurodegenerative disorder. In particular, the identification of the component proteins has changed our concepts about several neurodegenerative disorders. For example, the common identification of synuclein in several types of neurodegenerative diseases has led them to be known as synucleinopathy, including olivopontocerebellar degeneration, striatonigral degeneration, Parkinson disease and diffuse Lewy body disease. Recently, the identification of trans-activation response DNA protein 43 (TDP-43) as a component protein in ubiquitin-positive inclusions in ALS and frontotemporal lobar degeneration, has led to the classification of TDP-43 proteinopathy.[4,5] The identification of the *TARDBP* gene for TDP-43 mutation in both familial and sporadic ALS patients whose neuropathological findings are identical to those in sporadic ALS indicates that TDP-43 plays a fundamental role in the pathogenesis of not only ALS with *TARDBP* mutation but also that of sporadic ALS.[6–8]

In healthy cells, TDP-43 is a ubiquitously expressed nuclear protein that forms some bodies in the nucleus.[9,10] Under stress conditions, some TDP-43 moves to stress granules in the cytoplasm.[11] In ALS, TDP-43 forms cytoplasmic inclusions, which are phosphorylated, and then disappear from the nucleus.[12–14] These characteristic pathological findings may underlie the molecular pathogenesis of ALS. Although the molecular mechanism of the transport of TDP-43 to cytoplasm and the formation of inclusions is unclear, researchers have speculated that the disappearance of nuclear TDP-43 might precede the formation of visible cytoplasmic inclusions or abnormal modification, phosphorylation or ubiquitination of TDP-43.[13–15] These findings raise two possibilities regarding the pathogenesis of ALS: (i) the obtaining of toxic function by cytoplasmic inclusions; or (ii) the loss of the normal nuclear function of TDP-43.[14,15] The model animals deleting TDP-43 are embryonically lethal, indicating that TDP-43 is a fundamental protein in the maintenance of cell function and survival.[16] Therefore, the loss of nuclear function in affected neurons in ALS might cause cell dysfunction or cell loss. However, it is not clear how the loss of TDP-43 results in cell dysfunction or cell loss.

RNA METABOLISM AND TDP-43

TDP-43 was first identified as a protein that binds to DNA, and it is now considered to regulate RNA metabolism.[17] Using a method that identifies the mRNA binding to a specific protein, many RNAs that might be regulated by TDP-43 have been identified.[18,19] These studies have shown that TDP-43 binds to long mRNA molecules with large introns and regulates the splicing and amounts of mRNA in several ways.[18,19] Consequently, the depletion of TDP-43 might alter pre-mRNA metabolism. Indeed, the alteration of RNA profiles has been reported from cultured cells and model animals with depleted TDP-43.

In ALS, alterations of mRNA expression profiles have been reported,[20–22] although the association between TDP-43 and these alterations of mRNA observed in ALS remain to be clarified. To our knowledge, *POLDIP3* is the only gene in which the splicing is directly regulated by TDP-43 and is altered in spinal motor neurons with ALS but not in brain with frontotemporal lobar degeneration.[23,24] In addition, immunohisotochemical analysis indicated that several genes processed by TDP-43 express key molecules for function or survival of spinal motor neurons and show decreasing amounts of products.[25] However, it is unclear how the function of TDP-43 correlates with the depletion of these products. Thus, the specific functions of TDP-43 have not been fully evaluated *in vitro* or in ALS patients. These disturbances of RNA metabolism might not be explained simply by the loss of TDP-43 function on pre-mRNA. Therefore, some researchers have speculated that TDP-43 serves another function associated with RNA metabolism.[26] TDP-43 forms foci in the nucleus and associates with several nuclear bodies, suggesting that TDP-43 plays a role in the functioning of nuclear bodies.

CAJAL BODIES AND GEMS

Nuclear bodies are classified and identified by their unique protein components.[27] In addition, most of these bodies are

tightly associated with a unique RNA and regulate that particular RNA metabolism.[28,29] In contrast to cytoplasmic organelles, nuclear bodies do not have a membranous structure that separates their contents from nucleoplasm. Thus, the components of nuclear bodies are frequently exchanged between the bodies and the nucleoplasm. The dynamism of the components is a unique characteristic of nuclear bodies. The protein components decrease their mobility in nuclear bodies as compared to that in nucleoplasm. Thus, the bodies are recognized based on the increased concentration of the component protein.

The nucleolus and Cajal bodies are the most well-known nuclear bodies. The nucleolus is the center for maturation of rRNA, whereas Cajal bodies are sites for the maturation of U snRNAs and consist of coilin.[30–33] In addition, the causative protein for spinal muscular atrophy (survival of motor neuron: SMN) forms a body beside the coilin immunopositive body. Therefore, the foci stained by anti-SMN antibody have been designated as Gemini of the Cajal body, or Gems. However, coilin and SMN are colocalized in most of the cell. Therefore, these bodies are indistinguishable in most cell types.[30]

DECREASING THE NUMBER OF GEMS IN SPINAL MOTOR NEURONS WITH ALS

It has been reported that Gems are partly colocalized with TDP-43 bodies in cultured cells.[9] In human spinal motor neurons, some Gems are stained with TDP-43, but not all of them.[34] In addition, the depletion of TDP-43 decreases the number of Gems in HeLa cells and mouse spinal motor neurons.[34,35] A decrease in the number of Gems is also observed in spinal muscular atrophy.[36] Thus, we hypothesized that the loss of nuclear TDP-43 may result in a decrease in the number of Gems in spinal motor neurons with ALS as well. Indeed, our group and others have found that the number of Gems decreased in spinal motor neurons with ALS.[34,37]

However, surprisingly we found that the number of Gems was decreased in spinal motor neurons that still contained nuclear TDP-43.[34] This result raises the possibility that the decreasing number of Gems precedes the alteration of TDP-43. However, in spinal motor neurons with spinal muscular atrophy, no alteration of TDP-43 has been reported, suggesting that the alteration of TDP-43 precedes the decrease in the number of Gems. Therefore, we propose that disturbance of a function of TDP-43 associated with the formation of Gems precedes the disappearance of TDP-43 from the nucleus (Fig. 1a–c). Accumulating evidence suggests that the disappearance of nuclear TDP-43 precedes the inclusion formation of TDP-43 (Fig. 1d,e).[14] Although the mechanism for the disappearance of nuclear TDP-43 is unclear, the dysfunction of TDP-43 might precede their disappearance from the nucleus. Research has shown that TDP-43 regulates its own amounts of product by affecting its own mRNA.[18,38] Thus, the decreasing amount of nuclear TDP-43 should induce the production of more TDP-43. However, in spinal motor neurons with ALS, nuclear TDP-43 disappears. Therefore, these cells lose TDP-43 function, which is associated with pre-mRNA splicing, including the autoregulation mechanism (Fig. 1a–g).

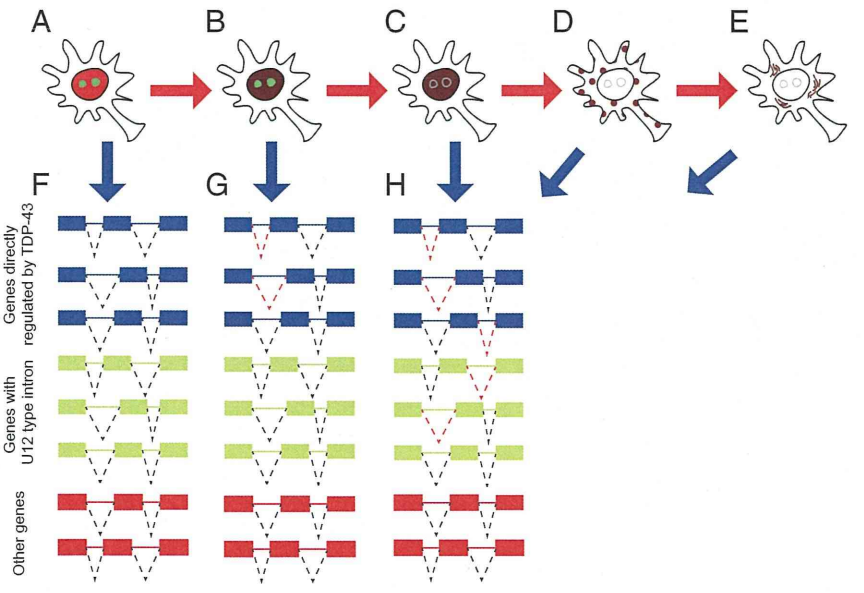

Fig. 1 Splicing alteration and nuclear pathology in ALS. (a–e) TAR-DNA binding protein 43 kDa (TDP-43) and Gem pathology in ALS. (a) Normal neurons show (TDP-43 immunoreactivity (shown in red) with Gems (green circles). (b) TDP-43 alters its property and loses its normal functions of splicing pre-mRNAs and facilitating the formation of Gems (altered TDP-43 is shown in brown). (c) Gems disappear (gray circles). (d) So-called pre-inclusions, which consist of granular cytoplasmic aggregates that are positive for phospho-TDP43 epitopes (p409 and p410), are formed (brown circles), and nuclear TDP-43 disappears. (e) Skein-like inclusions in cytoplasm (brown lines). (f–h) Alteration in the splicing at each stage. (f) Genes in which splicing is directly regulated by TDP-43 (blue), genes containing U12 type introns (green), and the other genes (red). Boxes represent exons and lines represent introns. Dashed lines represent splicing. (g) In neurons with altered TDP-43 (b), some of the splicing regulated by TDP-43 is disturbed (red dashed lines for blue exon and introns). (h) In neurons that lose Gems (c–e), some of the splicing of U12-type introns is also disturbed (red dashed lines for green exons and introns).

We must consider the possibility that the decreasing number of Gems results from the decreasing number of large motor neurons in ALS, because the number of Gems is positively correlated with the size of the cell.[39,40] Moreover, large motor neurons are more vulnerable to ALS than small ones.[41] To rule out this possibility, multiple regression analysis should be conducted to investigate whether ALS is an independent factor determining the number of Gems regardless of cell size.

THE MOLECULAR MECHANISM FOR THE DECREASE IN THE NUMBER OF GEMS

If our hypothesis is correct, the next question is whether the decreasing number of Gems results from a direct or indirect function of TDP-43. The number of Gems also declines due to decreasing transcriptional activity.[39,42,43] Therefore, it is possible that the alteration of TDP-43 suppresses the transcriptional activity, resulting in the decreasing number of Gems. However, in contrast to ALS, the number of Gems does not decrease in the spinal motor neurons in other motor neuron diseases.[34] Thus, in human spinal motor neurons, the nonspecific alteration of Gems resulting from the suppression of transcriptional activity is less likely. Therefore, we speculate that the alteration of TDP-43 directly decreases the number of Gems.

Another important question is how TDP-43 is associated with the formation of Gems. Two hypotheses have been proposed for the formation of nuclear bodies: (i) ordered assembly of the component proteins; or (ii) stochastic assembly, in which component proteins accumulate in an unordered manner at specific RNA or the complex of core proteins.[27–29,44,45] Although the process of how nuclear bodies are formed remains unclear, there are several indispensable component proteins in each body. Thus, two possible molecular mechanisms exist for decreasing the number of Gems by depletion of TDP-43: (i) the depletion of TDP-43 alters the mRNA of the component proteins of Gem; or (ii) TDP-43 directly contributes to the formation of Gems, such that its depletion results in fewer Gems.

With regard to the first possibility, it has been reported that TDP-43 regulates the alternative splicing of SMN. The depletion of TDP-43 increased the SMN splicing variant excluding exon 7 in a reporter system.[46] The SMN excluding exon 7 is less stable than SMN with exon 7, resulting in less SMN product.[47] Indeed, we found that the amount of SMN proteins decreased due to the depletion of TDP-43.[34] However, we were unable to confirm the increase in the SMN splicing variants excluding exon 7 in intrinsic SMN mRNA by depletion of TDP-43. Instead of the alteration of splicing variants, we found that the SMN mRNA decreased in the cells with depleted TDP-43, suggesting that the depletion of TDP-43 induces additional splicing, and the splicing isoform may be less stable than canonical SMN mRNA. However, we were unable to detect the additional splicing variants, which may contribute to the reduced amount of SMN mRNA. Moreover, researchers have not fully evaluated whether the SMN protein or mRNA are reduced in tissues affected with ALS.[48] Therefore, although the intrinsic SMN protein is reduced in cultured cells with the depletion of TDP-43, it is not clear that this is the mechanism underlying the reduction of SMN in tissue affected by ALS.

Next, we hypothesized that TDP-43 binds to the component proteins of Gem and increases their stability. Indeed, the protein–protein interaction between TDP-43 and SMN has been reported in a forced expression system,[9,37,49] although the result is controversial.[34] In addition, comprehensive analysis of binding proteins to TDP-43 using mass spectrometry failed to identify SMN or other component proteins of Gem.[50] Therefore, if an association exists between TDP-43 and Gems, it might be temporary, as is common in the components of nuclear bodies. Because these intranuclear structures do not have a membrane, the components of nuclear bodies and nuclear structures can rapidly interact. Many components of nuclear bodies change quickly, and an increased retention time of each component at a place represents foci.[27,51] Therefore, the interaction should be regulated temporally and rapid dissociation depends on the circumstance.

Finally, we examine the possibility that TDP-43 directly contributes to the formation of Gems. In TDP-43-depleted cells, a substantial number of Gems were still observed, whereas TDP-43 was not detected in the nucleus or Gems.[34] In addition, not all Gems include TDP-43 in cultured cells and normal spinal motor neurons.[34] Moreover, the size of each Gem was similar between control and ALS cells.[34] These results clearly indicate that TDP-43 is not a necessary component for all types of Gems. Thus, we propose two possibilities regarding the contribution of TDP-43 in the formation of Gems: (i) TDP-43 contributes to the formation of Gems only at a specific stage during their maturation (Fig. 2a); or (ii) TDP-43 is associated with only a subtype of Gems, but not all Gems (Fig. 2b). Interestingly, the overexpression of TDP-43 also decreased the number of Gems in the cultured cells,[34] indicating that the proper amount of each component is important for maintaining the number of Gems.

WHAT IS THE CONSEQUENCE OF THE DECREASING NUMBER OF GEMS?

One outcome of a decrease in the number of Gems can be speculated based on the molecular mechanism underlying

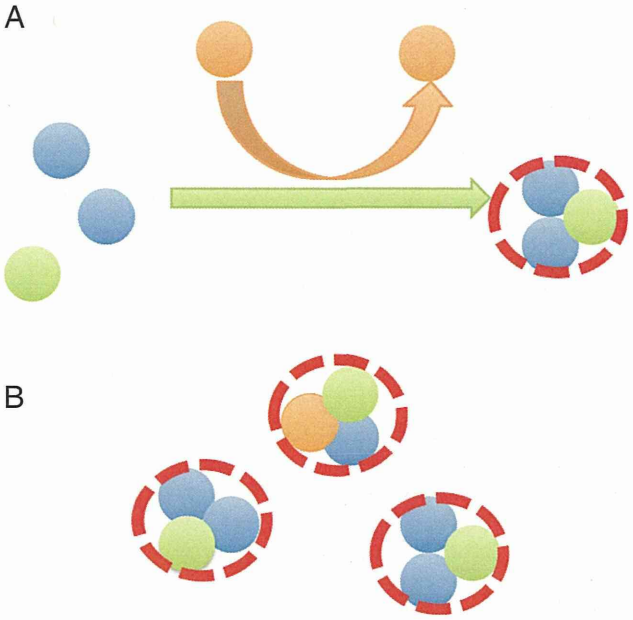

Fig. 2 Mechanisms of how TAR-DNA binding protein 43 kDa (TDP-43) contributes to Gem assembly. (a) TDP-43 contributes to the formation of Gems only at a specific stage during their maturation. TDP-43 may facilitate the formation of a Gem or associate with the maturation of a Gem. Thus TDP-43 temporarily binds to the Gem components. After Gems are formed, TDP-43 exits the complex. (b) The components differ among Gems, and TDP-43 is a component in only a subset of Gems. Gem (red dashed circle), TDP-43 (orange circle), survival of motor neuron (SMN) (green circle), other components (blue circle).

spinal muscular atrophy. Gems are the sites of assembly and maturation of snRNP.[29,31,52] In the assembly of snRNP, SMN first forms a dimer and directly binds to Gemin 2, 3 and 8 and indirectly binds to Gemin 4, 5, 6 and 7 and unrip.[53] This SMN complex then binds to the Sm complex and U snRNA and transports them into the nucleus.[47] At the Gems, additional proteins are assembled to snRNPs and U snRNAs are modified, consequently forming a spliceosome, which functions for pre-mRNA splicing. In addition, Gems accumulate at most U snRNA genes.[30] These findings suggest that the Gems may regulate the quality as well as the quantity of the U snRNA. Therefore, researchers have speculated that the depletion of SMN or Gems may result in decreasing amounts of SMN complex, snRNPs and U snRNAs. Indeed, Gemin 2, 3 and 8 are decreased in SMN-depleted cells and tissues.[54,55] In addition, the assembly of snRNP is also disrupted in these cells and tissues. Furthermore, a subset of U snRNA is decreased in the affected tissues in spinal muscular atrophy.[47,54] The U snRNAs are involved in the splicing machinery, the spliceosome, and are categorized into major and minor classes depending on the consensus sequences of the donor and acceptor splice sites of the introns.[56] Most of the splicing is regulated by major spliceosomes, whereas less than 1% is regulated by minor spliceosomes. In spinal muscular atrophy, U snRNA of the minor spliceosomes is markedly decreased in the CNS but not in other organs.[47,54]

Because we found decreased amounts of SMN in TDP-43-depleted cultured cells and fewer Gems in the spinal motor neurons with ALS, we speculated that the amounts of SMN complex, snRNPs and U snRNAs were decreased in TDP-43-depleted cells and tissues affected with ALS. As expected, a subset of Gemins were decreased in TDP-43-depleted cells and a subset of U snRNA was decreased in a subtype of cultured cells.[34] Among them, U12 snRNA, belonging to the minor spliceosome class, was decreased in the tissue with TDP-43 pathology but not in tissue without TDP-43 pathology. The repertoires of U snRNAs are not identical between cultured cells depleted of SMN and TDP-43, indicating that the contribution of each protein to the maturation of U snRNAs is different. Finally, immunohisotochemical analysis revealed that the amounts of snRNPs belonging to minor spliceosomes decreased in spinal motor neurons with ALS. These findings are consistent with the previous results obtained using a SMN-reduced mouse model.[54,55] However, another group reported that increased subtypes of U snRNAs and snRNPs accompanied the decreasing number of Gems in tissues affected with ALS.[37] Therefore, it is still unclear what type of alteration in U snRNA and snRNPs occurs in ALS.

The vulnerability of U snRNA belonging to the minor spliceosome class might be explained by the difference in the number of genes between U snRNAs belonging to major versus minor spliceosomes.[57] The genes for major spliceosomes are multicopy genes, whereas most of the genes encoding minor spliceosome U snRNAs have only a single copy. Therefore, because Gems contribute to the transcription and maturation of U snRNA, a decreasing number of Gems would have a proportionally greater effect on the expression of U snRNA belonging to the minor spliceosome class.

However, the specific decline of U snRNA in spinal muscular atrophy cannot be explained simply by the number of genes for U snRNA. Because the amount of SMN, which is a ubiquitously expressed protein, is decreased in all tissues in a spinal muscular atrophy model mouse, the minor spliceosome U snRNA is decreased selectively in the spinal cord.[54] Moreover, the disturbance of the repertoires of U snRNA differs depending on the cell type and tissues.[54] These results clearly indicate that the contribution of SMN to the regulation of U snRNA differs among cell types. These findings suggest that the maturation system for minor spliceosome snRNP is more

vulnerable to the depletion of SMN in cells of the motor neuron system as compared to other systems.

DOES DISTURBANCE OF U snRNA CAUSE DEGENERATION IN THE SELECTIVE SYSTEM?

How does the disturbance of U snRNAs belonging to the minor spliceosome class cause motor neuron death? The U snRNAs recognize the donor branch site sequence and contribute to pre-mRNA splicing. Although minor spliceosomes are involved in less than 1% of the splicing, they are involved in the splicing of "information function" genes, including genes associated with the replication, repair and translation of DNA: transcription and ion channels.[56] In addition, the splicing regulated by minor spliceosomes is a rate-limiting factor in the gene-splicing process.[56,58] The speed of splicing alters the splicing as well as the stability of mRNA. Therefore, the disturbance of minor spliceosomes may affect the quality and quantity of many genes (Fig. 1f–h). Indeed, the mutation of U4atac gene, the product of which is a key component of minor spliceosome, contributes to systemic developmental and degenerative disorders,[59–62] indicating that all tissues are vulnerable to the alteration of minor spliceosomes. However, patients with the U4atac gene mutation with a less severe phenotype do not show motor neuron disease.[63] This result clearly indicates that selectivity in the motor neuron system cannot be explained simply by the vulnerability of the motor neuron system to the alteration of minor spliceosomes.

Decreasing U12 snRNA may explain the selectivity in the motor neuron system. Interestingly, mutation of the U2 snRNA gene causes selective granule cell loss in mice.[64] This is surprising for two reasons. First, U2 snRNA is involved in the major spliceosome, which is fundamental machinery for pre-mRNA splicing. Second, although the gene for U2 snRNA is a multicopy, one of the U2 snRNA genes causes selective neurodegeneration. This may explain why the granular cell is more vulnerable to the depletion of U2 snRNA. However, the finding that the each U1 snRNA gene, which is also a multicopy, selectively regulates a subset of targeted genes suggests that each U2 snRNA gene may have a unique property for maintaining a specific type of splicing in specific cells.[65] Indeed, studies using a spinal muscular atrophy *Drosophila* model suggested that alteration of the splicing of U12 type intron in the specific gene in the intermediate and sensory neurons may result in selective motor neuron death.[66–68] Although the system selectivity in ALS may be explained by the limited TDP-43 pathology, it would be interesting to investigate whether alterations of the specific gene, which is regulated by minor spliceosomes, may underlie the pathogenesis of ALS.

CONCLUSION

Because the RNA-associated proteins have been identified as causative proteins for ALS as well as spinal muscular atrophy, the disturbance of RNA metabolism may underlie the pathogenesis of motor neuron diseases. In particular, the decline of minor spliceosome U snRNA in spinal muscular atrophy and ALS suggest the existence of a common molecular mechanism in motor neuron diseases. In addition, the evidence of alterations in the nuclear structure in ALS opens a new avenue for the study of neurodegenerative disease. Interestingly, it has been reported that product of *FUS*, another causative gene for ALS, interacts with SMN, and the number of Gems decreased in cultured cells depleted of FUS.[49] Moreover, the number of Gems decreased in fibroblasts with causative mutations in *FUS* and *TARDBP*.[49] Although the significance of the decreasing number of Gems in the affected tissues with *FUS* mutation has yet to be evaluated, this finding reinforces the importance of Gems in ALS.

The fine structure of the nucleus, including the nuclear bodies, might play an important role in regulating cell-specific RNA metabolism. For example, Hutchinson-Gilford progeria syndrome is caused by a mutation in *LMNA*.[69] Lamin A, a product of *LMNA*, is a dense network inside the nucleus and participates in chromatin organization.[70–72] Although the mutated lamin A may disturb the function of the nuclear membrane, the mutated lamin also affects chromatin organization and RNA metabolism, resulting in cell death.[69] In addition, the nuclear bodies have more diversity than expected. The diversity and dynamics of nuclear body components might be investigated more fully in each neuron, and neurons or glial cells in neurodegenerative disorders. In addition, the location of a nuclear body in association with other nuclear bodies may be important in the regulation of RNA metabolism. Little research has been conducted on the differences in the nuclear structure between various types of healthy and pathological cells. Closer investigation of the nucleus may help to elucidate the complex system underlying the regulation of cell identity and clarify the motor neuron system pathology of ALS.

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