



# Impaired Nucleoporins Are Present in Sporadic Amyotrophic Lateral Sclerosis Motor Neurons that Exhibit Mislocalization of the 43-kDa TAR DNA-Binding Protein

Hitoshi Aizawa<sup>a</sup>  
Takenari Yamashita<sup>b</sup>  
Haruhisa Kato<sup>a</sup>  
Takashi Kimura<sup>c</sup>  
Shin Kwak<sup>b</sup>

<sup>a</sup>Department of Neurology,  
Tokyo Medical University,  
Tokyo, Japan

<sup>b</sup>Division of Clinical Biotechnology,  
Center for Disease Biology and  
Integrative Medicine,  
Graduate School of Medicine,  
The University of Tokyo, Tokyo, Japan

<sup>c</sup>Department of Neurology,  
Asahikawa Medical Center,  
National Hospital Organization,  
Asahikawa, Japan

**Background and Purpose** Disruption of nucleoporins has been reported in the motor neurons of patients with sporadic amyotrophic lateral sclerosis (sALS). However, the precise changes in the morphology of nucleoporins associated with the pathology of the 43-kDa TAR DNA-binding protein (TDP-43) in the disease process remain unknown. We investigated the expression of nucleoporins that constitute the nuclear pore complex (NPC) in spinal motor neurons that exhibit sALS in relation to TDP-43 pathology, which is a reliable neuropathological hallmark of sALS.

**Methods** Paraffin-embedded sections of the lumbar spinal cord were obtained for immunofluorescence analysis from seven control subjects and six sALS patients. Anti-TDP-43 antibody, anti-nucleoporin p62 (NUP62) antibody, and anti-karyopherin beta 1 (KPNB1) antibody were applied as primary antibodies, and then visualized using appropriate secondary antibodies. The sections were then examined under a fluorescence microscope.

**Results** NUP62 and KPNB1 immunoreactivity appeared as a smooth round rim bordering the nuclear margin in normal spinal motor neurons that exhibited nuclear TDP-43 immunoreactivity. sALS spinal motor neurons with apparent TDP-43 mislocalization demonstrated irregular, disrupted nuclear staining for NUP62 or KPNB1. Some atrophic sALS spinal motor neurons with TDP-43 mislocalization presented no NUP62 immunoreactivity.

**Conclusions** Our findings suggest a close relationship between NPC alterations and TDP-43 pathology in the degenerative process of the motor neurons of sALS patients.

**Key Words** sporadic amyotrophic lateral sclerosis, nucleoporin, nuclear pore complex, 43-kDa TAR DNA-binding protein.

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## Correspondence

Hitoshi Aizawa, MD, PhD  
Department of Neurology,  
Tokyo Medical University,  
6-7-1 Nishishinjuku, Shinjuku-ku,  
Tokyo 160-0023, Japan  
**Tel** +81-3-3342-6111  
**Fax** +81-3-3342-6271  
**E-mail** haizawa@tokyo-med.ac.jp

## INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is the most common adult-onset motor neuron disease, and is characterized by progressive muscle atrophy and weakness that eventually results in respiratory failure. Sporadic ALS (sALS) comprises more than 90% of all ALS cases. Although more than 30 ALS-associated genes have been reported, the mechanism of motor neuronal degeneration remains unclear. Nuclear pore complexes (NPCs) are central to the process of nucleocytoplasmic transport, and they are scattered over the nuclear membrane. Each NPC is assembled from approximately 30 evolutionarily conserved proteins known as nucleoporins.<sup>1</sup> Recent studies have found that the disruption of nucleocytoplasmic transport through the NPC is a potential cause of neuronal death in *C9orf72* mouse

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models of ALS.<sup>2-4</sup> Moreover, morphological changes in the nuclear membrane are associated with the disruption of nucleoporins in the motor neurons of patients with sALS or SOD1-associated ALS.<sup>5-7</sup> In addition, mutations in the gene encoding GLE1 (a nucleoporin) have been reported in patients with ALS.<sup>8</sup>

Disruption of nucleoporins has been reported in an sALS mouse model (ADAR2<sup>fllox/fllox</sup>/VAcHT-Cre or AR2 mice),<sup>9</sup> in which the adenosine deaminase acting on RNA 2 (ADAR2) was conditionally knocked out.<sup>10</sup> Analogous changes in nucleoporins have also been observed in preliminary studies of the ADAR2-deficient motor neurons of sALS patients.<sup>10</sup> ADAR2 regulates Ca<sup>2+</sup> influx through  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptors via adenosine-to-inosine conversion at the glutamine/arginine site of GluA2 mRNA, which makes ADAR2 is a key factor in acquired Ca<sup>2+</sup> resistance in motor neurons. Disease-specific and site-selective deficiency of ADAR2 has been demonstrated in sALS motor neurons,<sup>11-13</sup> and ADAR2 deficiency is closely associated with the death of motor neurons in conditional ADAR2-knockout mice.<sup>9</sup> Notably, ADAR2 down-regulation along with pathology of the 43-kDa TAR DNA-binding protein (TDP-43) that is one of the most-reliable pathological hallmarks of ALS<sup>14,15</sup> have been observed concomitantly in the motor neurons of ALS patients.<sup>16</sup> The mechanism underlying the co-occurrence of these two molecular abnormalities within the same neurons involves an exaggerated Ca<sup>2+</sup> influx and activation of calpain (a Ca<sup>2+</sup>-dependent cysteine protease) in the ADAR2-deficient motor neurons, and the resultant calpain-dependent TDP-43 fragments serve as seeds for TDP-43 aggregation.<sup>17</sup> These findings suggest that ADAR2 deficiency is involved in the pathophysiology of motor neuron degeneration in sALS.

TDP-43 pathology is invariably observed in motor neurons that are devoid of ADAR2.<sup>16</sup> We suspect that the precise morphological relationship between the formation of TDP-43 aggregation and the alteration of nucleoporin expression in sALS motor neurons further reflects the role of nucleoporins in ALS. In this study we examined the expression of nucleoporin p62 (NUP62), a central nucleoporin containing phenylalanine-glycine repeats, and karyopherin beta 1 (KPNB1), a nucleocytoplasmic transport master regulatory protein,<sup>18</sup> in relation to TDP-43 pathology.

## METHODS

### Human subjects

Spinal cords were obtained from seven control cases and six sALS patients after autopsy. The cases used in this study are listed in Table 1. Written informed consent for performing

**Table 1.** sALS and control cases that provided specimens for this study

Case no.	Sex	Age (years)	Diagnosis
A1	Male	75	sALS
A2	Male	62	sALS
A3	Male	69	sALS
A4	Male	69	sALS
A5	Female	49	sALS
A6	Male	54	sALS
C1	Male	64	Cerebellar tumor
C2	Male	58	MD
C3	Male	70	LGM
C4	Male	73	NN
C5	Male	77	LGM
C6	Female	69	MD
C7	Male	78	Meningitis

LGM: limb-girdle muscular dystrophy, MD: myotonic dystrophy, NN: nonneurological condition, sALS: sporadic amyotrophic lateral sclerosis.

the autopsy and approval to use autopsy tissue specimens for research purposes was obtained from each patient or their family. The research protocol used in this study was approved by the Institutional Human Ethics Committee of Tokyo Medical University (No. 3374).

### Immunofluorescence analysis

Upper-, middle-, and lower-level lumbar spinal-cord sections were obtained from human ALS patients and controls, then fixed in 10% neutral buffered formalin for approximately 7 days. They were then embedded in paraffin, and 4- $\mu$ m-thick sections were deparaffinized in xylene and hydrated in a graded series of ethanol solutions. The sections were heated in citrate buffer (pH 6.0) at 120°C for 10 min to allow antigen retrieval, then washed with phosphate-buffered saline (PBS) and incubated with a primary antibody overnight at 4°C. The sections were then washed with PBS, and incubated with a secondary antibody at room temperature for 1 h.

The following primary antibodies were used: rabbit polyclonal and mouse anti-TDP-43 (1:3,000 dilution of 10782-2-AP, Proteintech Group Inc., Rosemont, IL, USA; and 1:3,000 dilution of H00023435-M01, Abnova Co., Taipei, Taiwan), mouse anti-NUP62 (1:200 610497, BD Biosciences Inc., Franklin Lakes, NY, USA) and rabbit anti-KPNB1 (1:200 sc-11367, Santa Cruz Biotechnology Inc., Dallas, TX, USA). The following species-appropriate secondary antibodies were used: Alexa Fluor 488, 594, or 633 secondary antibodies at 1:1,000 dilution (Invitrogen Inc, Waltham, MA, USA). The sections were stained with DAPI (D1306, Molecular Probes Inc., Eugene, OR, USA) for 5 min at room temperature for nuclear staining before being mounted onto slides with an antifade reagent (Fluoromount/Plus K048, Diagnostic Biosystems Inc.,

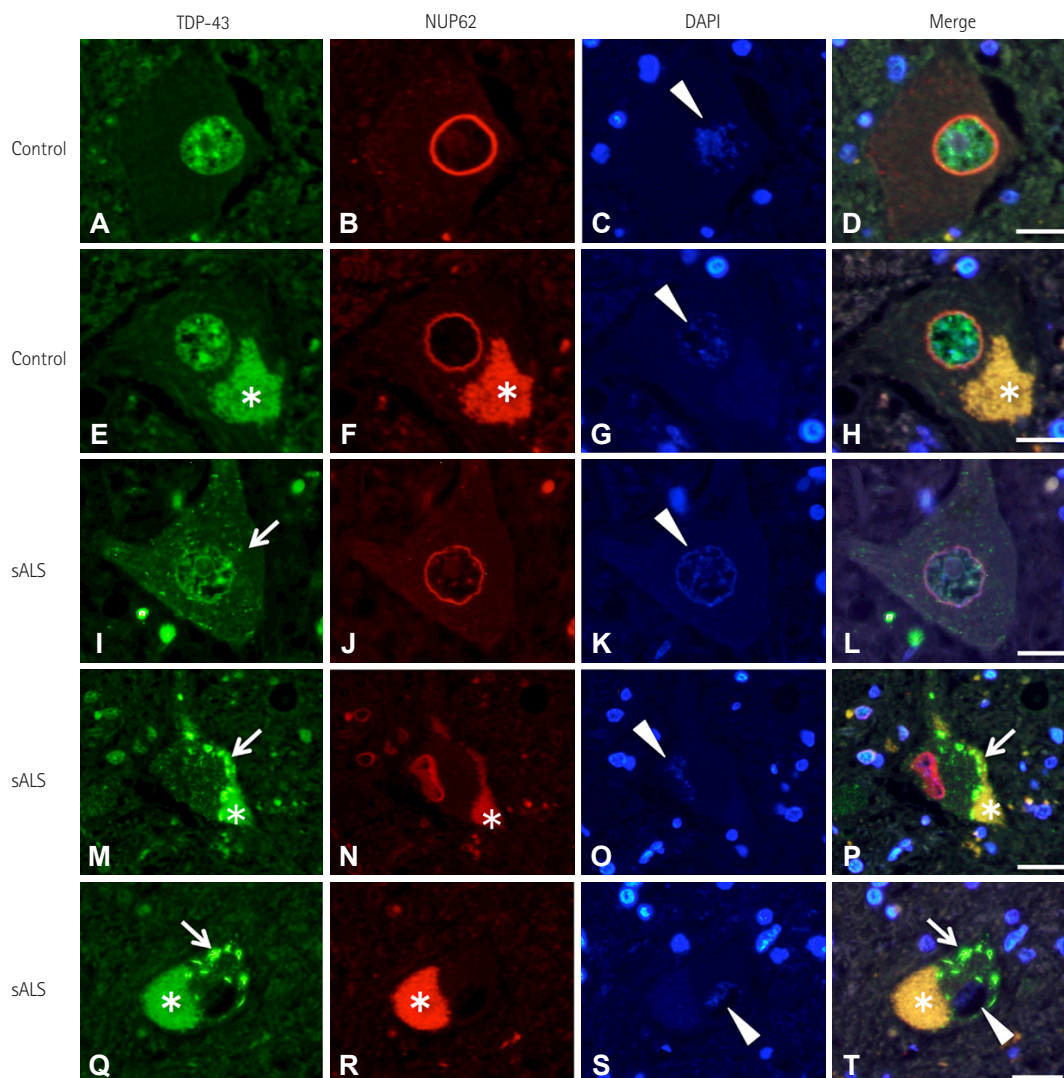
Pleasanton, CA, USA). The sections were then examined under a fluorescence microscope (BIOZERO BZ-8100, Keyence Corp., Osaka, Japan).

Three levels of lumbar sections from each subject were used to quantify TDP-43 and NUP62 immunoreactivity in the motor neurons. On each section, neurons with nucleoli in the anterior horns were counted separately. Statistical analyses (*t*-tests) were performed with IBM SPSS Statistics software (version 24; IBM Corp., Armonk, NY, USA). Data presented are mean±SD values.

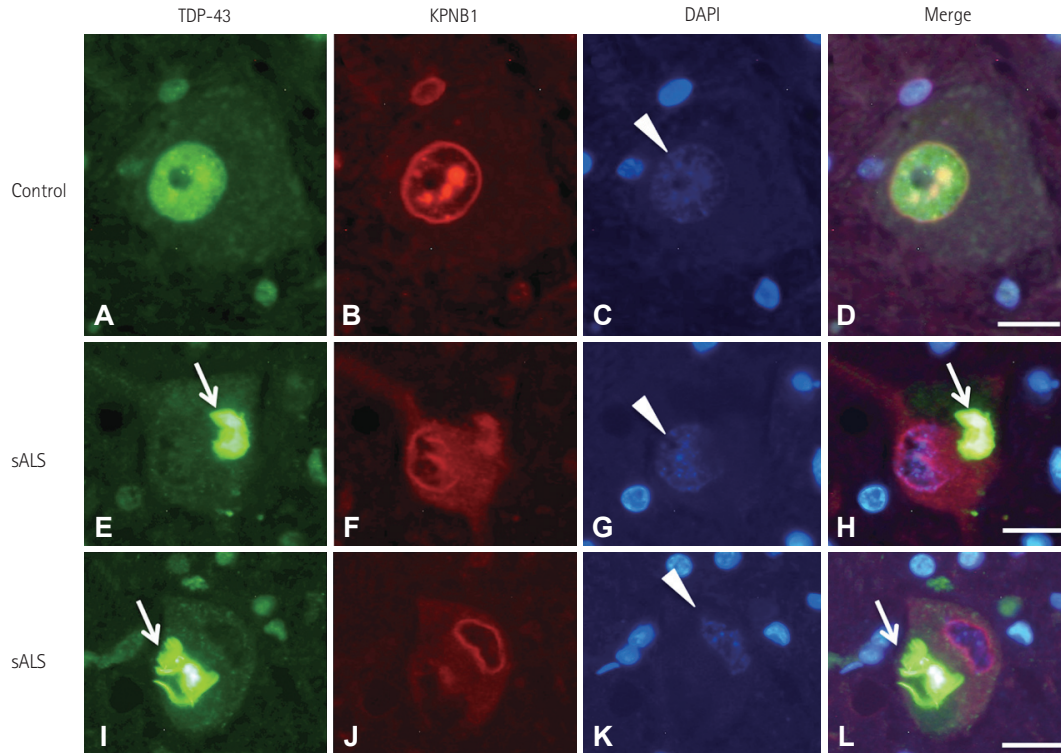
## RESULTS

### Detection of TDP-43 and NPC proteins by immunofluorescence staining

Immunofluorescence staining revealed that in the normal spinal motor neurons, TDP-43 was localized in the nucleus while NUP62 immunoreactivity appeared as a smooth round rim bordering the nuclear margin (Fig. 1A–H). In the sALS spinal anterior horn, some motor neurons exhibited decreased nuclear TDP-43 immunoreactivity with fine cytoplasmic TDP-43-positive inclusions (Fig. 1I); these neurons also demonstrated circular NUP62 immunoreactivity (Fig. 1J). Spinal motor neurons that had apparent TDP-43 mislocalization



**Fig. 1.** TDP-43 and NUP62 immunoreactivity in control and sALS spinal motor neurons. TDP-43 was localized to the nucleus (arrowheads) and NUP62 immunoreactivity was present along the nuclear membrane in control spinal motor neurons (A–H). Motor neurons with decreased nuclear TDP-43 (arrowhead) exhibited fine TDP-43-positive dots (arrow) within the cytoplasm in sALS (I–L). NUP62 was present in the disrupted nuclear membrane of sALS spinal motor neurons with apparent TDP-43 mislocalization (arrows, arrowhead) (M–P). NUP62 immunoreactivity was absent (arrowheads) in some of the atrophic sALS spinal motor neurons with apparent TDP-43 mislocalization (arrows) (Q–T). Scale bars indicate 30  $\mu$ m. \*Lipofuscin. DAPI: 4',6-diamidino-2-phenylindole, NUP62: nucleoporin p62, sALS: sporadic amyotrophic lateral sclerosis, TDP-43: 43-kDa TAR DNA-binding protein.



**Fig. 2.** TDP-43 and KPNB1 localization in control and ALS spinal motor neurons. TDP-43 was localized in the nucleus (arrowhead), and KPNB1 labeling was present along the nuclear membrane and appeared as dots in the nucleus of control spinal motor neurons (A–D). KPNB1 labeling was present in the disrupted or deformed nuclear membrane of sALS spinal motor neurons with TDP-43 mislocalization (arrows, arrowheads) (E–L). Scale bars indicate 30  $\mu$ m. DAPI: 4',6-diamidino-2-phenylindole, KPNB1: karyopherin beta 1, sALS: sporadic amyotrophic lateral sclerosis, TDP-43: 43-kDa TAR DNA-binding protein.

showed irregular, disrupted nuclear staining of NUP62 (Fig. 1M–P). Some atrophic spinal motor neurons that showed TDP-43 mislocalization demonstrated no NUP62 immunoreactivity (Fig. 1Q–T).

KPNB1 immunoreactivity was observed in the nuclear margin as a distinct round circle or dots in the nucleus of normal motor neurons (Fig. 2A–D), whereas KPNB1 immunoreactivity was observed as an irregularly twisted line in the disrupted or deformed nucleus, and was diffusely expressed within the cytoplasm of neurons with mislocalized TDP-43 (Fig. 2E–L). These observations suggest that mislocalization of TDP-43 is correlated with disruption of nuclear staining by anti-NUP62 and anti-KPNB1 antibodies.

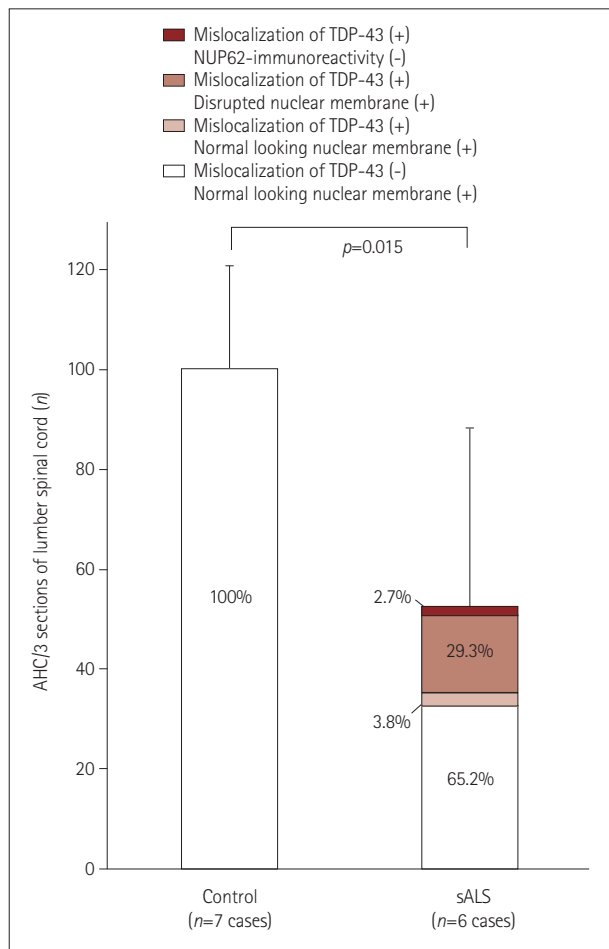
### Quantification of TDP-43 and NUP62 immunoreactivity in the motor neurons

Anterior horn cells (AHCs) with nucleoli on three levels of lumbar sections were counted separately. There were fewer AHCs in sALS patients ( $52.2 \pm 36.4$  cells) than in control subjects ( $104.6 \pm 15.8$  cells) ( $p=0.015$ ). All lumbar spinal motor neurons from control subjects exhibited normal nuclear TDP-43 immunoreactivity and normal ring-like NUP62 nuclear staining. Among the sALS samples, 65.2% of neurons showed normal nuclear TDP-43 immunoreactivity and normal nu-

clear NUP62 staining, while the remaining 34.8% exhibited TDP-43 mislocalization (Fig. 3): 3.8% exhibited normal nuclear NUP62 immunoreactivity, 29.3% demonstrated disrupted NUP62 nuclear staining, and 2.7% were negative for NUP62 immunoreactivity. The neurons with disrupted NUP62 nuclear staining or that were negative for NUP62 immunoreactivity were atrophic (Fig. 1M–T).

## DISCUSSION

This study found that disruption of the NPC was correlated with TDP-43 mislocalization in the spinal motor neurons of patients with sALS. TDP-43 pathology occurs in motor neurons that demonstrate abnormal activation of calpains, and so we suspect that disruption of the NPC in sALS is related to calpain activation.<sup>10</sup> NUP62 immunoreactivity was observed along the round-shaped nuclear membrane in normal motor neurons (Fig. 1A–H). Neurons that exhibited reduced nuclear TDP-43 immunoreactivity with fine cytoplasmic TDP-43-positive inclusions demonstrated circular NUP62 immunoreactivity (Fig. 1I–L), suggesting that these motor neurons are in the early stages of degeneration. The pattern of NUP62 immunoreactivity was irregular within the nucleus of motor neurons that exhibited TDP-43 mislocalization (Fig. 1M–P). Some



**Fig. 3.** Quantification of spinal motor neurons in lumbar spinal-cord tissue from control and sALS cases using double immunofluorescence labeling of NUP62 antibodies and TDP-43 antibodies. There were fewer AHC in sALS patients ( $52.2 \pm 36.4$ ) than in control subjects ( $104.6 \pm 15.8$ ) ( $p=0.015$ ). Spinal motor neurons in control spinal anterior horns exhibited nuclear membranes that were labeled with anti-NUP62 antibodies and did not show TDP-43 mislocalization. In sALS spinal anterior horns, approximately two-thirds of the motor neurons exhibited normal round membranes without TDP-43-positive cytoplasmic inclusions. The remaining 34.8% of neurons in sALS samples exhibited TDP-43 mislocalization: 3.8% exhibited normal nuclear NUP62 immunoreactivity, 29.3% demonstrated disrupted NUP62 nuclear staining, and 2.7% were negative for NUP62 immunoreactivity. AHC: anterior horn cells, NUP62: nucleoporin p62, sALS: sporadic amyotrophic lateral sclerosis, TDP-43: 43-kDa TAR DNA-binding protein.

atrophic spinal motor neurons with TDP-43 mislocalization demonstrated no NUP62 immunoreactivity (Fig. 1Q–T), which is indicative of the end stages of degeneration. The observed changes in NUP62 immunoreactivity seemed to be closely related to the stage of TDP-43 pathology. Similar to NUP62, the distribution of KPNB1 (a molecule involved in nucleocytoplasmic transport) was normal in motor neurons with correctly localized nuclear TDP-43, but was disrupted in motor neurons that exhibited TDP-43 mislocalization.

Activation of the  $\text{Ca}^{2+}$ -dependent cysteine protease calpain specifically cleaves TDP-43 at the C-terminal region to generate aggregation-prone N-terminal fragments.<sup>17</sup> ADAR2 deficiency induces the activation of calpain via the abnormal expression of  $\text{Ca}^{2+}$ -permeable AMPA receptors, thereby resulting in the abnormal mislocalization of TDP-43 in conditional ADAR2-knockout mice and sALS patients.<sup>17,19</sup> A particularly interesting observation was that nucleoporins were also cleaved by calpain, and were disrupted in ADAR2-negative neurons that exhibited positive staining for 136kf (a marker for activated calpain) in the conditional ADAR2-knockout mice.<sup>17</sup> In the present study, only a portion of the motor neurons with TDP-43 mislocalization exhibited normal nucleoporin staining, whereas all motor neurons with normal TDP-43 immunoreactivity exhibited normal nucleoporin staining. Thus, the calpain-dependent cleavage of TDP-43 in the cytoplasm may precede the cleavage of nucleoporins, as a result of a difference in subcellular localization or of a vulnerability to calpain cleavage between TDP-43 and nucleoporins. These findings suggest that calpain-dependent nucleoporin disruption participates in sALS pathogenesis.<sup>17</sup> The mechanism underlying how TDP-43 pathology induces cell death has not been resolved; however, disruption of the NPC induces cell death by inhibiting gene expression that is necessary for cell survival,<sup>17</sup> which has been reported to cause motor neuron death.<sup>20</sup>

Irregular nuclear membrane staining with nucleoporins and abnormal nuclear transporter proteins has been noted in sALS and SOD1-associated ALS, suggesting that nucleocytoplasmic transport is dysfunctional in ALS.<sup>4</sup> Morphological abnormalities and disrupted nucleocytoplasmic transport have recently been reported in both *in vitro* and *in vivo* models of *C9orf72*-related ALS<sup>2-4</sup> and sALS.<sup>7</sup> Combining these findings with those of the present study suggests that the disruption of nucleocytoplasmic transport underlies the pathomechanism of motor neuronal degeneration in sALS, as well as in some cases of genetic ALS. Further studies are required to elucidate the role of the NPC in nucleocytoplasmic transport in sALS.

#### Conflicts of Interest

The authors have no financial conflicts of interest.

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