Reduced PHOX2B stability causes axonal growth impairment in motor neurons with TARDBP mutations

Shio Mitsuzawa,1,12 Naoki Suzuki,1 Tetsuya Akiyama,1 Mitsuru Ishikawa,2 Takefumi Sone,2 Jiro Kawada,3,4 Ryo Funayama,5 Matsuyuki Shirota,6 Hiroaki Mitsuhashi,7 Satoru Morimoto,2 Kensuke Ikeda,1 Tomomi Shijo,1 Akiyuki Ohno,1 Naoko Nakamura,1 Hiroya Ono,1 Risako Ono,1 Shion Osana,8 Tadashi Nakagawa,5,9 Ayumi Nishiyama,1 Rumiko Izumi,1 Shohei Kaneda,4,10 Yoshiho Ikeuchi,4,11 Keiko Nakayama,5 Teruo Fujii,4 Hitoshi Warita,1 Hideyuki Okano,2 and Masashi Aoki,1,*

1Department of Neurology, Tohoku University Graduate School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan
2Department of Physiology, Keio University School of Medicine, 35 Shinanomachi, Shinjuku-ku, Tokyo 160-8582, Japan
3Jiksak Bioengineering Inc. 7-7 Shinkawasaki, Saiwai-ku, Kawasaki 212-0032, Japan
4Institute of Industrial Science, the University of Tokyo, 4-6-1 Komaba, Meguro-ku, Tokyo 153-8505, Japan
5Division of Cell Proliferation, United Centers for Advanced Research and Translational Medicine, Tohoku University Graduate School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan
6Division of Interdisciplinary Medical Science, United Centers for Advanced Research and Translational Medicine, Tohoku University Graduate School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan
7Division of Applied Biochemistry, School of Engineering, Tokai University, 4-1-1 Kitakanaame, Hiratsuka, Kanagawa 259-1292, Japan
8Division of Biomedical Engineering for Health and Welfare, Graduate School of Biomedical Engineering, Tohoku University, 2-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan
9Department of Clinical Pharmacology, Faculty of Pharmaceutical Sciences, Sanyo-Onoda City University, 1-1-1 Daigaka-Doori, Sanyo-Onoda, Yamaguchi 756-0884, Japan
10Department of Mechanical Systems Engineering, Faculty of Engineering, Kogakuin University, 1-24-2 Nishishinjuku, Shinjuku-ku, Tokyo, 163-8677, Japan
11Institute for AI and Beyond, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan
12Present address: Department of Neurology, Shodo-kai Southern Tohoku General Hospital, 1-2-5, Satonomori, Iwanuma, Miyagi 989-2483, Japan
*Correspondence: aokim@med.tohoku.ac.jp
https://doi.org/10.1016/j.stemcr.2021.04.021

SUMMARY

Amyotrophic lateral sclerosis (ALS) is an adult-onset incurable motor neuron (MN) disease. The reasons for selective MN vulnerability in ALS are unknown. Axonal pathology is among the earliest signs of ALS. We searched for novel modulatory genes in human MN axon shortening affected by TARDBP mutations. In transcriptome analysis of RNA present in the axon compartment of human-derived pluripotent stem cell (iPSC)-derived MNs, PHOX2B (paired-like homeobox protein 2B) showed lower expression in PHOX2B mutant axons, which was consistent with axon qPCR and in situ hybridization. PHOX2B mRNA stability was reduced in TARDBP mutant MNs. Furthermore, PHOX2B knockdown reduced neurite length in human MNs. Finally, phox2b knockdown in zebrafish induced short spinal axons and impaired escape response. PHOX2B is known to be highly express in other types of neurons maintained after ALS progression. Collectively, TARDBP mutations induced loss of axonal resilience, which is an important ALS-related phenotype mediated by PHOX2B downregulation.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is an incurable, progressive, adult-onset neurodegenerative disease, in which cortical, brainstem, and spinal motor neurons (MNs) are slowly destroyed, resulting in muscle weakness, atrophy, and finally death due to respiratory and swallowing dysfunction (Wijesekera and Leigh, 2009). The development of effective treatments has been hindered by a poor understanding of disease pathogenesis. The reasons for selective MN vulnerability in ALS are unknown.

Most cases of ALS are sporadic; whereas, about 10% of ALS patients have a family history (Taylor et al., 2016). Mutations of the TARDBP gene, encoding transactive response DNA binding protein 43 kDa (TDP-43), are found in 3% of familial ALS and 1.5% of sporadic ALS cases. Among Japanese familial ALS patients, TARDBP mutations are the third most common after SOD1 (Cu/Zn superoxide dismutase and FUS (fused in sarcoma) mutations (Nishiyama et al., 2017). Moreover, TDP-43 aggregates within spinal anterior horn neurons are observed in the majority of sporadic ALS patients (Mackenzie et al., 2007), suggesting a major contribution of TDP-43 to ALS pathogenesis.

TDP-43 is an RNA-binding protein of the heterogeneous nuclear ribonucleoprotein (hnRNP) family expressed mainly in the nucleus and partially in the cytoplasm, where it regulates RNA stability and translation (Buratti and Bartell, 2001; Coyne et al., 2014; Strong et al., 2007). In ALS with TARDBP mutations, mutant TDP-43 has been reported to cause abnormalities in various RNA metabolic functions such as splicing, stability, and transport (Kapeli et al., 2017). These processes are particularly critical for projection neurons such as MNs because the distal axon and synapses may be up to 1 m or more from the somas. The mechanisms contributing to the resilience of these structures throughout life have not been fully elucidated. In the SOD1 mutant
Figure 1. Phenotype evaluations of newly established iPSCs and iPSC-derived MNs

(A) Protocol for establishing iPSCs from the peripheral blood mononuclear cells (PBMCs) of a familial ALS patient harboring the TARDBP p.G376D mutation. The panel shows the selected colony. Scale bar, 500 μm.

(B) Reprogrammed PBMCs that formed colonies carried the same TARDBP p.G376D mutation as the familial ALS patient. The panel shows the heterozygous c.1127G > A change (black arrow) revealed by Sanger sequencing.

(C) Karyotype analysis of iPSCs harboring TARDBP p.G376D. No karyotype abnormality was detected.

(D) The established iPSCs expressed pluripotency markers as evidenced by immunocytochemistry. Scale bar, 100 μm.

(legend continued on next page)
mouse model of ALS, structural abnormalities in the axon have been reported to precede MN death (Tian et al., 2016). Moreover, MNs differentiated from ALS patient-derived induced pluripotent stem cells (iPSCs) with TARDBP mutations had shorter axons than healthy control MNs (Egawa et al., 2012; Fujimori et al., 2018). On the basis of these findings, we speculated that TARDBP mutations reduce the capacity to bind and stabilize mRNAs involved in axon homeostasis, resulting in functional and structural axonal abnormalities and ensuing neurodegeneration.

In this study, a transcription factor, PHOX2B, was identified by RNA sequencing (RNA-seq) analysis to be downregulated in iPSC-derived MN axons. The knockdown (KD) of PHOX2B shortened the neurite length of MNs compared with the control, consistent with an iPSC-derived MN phenotype in ALS patients with TARDBP mutations. In zebrafish, the KD of endogenous phox2b also reduced MN axon length and increased axon branching. These results indicated that reduced expression of PHOX2B may be associated with MN degeneration in ALS pathology.

RESULTS

Generation and phenotyping of MNs from iPSCs
iPSCs harboring the TARDBP p.G376D mutation were established from a 36-year-old male familial ALS patient with progressive muscle weakness of the upper extremities and head drop who died 1 year from onset (Mitsuzawa et al., 2018; Nishiyama et al., 2017). The iPSCs were validated by Sanger sequencing, karyotyping (normal), detection of pluripotency markers by immunocytochemistry, and three germ layer differentiation potential (Figures 1A–1E and S1A). All iPSCs used in this study (healthy control- and ALS-derived) were differentiated into MNs exhibiting a transitional embryonic body-like state, as described previously (Fujimori et al., 2018; Fujimori et al., 2017). Phenotype was confirmed by immunocytochemistry for the neuron-specific markers βIII tubulin, SMI32, and microtubule-associated protein 2 (MAP2), and for the MN-specific markers choline acetyltransferase (ChAT) and Islet1 (Figure 1F). The iPSCs used in this study are listed in Table S1.

TARDBP mutations reduce neurite length in iPSC-derived MNs
It has been reported that iPSC-derived MNs harboring TARDBP mutations (such as p.M337V) from ALS patients have shorter neurites than MNs derived from the iPSCs of healthy controls (Egawa et al., 2012; Fujimori et al., 2018). In this study, neurite length was measured by fluorescence signals that originate from the edge of MN precursor mass to the neurite ends upon transfection with an EF-1a::Venus, HB9::Venus, or HB9::RFP (red fluorescent protein) lentivirus vector. During culture, the average neurite length of TARDBP mutant MNs was significantly shorter than that of healthy control MNs by 33 days after differentiation (14 days post plating [dpp]) and remained shorter at the measurement endpoint when the neurites reached the well wall 17–21 dpp (Figures 2A and 2B).

Identification of genes associated with TARDBP mutation-induced axonal pathogenesis by RNA-seq
To identify molecules potentially related to TARDBP mutation-induced axonal pathology, we first conducted RNA-seq analysis using the axonal fractions of iPSC-derived MNs (Figure 3A). Candidate genes were identified based on the selection criteria outlined in the experimental procedures (Figures 3B; Tables S4, S5, S6, and S7). Enrichment analysis revealed that the transcripts of 252 genes associated with chloride transmembrane transport, MAPK cascade, and cell or neuron fate commitment were all significantly upregulated in axons derived from the iPSCs with a TARDBP mutation. In contrast, the transcripts of 220 genes associated with memory, cell motility, postsynaptic membrane potential, and axon or actin-related functions were significantly downregulated (Figure 3C). Enrichment analysis revealed that 16 of the downregulated transcripts were associated with significant changes in the neuronal soma, while 150 transcripts, 103 upregulated and 47 downregulated, were associated with the axon. We then focused on gene ontology (GO) categories, including terms like nerves, axons, transport, cytoskeleton, and inflammation. We also focused on those including the TDP-43 binding sequence UGUGUG (Tollervey et al., 2011) within the coding sequences or the 3’ untranslated regions (UTR) by RBP map (http://rbpmap.technion.ac.il). These strategies facilitated the identification of 61 candidate genes associated with TARDBP mutation-induced axonal pathology. Among these, we focused on PHOX2B, previously identified as essential for forming the neural crest and inducing MN differentiation (Nagashimada et al., 2012; Pattyn et al., 1999). Moreover, it was reported that NEFL mRNA was decreased and apoptosis increased in PHOX2B KD cells (Huber et al., 2005), suggesting that
PHOX2B is related to MN vulnerability and axonal morphology. Furthermore, PHOX2A, a paralogous gene product of PHOX2B in structure, was reported to form heterodimers with PHOX2B in vitro (Di Lascio et al., 2016) and was also among the 61 differentially expressed genes from RNA-seq axon fraction. PHOX2B mRNA has TDP-43 binding consensus sequence in 3' UTR (NCBI Reference Sequence: NM_003924.4) and its expression was reduced in TARDBP mutant axons with RNA-seq and qPCR (Figures 3D and S1B for qPCR results of TARDBP p.N345K [N345K] [Leventoux et al., 2020]), it was selected for further investigation. PHOX2B protein expression was significantly reduced in TARDBP mutant MNs compared with that in MNs derived from healthy controls (Figure 3E).

Confirmation of reduced PHOX2B expression using isogenic TARDBP-mutated iPSCs

To confirm the specificity of PHOX2B downregulation by ALS-causative TARDBP mutations, we examined expression changes in wild-type 1 (WT1) (CiRA 201B7)-derived isogenic lines harboring these mutations. Heterozygous and homozygous mutations of p.M337V and p.G298S were generated in iPSCs, and pluripotency was verified by selective marker expression (Figure S2A). Moreover, MN phenotype was verified by selective marker expression following the differentiation protocol (Figure 4A). Like MNs derived from ALS patients, these isogenic TARDBP mutant MNs also exhibited shorter neurites than did MNs derived from healthy controls under HB9::RPF-infected live cell neurite staining (Figure 4B). Moreover, PHOX2B mRNA expression was also downregulated (Figure 4C), suggesting that normal PHOX2B expression depends on conserved TDP-43 function. Axon length and PHOX2B mRNA expression did not change between control MNs and genome-editing non-targeting control MNs that only had silent mutations (Figure S2B). Axon branching of iPSC-derived TARDBP mutant MNs were increased compared with those of healthy control MNs in live cells, which were consistent with our previous report (Akiyama et al., 2019) (Figure S2C).

TDP-43 binds and stabilizes PHOX2B mRNA in neurites

RIP (RNA immunoprecipitation) with anti-TDP-43 antibodies revealed that TDP-43 was bound to PHOX2B mRNA (Figures 5A and 5B). The GFP-tagged WT and mutant TDP-43 (p.A315T, p.M337V, and p.G376D) immunoprecipitated using anti-GFP antibodies were found to bind PHOX2B mRNA (Figures S3A and S3B), suggesting that the ALS-associated mutations did not eliminate TDP-
A 43’s binding ability to the PHOX2B mRNA. Therefore, PHOX2B mRNA downregulation is not likely to result from the lack of recognition by mutant TDP-43. Moreover, TARDBP mRNA KD using small interfering RNA (siRNA) (si-TARDBP, siT) also decreased the level of PHOX2B mRNA in SH-SY5Y cells (Figure 5C).

To further investigate the relationship between PHOX2B mRNA downregulation and TARDBP mutations, we compared the subcellular localization of PHOX2B mRNA between WT and mutant MNs by single-molecule RNA fluorescence in situ hybridization (smFISH). Although some PHOX2B mRNA red fluorescence was localized in the axons of healthy control MNs (WT1), this signal was absent from the axons of mutant MNs (Q343R, M337V, and G376D) (Figure 5D, inset). To determine whether this change in subcellular expression is due to PHOX2B mRNA instability, mRNA stability assays were conducted using actinomycin D. Concentration and treatment time of actinomycin D were the same between control and TARDBP mutant MNs, and there was no clear damage to the cells. After 1 h of actinomycin D treatment, expression of endogenous PHOX2B mRNA in TARDBP mutant MNs was significantly reduced, whereas it was maintained in control MNs (Figure 5E).

**Figure 3.** Expression of PHOX2B was reduced in TARDBP mutant MNs compared with control MNs

(A) Schema illustrating isolation of somas and axons from iPSC-derived MNs using microfluidic chambers.

(B) Selection of candidate genes associated with TARDBP mutation-induced axonal pathology based on RNA-seq. The number of candidates was reduced from 26,254 to 231 in somas and 472 in axons on normalized fragments per kilobase of the exon model per million mapped fragments (FPKM) values | log2 | > 1 and p < 0.1 (Student’s t test). From enrichment analysis, 16 genes (all genes were downregulated in the mutant) were significantly changed in the soma fraction, and 150 genes (103 genes were upregulated and 47 genes were downregulated in the mutant) were in the axon fraction. PHOX2B was selected from GO term (related to nerves, axons, transport, cytoskeleton, and inflammation), RBP map, and qPCR. See also Tables S4, S5, S6, and S7.

(C) Enrichment analysis revealed significant upregulation of transcripts associated with chloride transmembrane transport, the MAPK cascade, and cell or neuron fate commitment in TARDBP mutant axons (252 genes, upper panel). By contrast, transcripts associated with memory, cell motility, postsynaptic membrane potential, and axon- or actin-related genes were significantly downregulated (220 genes, lower).

(D) PHOX2B was selected from RNA-seq results. The upper graph shows RNA-seq results (normalized FPKM) and the lower graph shows qPCR results (PHOX2B/ACTB [β-actin]). PHOX2B normalized FPKM were decreased in TARDBP mutant axons. PHOX2B mRNA expression was also equal or decreased in the mutant axons with qPCR using RNA samples for RNA-seq under independent triplicates per sample. See also Figure S1B for qPCR results of N345K.

(E) PHOX2B protein expression was examined by western blotting. Expression of PHOX2B (32 kDa, arrowhead) was substantially lower in TARDBP mutant MNs (M337V) compared with that in healthy control MNs (WT2), consistent with the mRNA expression pattern measured by RNA-seq and qPCR. GAPDH (37 kDa, arrowhead) was used as the gel loading control.
On the contrary, GAPDH mRNA as the negative control gene did not decrease during 6 h of actinomycin D treatment in both control and TARDBP mutant MNs (Figure S3C). These results indicate that TARDBP mutations reduce PHOX2B mRNA stability.

**PHOX2B regulates iPSC-derived MN neurite length**

To investigate whether PHOX2B can regulate neurite length, iPSC-derived MNs were transfected with siRNA for PHOX2B KD (siPHOX2B, siP) and their neurites were compared with those of a negative control siRNA.
Figure 5. TDP-43 bound and stabilized PHOX2B mRNA in neurites
(A) Endogenous TDP-43 (43 kDa, black arrowhead) was detected by western blotting in SH-SY5Y cell immunoprecipitation samples using anti-TDP-43 antibody. The upper band indicates the immunoglobulin G (IgG) heavy chain (50 kDa, gray arrowhead). The lower IgG light chain band (25 kDa) was not shown.

(B) PHOX2B mRNAs (black arrowhead) were amplified by PCR from SH-SY5Y cell RIP samples using anti-TDP-43 antibody. PHOX2B mRNA bound to TDP-43.

(C) Transduction of siRNA (siTARDBP, siT) knocked down TARDBP mRNA in SH-SY5Y cells (upper graph), wherein PHOX2B mRNA also decreased (lower graph). n = 3 independent triplicates. One-way ANOVA with post hoc Tukey HSD tests. ***p < 0.001, **p < 0.01.

(legend continued on next page)
(siControl, siC) transfected MNs. Transfection of siP to healthy control MNs (WT1) substantially reduced PHOX2B expression at both mRNA and protein levels compared with WT1 MNs with siControl (Figures 6A and 6B). Furthermore, siP but not siC transfection substantially reduced neurite length compared with healthy control MNs (WT1 and WT2) at 21 dpp (Figures 6C and 6D).

**phox2b affects zebrafish spinal axon length, morphology, and motor functions**

To examine if PHOX2B can also influence MN neurite length in vivo and influence motor function, we compared MN morphology and escape behavior between control and phox2b KD zebrafish. Because zebrafish embryos are transparent, spinal MNs are easy to observe by fluorescent protein labeling with motor neuron-specific Hb9 promoter (Stil and Drapeau, 2016), and zebrafish phox2b is highly homologous to the human PHOX2B (Pei et al., 2013). phox2b KD was induced by injection of a splice-blocking morpholino into zebrafish fertilized eggs (Figure S4A). The splice-blocking morpholino oligonucleotide (MO) targets splice junction of phox2b second exon and blocks splicing of the intron 2, including a stop codon, resulting in an incomplete phox2b translation and phox2b KD (Pei et al., 2013). Consistent with cell culture studies, zebrafish injected with the splice-blocking MO exhibited significantly shorter spinal axons (and more axonal spines) than controls injected with mismatch control MO at both 2 and 3 days post fertilization (dpf) (Figures 7A–7C). Moreover, axonal spines were thicker and abnormal ectopic axons were increased in KD zebrafish at 3 dpf (Figures 7A and 7D). Finally, motor functions of MO-injected zebrafish were analyzed by comparing touch-evoked escape responses at 2 dpf. Consistent with disruption of MN morphology, zebrafish injected with the splice-blocking MO were unresponsive to tail stimulation (Figures 7E, 7F, and S4B). Moreover, Phox2b expression in hsODT<sup>G93A</sup> transgenic (Tg) rats was reduced in the lumbar spinal cord anterior horn cells compared with the non-Tg rats (Figure 7G). The in vivo expression of Phox2b was decreased in the MNs of the ALS model.

**DISCUSSION**

In this study, RNA-seq analysis revealed diminished transcription of PHOX2B in iPSC-derived MNs that include the ALS-associated TARDBP mutation; this finding was associated with neurite length shortening and reduced motor function both in vivo and in vitro. Both neurite length shortening and reduced motor functions were reported previously in experimental models of ALS (Akiyama et al., 2019; Babin et al., 2014; Egawa et al., 2012; Fujimori et al., 2018) and these findings were reproduced in this study. Reduced neurite length in MNs derived from the iPSCs of ALS patients harboring TARDBP mutations, such as p.M337V or p.Q343R, has been observed previously (Fujimori et al., 2018). To confirm and extend these findings, neurite length was significantly shorter in MNs derived from both patient-derived and isogenic mutant iPSCs than that from healthy control iPSCs, indicating that reduced neurite length is a common feature of MNs with ALS-associated TARDBP mutations. Similarly, phenotypes associated with the phox2b KD zebrafish were similar to those identified in our previous reports describing the impact of Fos-B overexpression (Akiyama et al., 2019). Shorter axon length and increased axon branching have been reported as pathological hallmarks of ALS-associated gene mutations in zebrafish (Babin et al., 2014).

PHOX2B is the causative gene for congenital central hypoventilation syndrome (Cain et al., 2017) but has not been directly linked to ALS. PHOX2B protein is a highly conserved transcription factor expressed mainly in neural cells and is essential for neural crest formation (Pattyn et al., 1999). Phox2b, a transcription factor that is expressed in intracranial and upper cervical MNs of embryonic mice, was also reported to induce neural differentiation from neural progenitor cells (Mazzoni et al., 2013; Nagashimada et al., 2012; Song et al., 2006). In adults, Phox2b mRNA is expressed in mouse spinal anterior horn cells according to the Allen Brain Atlas (http://portal.brain-map.org/). In this study, it was revealed that Phox2b is expressed in normal rat lumbar spinal cord anterior horn cells (Figure 7G). These characteristics suggest potential functions in the ALS model.
of PHOX2B in MNs, a notion supported by reduction of MN axonal length and motor dysfunction by PHOX2B KD in this study. As per our knowledge, PHOX2B has not been mentioned in the published dataset using ALS autopsies samples in the Gene Expression Omnibus. This might be because the autopsy samples reflect the disease’s end stage, whereas PHOX2B suppression and axonal pathology reflect the disease’s early stage. Moreover, it has been reported that oculomotor and trochlear MNs highly express PHOX2A (Allodi et al., 2019), which is structurally similar to PHOX2B. Oculomotor and trochlear motor neurons show a better survival rate than spinal cord MNs in ALS (An et al., 2019), thus high PHOX2A expression in these cells might serve to protect them from cell death associated with ALS. In this study, expression of PHOX2A was also downregulated in TARDBP mutant axons (RNA-seq; fold change = 0.1085, p = 0.0104, Table S5); interestingly, the expression of PHOX2A protein in mutant MNs was indistinguishable from that detected in the WT (data not shown). PHOX2B is highly expressed in noradrenergic neurons or oculomotor neurons that are maintained for a long time during ALS progression (Allodi et al., 2019; Fan et al., 2018). These results suggest that PHOX2B may normally serve to reduce MN vulnerability and to increase resilience of noradrenergic neurons or oculomotor neurons in patients with ALS.

TDP-43 mutations are known to be involved in the pathogenesis of ALS through a variety of mechanisms (Ling et al., 2013). In this study, TDP-43 mutations seem to be involved in the decreased stability of PHOX2B mRNA, consistent with the presence of a TDP-43 binding consensus sequence in its 3’ UTR. However, the mechanisms underlying the stability of PHOX2B mRNA remain uncertain. While we observed no changes with respect to TDP-43 and PHOX2B mRNA binding interactions, there may be differential expression of specific mRNA isoforms in the WT compared with the mutant MNs. There are at

Figure 6. PHOX2B knockdown in control MNs reduced neurite length

(A) Control MNs were transfected with siP or siC at 3 dpp and RNA extracted at 7 dpp for expression analysis by qPCR. MNs transfected with siP showed an approximately 50% reduction in PHOX2B expression compared with MNs transfected with siC. Statistical analysis performed on n = 3 independent triplicates. One-way ANOVA with post hoc Tukey HSD tests. **p < 0.01, *p < 0.05.

(B) Knockdown efficiency of PHOX2B by siRNA was examined by western blotting. The reduction in PHOX2B (32 kDa, arrowhead) was comparable with that observed in M337V TARDBP mutant MNs. GAPDH (37 kDa, arrowhead) was used as the gel loading control.

(C) Knockdown of PHOX2B in healthy control MNs (WT1 and WT2) reduced neurite length compared with MNs transfected with siC at 21 dpp. The white arrowheads indicate the most distal neurite ends. Scale bar, 300 μm.

(D) The upper graph shows neurite length changes over time. Twenty-five to 80 neurites were analyzed per group for one experiment, depending on the time post differentiation. A comparison between the siC and siP groups showed that the neurite length significantly decreased in the siP group from day 14. The lower graph shows quantitative analysis at 21 dpp. MNs transfected with siP significantly reduced neurite length compared with MNs transfected with siC at 21 dpp. Repeated measures ANOVA with post hoc Tukey HSD tests for comparison between siC and siP. Two-way ANOVA with post hoc Tukey HSD tests for among each cell lines. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. See also Tables S2 and S3.
Figure 7. *phox2b* knockdown in zebrafish reduces spinal axon length and impairs motor function

(A) Zebrafish embryos injected with a splice-blocking MO for *phox2b* (lower column) have shorter spinal axons and increased axonal spines compared with those injected with mismatch control MO (upper column) at 3 dpf. In zebrafish injected with the splice-blocking MO, axonal spines also became thicker (yellow arrow heads), and abnormal ectopic axons (white arrow heads) were detected. Scale bar, 100 μm.

(legend continued on next page)
least two isoforms of PHOX2B that can be distinguished by the lengths of their respective 3’ UTRs (Cardani et al., 2018); the biphasic degradation of PHOX2B mRNA observed here (Figure 5E) may be directly related to factors distinguishing these mRNAs. Likewise, isoforms encoding characteristic sequences of the mutant MNs may be more easily degraded than those identified in the WT.

This study has several limitations. First, the selection of candidate genes associated with TARDBP mutation-induced axonal pathology did not consider some genes associated with other pathological hypotheses. Second, both iPSC-derived MNs and zebrafish were observed at the early stages of development, while ALS is an adult-onset disease. Third, phox2b KD in zebrafish was not specific to MNs. In addition, PHOX2B was reported to be expressed mainly in the neural crest during differentiation (Fan et al., 2018), so motor deficits in zebrafish embryos may be due to restricted development of other neural pathways. Moreover, although we attempted to overexpress PHOX2B by transfection with lentiviral vectors expressing PHOX2B with a C-terminal GFP tag under the cytomegalovirus promoter, the total amount of PHOX2B mRNA remained unchanged, possibly because the total mRNA level of PHOX2B in the MNs is strictly controlled or due to the effects of lentiviral transfection (data not shown). In neuroblastoma, PHOX2B mRNA has been reported to transactivate itself by binding to its own promoter region (Cargnin et al., 2005). However, this transactivation disappears in the presence of PHOX2B mutations due to their dominant-negative effect (Di Lascio et al., 2018). The sequence of lentiviral vectors expressing GFP-tagged PHOX2B was confirmed as expected (data not shown), thus ruling out the possibility of a dominant-negative effect. The mutant TDP-43 knockin mouse model with early MN-selective degeneration (Ebstein et al., 2019) should be used in future studies for analyzing expression and localization changes of PHOX2B.

In conclusion, PHOX2B downregulation found in RNA-seq using iPSCs derived MNs mediates TARDBP mutation-induced loss of axonal resilience, suggesting PHOX2B pathways as potential therapeutic targets for ALS. We speculate that the decreased PHOX2B mRNA and protein expression in TARDBP mutant MNs reduces this local translation and retrograde transport in response to various stimuli, which in turn leads to deficient expression of the still unknown PHOX2B target genes required for neurite length elongation, axonal maintenance, and/or MN survival. Further studies should explore downstream factors of PHOX2B that are related to MN vulnerability of ALS.

**EXPERIMENTAL PROCEDURES**

**Creation of iPSC lines**
The creation of human iPSC lines was approved by the Tohoku University ethics review board (Nos. 2014-1-733 and 2016-1-814) and Keio University ethics review board (No. 20080016). See also supplemental experimental procedures.

**Collection and maintenance of iPSCs**
All iPSC lines used in this study (Table S1) were cocultured with SNL 76/7 murine fibroblast feeder cells (ECACC) pretreated with 10 μg/mL of mitomycin C (Sigma) and maintained at 37°C and 3% CO2.

**Differentiation into MNs**

iPSCs were differentiated into MNs according to a previous report (Fujimori et al., 2017) (see also supplemental experimental procedures). The second MPCs were cultured in 96-well v-bottom plates (Sumitomo Bakelite) at 1 × 104 cells/well to generate neurospheres.
for performing neurite length measurements and RNA-seq analysis. The second MPC neurospheres were then cultured on 24-well thin-bottom plates (Iwaki) coated as above to measure neurite length or in microfluidic devices (Jiskak Bioengineering) (Kawada et al., 2017) for axon/soma isolation and analysis by RNA-seq. The microfluidic devices were obtained from Prof. Teruo Fujii and Dr. Jiro Kawada.

**Neurite length analysis of iPSC-derived MNs**

iPSC lines were differentiated into MNs as described above and infected with lentivirus vector (HB9::Venus, FE-1::Venus, or HB9::RFP). Neurite length was measured every 2–4 days from the neurite end to the sphere edge using the BZ-X700 measurement tool (Keyence). Transfection of siRNA siPHOX2B (Ambion) were performed every 3 days starting on day 22 of differentiation using RNAiMax (Thermo Fisher). Silencer Select siRNA (Ambion) was expected of degrading RNA (e.g., the expression pattern of only the 3' UTR was displayed) in the evaluation using IGV (Integrative Genomics Viewer: https://software.broadinstitute.org/software/igv/home) were excluded from the analysis. The results were analyzed using Subio for each of the axons and somas (see also supplemental experimental procedures). Normalized FPKM values were compared between controls and TARDBP mutations in axon or soma fractions, and differentially expressed genes were picked up with | log2 | > 1 (fold changes > 0.5, 2 < fold changes) and p < 0.1 (Student's t test). Enrichment analysis was also performed using Subio software (v.1.24), and enriched GO term categories were selected by number in Category < 100, number Overlaps > 3, and p < 0.05. TDP-43 binding sequences were searched using RBP map (http://rbpmap.techion.ac.il) (Figure 3B).

**qPCR**

Extracted RNA was reverse transcribed to cDNA using the Quantitect Reverse Transcription Kit (QIAGEN). Quantitative real-time PCR was performed using the SsoFast EvaGreen Supermix (Bio-Rad) and analyzed using a CFX96 Real-Time PCR Detection System (Bio-Rad). Primer sets used for each target gene are listed in Table S2. ACTB was used as the internal control gene. All qPCRs were performed in triplicate for each sample. Candidate genes were quantified using a standard curve. Expression levels are shown as mean and standard error.

**Western blotting**

Western blotting was performed as described in our previous report (Akiyama et al., 2019). See also supplemental experimental procedures.

**RNA stability assay**

To determine the impact of endogenous mutations on the stability of PHOX2B mRNA, iPSC-derived MNs derived from TARDBP isogenic lines were treated with 10 μg/mL actinomycin D for 0, 1, 3, and 6 h; three samples were collected for each line and in response to 0 and 1 h treatment conditions. RNA extraction and qPCR protocols were as described above.

**Construction of lentivirus vectors**

We used lentivirus vectors for fluorescent marking of MNs. Lentivirus vectors HB9::Venus, FE-1::Venus, and HB9::RFP were constructed as described in our previous report (Akiyama et al., 2019). The self-inactivating (SIN) vector plasmid and the packaging vector plasmid were provided by Dr. Hiroyuki Miyoshi (Shimozjo et al., 2015). Venus is a modified GFP developed by Dr. Atsushi Miyawaki of RIKEN (Nagai et al., 2002). RFP was permitted for use by UCSD (University of California San Diego).

**Axon length and spine-counting analyses in phox2b KD zebrafish**

Zebrafish were maintained at Tokai University under approved protocols (approval No. 195001). Endogenous *phox2b* KD zebrafish were created by injection of a splice-blocking MO that was designed to induce aberrant splicing of zebrafish *phox2b* as described previously (Pei et al., 2013). The transgenic zebrafish line hb9:Venus (Nakano et al., 2010) was used to visualize MNs in vivo. The

**RNA-seq sample collection from microfluidic devices**

Prepared samples for RNA-seq were iPSC-derived MNs of healthy controls (WT1, WT2, and WT3, which was a WT2 technical duplicate), familial ALS with TARDBP mutations (M337V and G376D), and TARDBP isogenic MNs constructed from WT1 (TARDBP^{M337V/WT}, TARDBP^{G376D/WT}, TARDBP^{G298S/V298S}, and TARDBP^{G298S/WT}). In brief, iPSCs were differentiated into MPC spheres as described above and plated in one well of a microfluidic device containing two wells at opposite ends. With this plating strategy, only axons extended to reach to the opposite well, usually within 21 days. Neurites 450 μm or more distant from the cell body, defined as axons according to a previous report (Taylor et al., 2005), were separated from soma using medical scalpels, and both axon and soma fractions were collected. In total, 8–16 wells were obtained from each cell line.

**RNA extraction**

RNA was extracted for RNA-seq and qPCR using the QIAGEN RNA Micro Kit according to the manufacturer's protocol.

**RNA-seq and analysis**

The TruSeq Stranded mRNA LT Sample Prep Kit (Illumina) was used for preparation of the soma RNA-seq library and the SMARTER Seq v4 Ultra Low Input RNA Kit for the axonal RNA-seq library (TruSeq). RNA-seq was performed using the HiSeq 2500 system (Illumina) in rapid mode with 51 bp single-end sequencing. UCSC hg19 and RefSeq were used for the reference human genome and gene model. For gene expression analysis, sequence FASTQ files were mapped to the reference human genome using TopHat (v.2.1.1). FPKM (fragments per kilobase of the exon model per million mapped fragments) was calculated as the gene expression levels from the mapping file using Cufflinks (v.2.2.1).

**Selection of disease-related candidate genes from RNA-seq**

Two batches of RNA-seq (the non-isogenic MNs and isogenic MNs) were integrated and analyzed. Samples containing some genes suspected of degrading RNA (e.g., the expression pattern of only the 3'...
fertilized eggs of the hb9:Venus × RIKEN WT zebrafish strain were injected with 4 pg of the splice-blocking MO or mismatched MO as a control. Venus-positive axon length was measured from the beginning of the spinal cord to the end. Ectopic axons, axons connecting to ectopic axons, and axons with thicker spines than caudal MNs were defined as abnormal. Data are expressed as mean and standard error and were compared by two-way ANOVA with post hoc Tukey HSD tests.

**Motor function analyses of phox2b KD zebrafish**

The effects of phox2b KD on motor function were assessed by comparing touch-evoked escape responses in WT zebrafish injected with mismatch control MO at 2–3 dpf. Touch-evoked escape responses were graded (0, no movement; 1, flicker of movement but no swimming; 2, movement away from probe but with impaired swimming; 3, normal swimming) as described previously (Teller et al., 2010) and performed in triplicate for each subject (n = 20 per treatment group). Data are expressed as mean and standard error and were compared by two-way ANOVA with post hoc Tukey HSD tests.

**Statistical analysis**

GraphPad Prism 7.04 (MDF) was used for all statistical analyses. Data are expressed as mean and standard error and were compared among treatment groups by one-way, two-way, or repeated measures ANOVA as indicated with post hoc Tukey HSD tests for pairwise comparisons. A p value of < 0.05 (two-tailed) was considered statistically significant for all tests. Data with no significant differences are described as “n.s.” The level of significance is indicated by the number of asterisks (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

**Data and code availability**

The accession number for the RNA-seq data reported in this paper is DDBJ (DNA DataBank of Japan): DRA011838.

**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.stemcr.2021.04.021.

**AUTHOR CONTRIBUTIONS**

S. Mitsuzawa, N.S., and M.A. wrote and revised the manuscript. All authors read the final manuscript. S. Mitsuzawa, N.S., T.A., and M.A. designed the experiments and interpreted the results. S. Mitsuzawa performed all experiments, except for iPSC establishment and rat experiments. A.T. established hiPSCs from ALS patients with TARDBP p.G376D mutation and S. Morimoto established and provided hiPSCs from ALS patients with TARDBP p.N345K mutation (Leventoux et al., 2020). M.I., T.S., and H. Okano helped in iPSC establishment. J.K., S.K., Y.I., and T.F. developed the microfluidic devices. T.A., K.I., and A.O. helped in maintenance of iPSCs and differentiation to MNs. N.N., H. Ono, R.O., and S.O. discussed and advised about all experiments. T.A., R.F., M.S., T.N., and K.N. helped in RNA-seq. N.S., T.A., A.N., and R.I. helped in genetic analysis of ALS patients. H.M. performed the zebrafish experiments. T.S. and H.W. performed the rat experiments.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.

**ACKNOWLEDGMENTS**

The authors thank N. Sugeno, T. Hasegawa, Y. Takai, T. Misu, and M. Kato of the Department of Neurology, Tohoku University, for advice regarding this research; M. Suzuki, N. Shimakura, H. Shigihara, and A. Machii of the Department of Neurology, Tohoku University, for their technical assistance; K. Kuroda, M. Kituchi, and M. Nakagawa of the Division of Cell Proliferation, Tohoku University, and A. Tabe of Subio for assistance with the RNA-seq procedure; S. Koyama, T. Kato, and Y. Suzuki for ongoing care of the G376D proband; H. Inoue, S. Yamamaka, and M. Nakagawa (Kyoto University) for donating hiPSC clones 409B2, 201B7, and CiRA00026; S. Nakamura and F. Ozawa for cells shipping and maintenance in Department of Physiology, Keio University; T. Nihori and Y. Aoki for genetic analysis; T. Kamei, T. Yamashita, and T. Matsuo of Takeda Pharmaceutical Company for their gift of TARDBP mutant isogenic iPSCs; A. Miyawaki and H. Miyoshi for donating lentivirus vectors; and University of California, San Diego ([UCSD], San Diego, USA) for permission to use their RFP; H. Momma for statistical advice; Y. Akiyama for the illustrations. We also thank the Biomedical Research Unit of Tohoku University Hospital for providing the locations to maintain hiPSCs. Zebrafish line Tg [hb9:Venus] and wild-type strain RIKEN WT were obtained from National BioResource Project, Zebrafish Core Institution, Japan. Sanger sequencing of zebrafish phox2b DNA constructs was performed by the Support Center for Medical Research and Education, Tokai University. This work was supported by JSPS KAKENHI grant No. JP20K16593. The authors would also like to thank Enago (www.enago.jp) for the English language review.

Received: July 17, 2020
Revised: April 26, 2021
Accepted: April 28, 2021
Published: May 27, 2021

**REFERENCES**

Akiyama, T., Suzuki, N., Ishikawa, M., Fujimori, K., Sone, T., Kawada, J., Funayama, R., Fujishima, E., Mitsuzawa, S., Ikeda, K., et al. (2019). Aberrant axon branching via Fos-B dysregulation in FUS-ALS motor neurons. EBioMedicine 45, 362–378.

Allodi, I., Nijssen, J., Benitez, J.A., Schweingruber, C., Fuchs, A., Bonvicini, G., Cao, M., Kiehn, O., and Hedlund, E. (2019). Modeling motor neuron resilience in ALS using stem cells. Stem Cell Reports 12, 1329–1341.

An, D., Fuji, R., Iannitelli, D.E., Smerdon, J.W., Maity, S., Rose, M.F., Gelber, A., Wanasseljka, E.K., Yagudayeva, I., Lee, J.Y., et al. (2019). Stem cell-derivered cranial and spinal motor neurons reveal proteostatic differences between ALS resistant and sensitive motor neurons. Elife 8, e44243.

Babin, P.J., Goizet, C., and Raidua, D. (2014). Zebrafish models of human motor neuron diseases: advantages and limitations. Prog. Neurobiol. 118, 36–58.
The homeobox gene Phox2B is essential for the development of autonomic neural crest derivatives. Nature 399, 366–370.
Pei, D., Luther, W., Wang, W., Paw, B.H., Stewart, R.A., and George, R.E. (2013). Distinct neuroblastoma-associated alterations of PHOX2B impair sympathetic neuronal differentiation in zebrafish models. PLoS Genet. 9, e1003533.

Shimojo, D., Onodera, K., Doi-Torii, Y., Ishihara, Y., Hattori, C., Miwa, Y., Tanaka, S., Okada, R., Ohyama, M., Shoji, M., et al. (2015). Rapid, efficient, and simple motor neuron differentiation from human pluripotent stem cells. Mol. Brain 8, 79.

Song, M.R., Shirasaki, R., Cai, C.L., Ruiz, E.C., Evans, S.M., Lee, S.K., and Pfaff, S.L. (2006). T-Box transcription factor Tbx20 regulates a genetic program for cranial motor neuron cell body migration. Development 133, 4945–4955.

Stil, A., and Drapeau, P. (2016). Neuronal labeling patterns in the spinal cord of adult transgenic zebrafish. Dev. Neurobiol. 76, 642–660.

Strong, M.J., Volkening, K., Hammond, R., Yang, W., Strong, W., Leystra-Lantz, C., and Shoesmith, C. (2007). TDP43 is a human low molecular weight neurofilament (hNFL) mRNA-binding protein. Mol. Cell. Neurosci. 35, 320–327.

Taylor, A.M., Blurton-Jones, M., Rhee, S.W., Cribbs, D.H., Cotman, C.W., and Jeon, N.L. (2005). A microfluidic culture platform for CNS axonal injury, regeneration and transport. Nat. Methods 2, 599–605.

Taylor, J.P., Brown, R.H., Jr., and Cleveland, D.W. (2016). Decoding ALS: from genes to mechanism. Nature 539, 197–206.

Telfer, W.R., Busta, A.S., Bonnemann, C.G., Feldman, E.L., and Dowling, J.J. (2010). Zebrafish models of collagen VI-related myopathies. Hum. Mol. Genet. 19, 2433–2444.

Tian, F., Yang, W., Mordes, D.A., Wang, J.Y., Salameh, J.S., Mok, J., Chew, J., Sharma, A., Leno-Duran, E., Suzuki-Uematsu, S., et al. (2016). Monitoring peripheral nerve degeneration in ALS by label-free stimulated Raman scattering imaging. Nat. Commun. 7, 13283.

Tollervey, J.R., Curk, T., Rogelj, B., Briese, M., Cereda, M., Kayikci, M., Konig, J., Hortobagyi, T., Nishimura, A.L., Zupunski, V., et al. (2011). Characterizing the RNA targets and position-dependent splicing regulation by TDP-43. Nat. Neurosci. 14, 452–458.

Wijesekera, L.C., and Leigh, P.N. (2009). Amyotrophic lateral sclerosis. Orphanet J. Rare Dis. 4, 3.