Intracellular aggregates of phosphorylated TDP-43 are a major component of ubiquitin-positive inclusions in the brains of patients with frontotemporal lobar degeneration and ALS and are considered a pathological hallmark. Here, to gain insight into the mechanism of intracellular TDP-43 accumulation, we examined the relationship between phosphorylation and aggregation of TDP-43. We found that expression of a hyperactive form of casein kinase 1 δ (CK1δ1-317, a C-terminally truncated form) promotes mislocalization and cytoplasmic accumulation of phosphorylated TDP-43 (ubiquitin- and p62-positive) in cultured neuroblastoma SH-SY5Y cells. Insoluble phosphorylated TDP-43 prepared from cells co-expressing TDP-43 and CK1δ1-317 functioned as seeds for TDP-43 aggregation in cultured cells, indicating that CK1δ1-317-induced aggregated TDP-43 has prion-like properties. A striking toxicity and alterations of TDP-43 were also observed in yeast expressing TDP-43 and CK1δ1-317. Therefore, abnormal activation of CK1δ causes phosphorylation of TDP-43, leading to the formation of cytoplasmic TDP-43 aggregates, which, in turn, may trigger neurodegeneration.

Frontotemporal lobar degeneration (FTLD)2 and ALS are well known neurodegenerative disorders. FTLD is the second most common form of cortical dementia in the population below the age of 65 years. ALS is the most common of the motor neuron diseases and is characterized by progressive weakness and muscular wasting, resulting in death within a few years. Abnormal protein aggregates positive for ubiquitin are observed as a pathological hallmark in the brains of FTLD and ALS patients. TAR DNA-binding protein of 43 kDa (TDP-43) is the major component protein of ubiquitin-positive inclusions observed in the brains of patients with FTLD (FTLD-TDP) and ALS (1, 2). TDP-43 is expressed ubiquitously mainly in nuclei and has been reported to be involved in exon splicing, gene transcription, regulation of mRNA stability and biosynthesis, and formation of nuclear bodies (3–7). This protein is composed of 414 amino acids and includes two highly conserved RNA recognition motifs and a glycine-rich region mediating protein-protein interactions at the C terminus (8–11).

Intracellular aberrant protein aggregates in affected neurons are one of the neuropathological features of neurodegenerative diseases, and the formation of intracellular aggregates is believed to be associated with neurodegeneration leading to the onset of disease. Cytoplasmic proteins such as tau in Alzheimer disease and α-synuclein in Parkinson disease are accumulated in insoluble inclusions consisting of abnormal filaments with the fine structure of amyloid (12). In most cases, these proteins are hyperphosphorylated abnormally and aggregated in neuronal cells. Therefore, abnormal hyperphosphorylation is one of the characteristic posttranslational modifications of aggregated proteins in most neurodegenerative diseases, and phosphorylation is thought to be a key event in the formation of toxic intracellular protein aggregates. Various changes of TDP-43, including cytoplasmic localization, cleavage to produce C-terminal fragments, aggregation, and phosphorylation at the Ser-379, Ser-403/404, and Ser-409/410 residues of TDP-43 (1, 13) have been linked with TDP-43 proteinopathies, including FTLD-TDP and ALS. Cytoplasmic translocation and cleavage of TDP-43 have been reported to elicit intracellular TDP-43 accumulation (14–19). Regarding phosphorylation, various kinases have been suggested to be involved in the phosphorylation of TDP-43 (13, 20–24), but it is not clear whether any of them induce mislocalization and aggregate formation of TDP-43.

In this study, we examined which kinase is mainly involved in the formation of intracellular phosphorylated TDP-43 aggregates in cultured cells and yeast. We found that the hyperactive form of casein kinase 1 δ (CK1δ1-317, a C-terminally truncated form of CK1δ) promotes not only phosphorylation but also cytoplasmic localization and aggregation of TDP-43 most effectively among the tested kinases. CK1δ1-317-induced intracellular phosphorylated TDP-43 aggregates were found to serve as seeds for TDP-43 aggregation in cells. Significant tox-
Phosphorylated TDP-43 Aggregation Caused by Truncated CK1ε

icity and alterations of TDP-43 were also observed in yeast expressing TDP-43 and CK1ε. Our results clearly show that phosphorylation of TDP-43 by abnormally activated CK1ε causes both cytoplasmic aggregation of TDP-43 and cytotoxicity in vitro and in vivo, establishing a novel mechanism of neurodegeneration that is likely to be relevant to the pathogenesis of diseases such as FTLD and ALS.

Experimental Procedures

Antibodies—Monoclonal and polyclonal (anti-Ser(P)-409/410) antibodies against a synthetic phosphopeptide of TDP-43 have been reported previously (13, 25). The following antibodies against a synthetic phosphopeptide of TDP-43 have been reported previously (13, 25). The following antibodies against a synthetic phosphopeptide of TDP-43 have been reported previously (13, 25). The following antibodies against a synthetic phosphopeptide of TDP-43 have been reported previously (13, 25). The following antibodies against a synthetic phosphopeptide of TDP-43 have been reported previously (13, 25).

Cell Culture and Transfection of Expression Vectors—Human neuroblastoma SH-SY5Y cells obtained from the ATCC (Manassas, VA) were cultured in DMEM/F12 medium (Sigma) supplemented with 10% (v/v) fetal calf serum, penicillin-streptomycin-glutamine (Life Technologies), and MEM non-essential amino acid solution (Life Technologies), and MEM non-essential amino acid solution (Life Technologies). The cells were maintained at 37 °C under a humidified atmosphere of 5% (v/v) CO₂ in air. They were grown to 50% confluence in 6-well culture dishes for transient expression and then transfected with each expression vector (usually 1 μg) using XtreXGENE9 (Roche) according to the instructions of the manufacturer. Under our conditions, the efficiency of transfection using the pEGFP-C1 vector was 20–30%.

The expression vectors for the SH-SY5Y cells used in this study were as follows: pcDNA3.1-TDP-43 WT, pcDNA3.1-TDP-43 lacking a nuclear localization signal (78–84 residues, ΔNLS), pcCS2-Myc-CK1α1, pcCS2-Myc-CK1α2, pcCS2-Myc-CK1θ, pcCS2-Myc-CK1ε, pcDNA3.1-FLAG-CK1θ-317, pcDNA3.1-HA-CK2, pME18S-Cdc7-HA, and pME18S-ASK-FLAG. The pcCS2-Myc vectors were provided by Drs. Cheong Jit Kong and David M. Virshup (Duke-NUS Graduate Medical School Singapore).

Fractionation of Cellular Proteins and Immunoblotting—SH-SY5Y cells grown in a 6-well plate were transfected with several expression vectors. After incubation for 1–3 days, the cells were harvested and lysed in 300 μl of homogenization buffer (HB buffer: 10 mM Tris-HCl (pH 7.5) containing 0.8 M NaCl, 1 mM EGTA, 1 mM DTT, and 1% N-lauroylsarcosine sodium salt (Sarkosyl)) by brief sonication. The lysates were centrifuged at 100,000 × g for 20 min at room temperature. The supernatant was recovered as Sarkosyl-insoluble fraction (Sar-sup). The pellet was suspended in 100 μl SDS-sample buffer and sonicated. The resulting samples were used as the Sar-insoluble fraction (Sar-ppt). Each sample was separated by SDS-PAGE and immunoblotted with the indicated antibodies as described previously (26).

Immunofluorescence Analysis—SH-SY5Y cells were grown on coverslips and transfected as described above. After incubation for the indicated times, cells were fixed with 4% paraformaldehyde and stained with primary antibody at 1:500–1000 dilution. The cells were washed and incubated further with anti-mouse IgG-conjugated Alexa Fluor 488 (1:1000) or anti-rabbit IgG-conjugated Alexa Fluor 568 (1:1000) and then with Hoechst 33342 (Life Technologies) to counterstain nuclear DNA. The samples were analyzed using a LSM780 confocal laser microscope (Carl Zeiss).

Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) Exon 9 Skipping Assay—SH-SY5Y cells grown in 6-well plates were transfected with 0.5 μg of reporter plasmid pSPL3-CFTR exon 9, including the repeat sequence of TG11T7 (16), pcDNA3.1-TDP-43, and/or pcDNA3.1-CK1θ-317 (total 1.5 μg of plasmids), using XtreXGENE9 (Roche). The cells were harvested 48 h after transfection, and total RNA was extracted with TRIzol (Invitrogen). The cDNA was synthesized from 1 μg of total RNA using the Superscript II system (Invitrogen). Primary and secondary PCRs were carried out according to the instruction manual of the exon-trapping system (Life Technologies).

Real-time PCR—SH-SY5Y cells grown in 6-well plates were transfected with 1 μg of pcDNA3.1-TDP-43 and/or pcDNA3.1-CK1θ-317 (total 2 μg of plasmids), using XtreXGENE9 (Roche). Cells were harvested 48 h after transfection, and total RNA was isolated with TRIzol (Invitrogen). First-strand cDNA was synthesized with SuperScript II reverse transcriptase (Invitrogen). PCR reactions for Homo sapiens histone deacetylase 6 (HDAC6, NM_006044.2, 5’-CCCATTTGGTGGCAGTATG-3’ (forward) and 5’-CACAAAGGTTGGTCCATC-GTC-3’ (reverse)) and hypoxanthine-guanine phosphoribosyltransferase (internal standard, NM_000194.2, 5’-TGACCTTGATTATTTGTGCATACC-3’ (forward) and 5’-CGAGC-AAACGCTTCAGTCC-3’ (reverse)) were performed with Thundherbird SYBR quantitative PCR mixture (Toyobo) and CFX96 (Bio-Rad). The PCR reactions were carried out as follows: 1 min at 95 °C for the initial denaturation followed by 40 cycles of amplification at 95 °C for 15 s and 60 °C for 60 s.

Mutagenesis—Site-directed mutagenesis of the CK1θ-317 gene was performed to switch Lys-38 to alanine and arginine by using a site-directed mutagenesis kit (Agilent Technologies). All constructs were verified by DNA sequencing.

Mass Spectrometric Analysis of Phosphorylation Sites of Intracellular TDP-43 Aggregates—Sarkosyl-insoluble fraction prepared from cells expressing TDP-43 and CK1θ-317 was subjected to 12% SDS-PAGE. After electrophoresis, the pS409/410-positive, ~46-kDa bands were dissected and digested in gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC gel with trypsin. The digests were applied to a DiNa HPLC g
**Phosphorylated TDP-43 Aggregation Caused by Truncated CK1δ**

**FIGURE 1. Biochemical evidence of phosphorylated TDP-43 aggregation by CK1δ-317.** Shown are immunoblot analyses of proteins extracted from SH-SY5Y cells co-expressing both TDP-43 and either empty vector, Myc-tagged CK1α1, Myc-tagged CK1α2, Myc-tagged CK1β, Myc-tagged CK1ε, FLAG-tagged CK1δ-317, HA-tagged CK2, HA-tagged Cdc-7, or FLAG-tagged ASK. Proteins were extracted from cells with 1% Sarkosyl, and the Sar-sup and Sar-ppt were subjected to immunoblot analyses. The blots were probed using anti-phosphorylated TDP-43 (anti-pS409/410) polyclonal and anti-TDP-43 monoclonal (anti-TDP mono) antibodies, a mixture of anti-Myc monoclonal and anti-FLAG monoclonal antibodies, a mixture of anti-HA monoclonal and anti-FLAG monoclonal antibodies, and anti-tubulin α antibody. Because FLAG-CK1δ-317 is predicted to be ~35 kDa, the band of ~43 kDa may correspond to its ubiquitinated form. Note that the band of phosphorylated TDP-43 is observed in the Sar-sup and Sar-ppt of cells expressing CK1δ-317 (arrowheads).

**Introduction of Protein Aggregates as Seeds into Cultured Cells**—Cells co-expressing TDP-43 and CK1δ-317 were incubated for 3 days and then harvested. The Sar-ppt was prepared as described above and used as seeds. The Sar-ppt was resuspended in 100 μl of PBS and sonicated briefly. The resulting suspension (10 μl) was mixed with 120 μl of Opti-MEM (Life Technologies) and 62.5 μl of Multifectam reagent (Promega). After incubation for 30 min at room temperature, 62.5 μl of Opti-MEM was added, and the incubation continued for 5 min at room temperature. Then the mixtures were added to cells expressing TDP-43, and incubation continued for 6 h in a CO₂ incubator. After incubation, the medium was replaced with fresh DMEM/F12, and culture continued for the indicated period in each case. The cells were prepared for immunofluorescence and/or immunoblotting analyses as described above. Under our conditions, the efficiency of introduction of Sar-ppt seeds was ~10%.

**Yeast Experiments**—Standard yeast medium and transformation technology were used. Yeast cells were grown at 30 °C. The human TDP-43 gene with or without GFP was inserted into a pYES2/CT expression vector (Life Technologies). The human full-length CK1δ and CK1δ-317 genes were inserted into a pRS315 vector under the GAL1 promoter. The wild-type yeast strain BY4741 was transformed with these plasmids and plated on SD (synthetic complete containing dextrose)-Ura-Leu plates to isolate double-transfected yeast cells. For the cell toxicity assay, these cells were cultured in SD-Ura-Leu medium, washed, plated on SD-Ura-Leu (expression off) or SG (synthetic complete containing galactose)-Ura-Leu (expression on) plates and incubated for 2 days. For Western blotting, these cells were cultured in SD-Ura-Leu medium, washed, and then cultured in SG-Ura-Leu medium for 2 days. Cells were collected and broken with glass beads in HB buffer using a bead shaker. The lysates were centrifuged at 100,000 × g for 20 min at room temperature. The supernatant was recovered as the Sar-sup. The pellet was suspended in 100 μl of SDS-sample buffer and sonicated. The resulting samples were used as the Sar-ppt. For microscopic analysis, cells were cultured in SD-Ura-Leu medium, washed, and then cultured in SG-Ura-Leu medium for 10 h. Cells were fixed and mounted on slide glasses with Hoechst 33342 (Life Technologies) and observed with a fluorescence microscope (BZ-X710, Keyence).

**Statistical Analysis**—Statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software). Data were analyzed statistically using unpaired two-tailed Student’s t test. A p value of 0.05 or less was considered to be statistically significant.

**Results**

**Expression of Truncated CK1δ Causes Mislocalization and Aggregation of TDP-43 in Cultured Cells**—Although recent studies have shown that several protein kinases, including CK1ε, CK2, and Cdc7, are involved in phosphorylation of TDP-43 in vitro, in cultured cells, fly or Caenorhabditis elegans (13, 20–24), it remains unknown whether phosphorylation of TDP-43 is associated with intracellular aggregation of itself or whether a kinase elicits the formation of phosphorylated TDP-43 inclusions.
To address these questions, we co-expressed each kinase and TDP-43 in cultured neuroblastoma SH-SY5Y cells. The cells were transiently transfected with each expression vector for 2–3 days and then harvested. The cells were lysed, and the lysates were fractionated and subjected to immunoblot analysis. As shown in Fig. 1, intracellular expression of each kinase and TDP-43 was confirmed. In cells transfected with both the hyperactive form of CK1δ \((\text{CK1}\delta^{1-317})\), which lacks the C-terminal domain \((27, 28)\), and TDP-43, a band of phosphorylated TDP-43 (Fig. 1, red arrowheads) was detected in the Sar-ppt using anti-phosphorylated TDP-43 antibody (Ser(P)-409/410), clearly indicating that expression of CK1δ^{1-317} induces intracellular aggregation of TDP-43 in cultured cells. On the other hand, in cells transfected with TDP-43 and any one of CK1α, CK1ε, CK2, and Cdc7/ASK (Dbf4/activator of S phase kinase, known as the activator of Cdc7 \((29, 30)\)), phosphorylated TDP-43 was not found in the Sar-ppt (Fig. 1), indicating that none of these kinases other than \(\text{CK1}\delta^{1-317}\) can elicit phosphorylated TDP-43 aggregation.

To monitor the localization and aggregation of TDP-43, we performed immunofluorescence analyses of transfected cells. In confocal microscopic analyses of cells transfected with FLAG-tagged \(\text{CK1}\delta^{1-317}\) alone, endogenous TDP-43 was phosphorylated and aggregated in the cytoplasm (Fig. 2). In cells expressing both TDP-43 and FLAG-tagged \(\text{CK1}\delta^{1-317}\) alone, cytoplasmic inclusions composed of phosphorylated TDP-43 were observed, and these inclusions were also stained with anti-ubiquitin and anti-p62 antibodies. Therefore, their characteristics are very similar to those of the phosphorylated TDP-43 inclusions positive for ubiquitin and p62 seen in the brains of patients with TDP-43 proteinopathy.

Next we performed time course experiments with cells expressing TDP-43 and FLAG-tagged \(\text{CK1}\delta^{1-317}\). Cells were transfected with plasmids expressing TDP-43, FLAG-tagged \(\text{CK1}\delta^{1-317}\), or both, followed by immunoblotting analysis. In cells expressing TDP-43 alone, phosphorylated TDP-43 was not observed in the Sar-sup or Sar-ppt (Fig. 3). In cells transfected with TDP-43 alone, endogenous phosphorylated TDP-43 was not observed in the Sar-sup or Sar-ppt (Fig. 3). In cells transfected with FLAG-tagged \(\text{CK1}\delta^{1-317}\) alone, phosphorylated TDP-43 was phosphorylated and aggregated on days 2 and 3. The level of FLAG-tagged \(\text{CK1}\delta^{1-317}\) in the Sar-ppt was slightly greater than that in the Sar-sup, indicating that FLAG-tagged \(\text{CK1}\delta^{1-317}\) is aggregation-prone (Fig. 3). We also observed that phosphorylation and aggregation of full-length TDP-43 preceded the fragmentation of phosphorylated

![Microscopic analyses of phosphorylated TDP-43 inclusions by CK1δ1-317.](http://example.com/fig2.jpg)

**FIGURE 2. Microscopic analyses of phosphorylated TDP-43 inclusions by CK1δ1-317.** Shown are confocal microscopic analyses of cells expressing TDP-43 alone, FLAG-CK1δ1-317 alone, or both. These cells were immunostained with anti-phosphorylated TDP-43 (pS409/410) polyclonal, anti-FLAG monoclonal, anti-ubiquitin (Ub) monoclonal, and anti-p62 monoclonal antibodies and counterstained with Hoechst 33342. Scale bars = 20 nm.
Phosphorylated TDP-43 Aggregation Caused by Truncated CK16

CK16-317 Kinase Activity Is Essential for Intracellular TDP-43 Aggregation—We tested whether kinase activity of CK16-317 is necessary for induction of intracellular aggregates of TDP-43. It has been reported that CK16 exon 2 encodes a portion of the ATP-binding domain essential for kinase activity and that the K38R and K38A mutants of CK16 have no kinase activity (33). We constructed inactive K38R and K38A mutants of CK16-317 and transfected one of these mutants and TDP-43 into SH-SY5Y cells. After 2 days of incubation, cells were harvested, and cell lysates were prepared for immunoblot analyses. As shown in Fig. 5, Sar-insoluble TDP-43 was detected in cells expressing wild-type CK16-317 alone, indicating that endogenous TDP-43 is phosphorylated and aggregated by expression of the WT. In cells transfected with both the WT and TDP-43, a strong band of phosphorylated TDP-43 was detected in the Sar-ppt. On the other hand, we hardly observed phosphorylated TDP-43 in the Sar-ppt of cells expressing either the K38R or K38A mutant of CK16-317 together with TDP-43. These results show that kinase activity of CK16-317 is required to elicit intracellular aggregate formation of phosphorylated TDP-43.

Identification of Phosphorylation Sites of Aggregated TDP-43 by CK16-317—To investigate whether phosphorylation of TDP-43 by CK16-317 is a key modification for intracellular accumulation, we attempted to identify phosphorylation sites of aggregated TDP-43 by CK16-317. The Sar-ppt from cells expressing TDP-43 and CK16-317 was prepared and subjected to mass spectrometric analyses. Finally, we identified Ser-92, Ser-292, Ser-305, Ser-317, Ser-333, Ser-389, Ser-393, Ser-395, Ser-403, Ser-404, Ser-409 and Ser-410 as phosphorylation sites of aggregated TDP-43 by CK16-317, as shown in Fig. 6A. Then, to evaluate the effects of phosphorylation of TDP-43 on its intracellular accumulation in cells, we prepared several Ser-to-Ala mutants of these CK16-317 phosphorylation sites and transfected them together with CK16-317 into SH-SY5Y cells. After incubation for 3 days, Sar-ppt fractions were prepared and subjected to immunoblot analyses. We observed that the band intensities of phosphorylated TDP-43 in the Sar-ppt of cells expressing S393A/S395A (Fig. 6, B, lane 4, and C, column 4), S403A/S404A (Fig. 6, B, lane 5, and C, column 5) and S393A/S395A/S403A/S404A (Fig. 6, B, lane 6, and C, column 6) were decreased compared with cells expressing TDP-43 wild-type (Fig. 6, B, lane 3, and C, column 3) using not only anti-Ser(P)-409/410 but also anti-TDP-43 monoclonal antibody (Fig. 6, B and C). On the other hand, the level of phosphorylated TDP-43 in the Sar-ppt of cells expressing S393A/S395A (Fig. 6, B, lane 4, and C, column 4) was not significantly different from that of cells expressing only CK16-317 (Fig. 6, B, lane 2, and C, column 2). In other words, the level of phosphorylated TDP-43 in the Sar-ppt of cells expressing S393A/S395A was reduced to a level similar to that of endogenous phosphorylated TDP-43, suggesting that phosphorylation of TDP-43 at Ser-393/Ser-395 by CK16-317 facilitates its accumulation. In the case of cells expressing S403A/S404A, the level of phosphorylated TDP-43 in the Sar-ppt (Fig. 6, B, lane 5, and C, column 5) was significantly higher than that in cells expressing only CK16-317 (Fig. 6, B, lane 2, and C, column 2), which exhibit the background phosphorylation level of the endogenous TDP-

TDP-43 in cells expressing both TDP-43 and FLAG-tagged CK16-317 (Fig. 3). Taken together, these results clearly indicate that expression of CK16-317 induces phosphorylation and mislocalization of TDP-43 and the formation of intracellular TDP-43 aggregates similar to those found in the brains of FTLD-TDP or ALS patients.

Physiological Activites of TDP-43 Are Suppressed in Cells Co-expressing TDP-43 and CK16-317—To investigate whether the phosphorylation and induced aggregation of TDP-43 by CK16-317 are accompanied by changes in the biological properties of TDP-43, we first performed an exon skipping assay of CFTR, which is a well known target of TDP-43 (31). As shown in Fig. 4A, CFTR exon 9 skipping activity was decreased significantly in cells expressing both TDP-43 and CK16-317 compared with cells expressing TDP-43 alone. Furthermore, we evaluated the mRNA levels of endogenous HDAC6, which has also been reported to be a target of TDP-43 (32), in these cells. Real-time PCR analyses confirmed that endogenous HDAC6 mRNA levels were reduced in cells transfected with both TDP-43 and CK16-317 compared with cells transfected with TDP-43 alone (Fig. 4B). These results suggest that the levels of soluble and functional TDP-43 are reduced in cells expressing TDP-43 and CK16-317 and, consequently, that physiological activities of TDP-43 are suppressed in these cells compared with normal cells.
These results suggest that phosphorylation of TDP-43 at Ser-393/Ser-395 and, to a lesser extent, at Ser-403/Ser-404 facilitates TDP-43 accumulation.

**Prion-like Seeding Activity of Insoluble Phosphorylated TDP-43 Aggregates**

We examined whether insoluble phosphorylated TDP-43 prepared from cultured cells has a prion-like seeding function. The Sarkosyl-insoluble fraction was prepared from cells expressing TDP-43 and CK1δ/H92541-317 as seeds (Sar-ppt seeds) and introduced into cells expressing TDP-43 wild-type or δ/H9004NLS. After incubation for 2 days, these cells were stained with anti-Ser(P)-409/410 antibody and analyzed by confocal microscopy. In cells expressing TDP-43 wild-type treated with Sar-ppt seeds, no aggregates positive for anti-Ser(P)-409/410 were observed (data not shown). In contrast, we found phosphorylated TDP-43 inclusions in cells expressing TDP-43 δ/H9004NLS treated with Sar-ppt seeds, as shown in Fig. 7. No aggregates were detected in cells expressing TDP-43 δ/H9004NLS alone or cells treated with Sar-ppt seeds alone (Fig. 7). These results indicate that insoluble phosphorylated TDP-43 aggregates can serve as seeds for the transformation of soluble TDP-43 into insoluble aggregates in cultured cells, suggesting that phosphorylated TDP-43 aggregates induced by CK1δ/H92541-317 have prion-like seeding properties.

**Alterations of TDP-43 Caused by Expression of CK1δ/H92541-317 Induce Toxicity in Yeast**

To examine cytotoxicity in cells expressing TDP-43, CK1δ/H92541-317, or both, cell viability was evaluated using the trypan blue exclusion method 2 days after the transfection of plasmids. Viability rates were as follows (n = 5): non-transfected cells, 89.6% ± 1.4%; cells expressing TDP-43, 85.6% ± 8.3%; cells expressing CK1δ/H92541-317, 92.2% ± 4.0%; cells 43. These results suggest that phosphorylation of TDP-43 at Ser-393/Ser-395 and, to a lesser extent, at Ser-403/Ser-404 facilitates TDP-43 accumulation.

**FIGURE 4.** The physiological properties of TDP-43 are altered in cells co-expressing TDP-43 and CK1δ/H92541-317. A, CFTR exon 9 skipping assay of transfected cells. Gel electrophoresis of the RT-PCR products of RNA from transfected cells was performed. The RNAs from SH-SY5Y cells transfected with the reporter plasmid pSPL3-CFTR exon 9 plus pcDNA3.1 expression vectors were used as templates for RT-PCR analysis. The products were analyzed by electrophoresis in 1.5% agarose gel. The band intensities (∼exon 9) were quantified and the results are expressed as mean ± S.E. (n = 3). ****, p < 0.0001 by Student’s t test. a.u., arbitrary unit. B, quantification of endogenous HDAC6 mRNA levels in several transfected cells by real-time PCR. The mRNA ratio of HDAC6/hypoxanthine-guanine phosphoribosyltransferase (HPRT) is expressed as mean ± S.E. (n = 3). *, p < 0.05 by Student’s t test.

**FIGURE 5.** Kinase activity of CK1δ/H92541-317 is required for the accumulation of phosphorylated TDP-43. Shown are immunoblot analyses of cells expressing FLAG-CK1δ/H92541-317 WT, K38R (KR), or K38A (KA) with or without TDP-43. Sar-sup and Sar-ppt were prepared from cells and subjected to immunoblot analyses. The blots were probed using anti-phosphorylated TDP-43 (anti-pS409/410) monoclonal, anti-FLAG monoclonal, and anti-tubulin α antibodies. Note that endogenous TDP-43 is phosphorylated and aggregated in cells expressing the WT alone. The **arrowhead** shows phosphorylated TDP-43.
expressing TDP-43 and CK1δ-317, 93.2% ± 2.3%. Therefore, no obvious toxicity was found in cells having phosphorylated TDP-43 aggregates.

Next we tried to develop a yeast model expressing human TDP-43 and CK1δ to examine whether alterations of TDP-43, such as mislocalization and intracellular aggregation, resulting in neurodegeneration are caused by expression of CK1δ-317 in vivo. We performed spotting assays to compare growth defects elicited by full-length CK1δ or CK1δ-317 in the presence or absence of TDP-43. As shown in Fig. 8A, co-expression of TDP-43 and CK1δ-317 resulted in the greatest toxicity. Co-expression of TDP-43 and CK1δ also showed considerably more toxicity than single expression of TDP-43, CK1δ, or CK1δ-317.

To test whether intracellular phosphorylated TDP-43 aggregation is caused by CK1δ-317 in yeast, we performed immunoblot analyses of yeast cells expressing TDP-43 with CK1δ or CK1δ-317. Yeast lysates were fractionated with 1% Sar, and Sar-sup and Sar-ppt were subjected to immunoblot analyses. As shown in Fig. 8B, the band corresponding to phosphorylated TDP-43 was detected not only in the Sar-sup but also the Sar-ppt of yeast cells co-expressing TDP-43 and CK1δ-317, clearly confirming that the formation of intracellular phosphorylated TDP-43 aggregates is induced by CK1δ-317 in yeast. Further more, immunofluorescence analyses of cells expressing TDP-43 and CK1δ-317 were carried out. When GFP-tagged TDP-43 (TDP-43-GFP) alone was transfected into yeast cells, TDP-43-GFP was expressed in nuclei (Fig. 8C). On the other hand, in cells expressing both TDP-43-GFP and CK1δ-317, we observed that TDP-43 is mislocalized considerably from the nucleus to the cytosol and is accumulated partly into dot-like inclusions, as shown in Fig. 8C (arrowheads). Taken together, these data show that, in yeast, CK1δ-317 can also induce mislocalization and aggregate formation of TDP-43, resulting in cytotoxicity.

**Discussion**

Several kinases, such as CK1ε, CK2, and Cdc7, have been reported to be involved in phosphorylation of TDP-43 in vitro and in vivo (13, 20–24). Among these kinases, we report here that the truncated and hyperactive form of CK1δ (CK1δ-317) has the
most striking ability to hyperphosphorylate TDP-43, leading to its accumulation in SH-SY5Y cells. However, we could not reproduce phosphorylation of TDP-43 by CK1ε, CK2, or Cdc7 in SH-SY5Y cells. A possible reason for this apparent discrepancy would be species or cell type differences between this cultured human neuroblastoma cell line and cells from fly and C. elegans.

Our results demonstrate that hyperactive CK1ε-317 causes TDP-43 mislocalization and accumulation of intracellular phosphorylated TDP-43 in cultured cells. We also found that expression of CK1ε-317 and TDP-43 causes mislocalization and aggregation of phosphorylated TDP-43 in yeast cells, ultimately resulting in cell death. It is particularly striking that mislocalization of TDP-43 from nuclei to the cytosol was induced in yeast cells expressing CK1ε-317 (Fig. 8C). Furthermore, we showed that physiological activities of TDP-43 were suppressed in cells including phosphorylated TDP-43 aggregates (Fig. 4). These observations suggest that loss of normal TDP-43 function is a causative factor of cytotoxicity, although further investigation is needed to elucidate the molecular mechanisms of cytotoxicity because of aggregation of phosphorylated TDP-43.

The mechanisms through which phosphorylation of TDP-43 by CK1ε-317 elicits intracellular aggregation of TDP-43 remain unclear, but it is interesting that multiple phosphorylation at Ser-393/395 and/or Ser-403/404 of TDP-43 is likely to trigger the intracellular accumulation. Autosomal-dominant missense mutations in the TARDBP gene have been identified in patients with ALS or FTLD-TDP. Interestingly, most mutations were reported to be located in the C-terminal portion of TDP-43, and those that are present in the C-terminal enhance aggregation of TDP-43 (17). It has also been reported that the C-terminal portion of TDP-43 shows sequence similarity to prion proteins (34). These findings suggest that conformational changes triggered by mutation in the C-terminal portion of TDP-43 are related to its aggregation. Therefore, hyperphosphorylation of the C-terminal portion of TDP-43 (at Ser-393/395 and/or Ser-403/404) may cause structural changes of full-length TDP-43 that promote intracellular aggregation.

We also observed that endogenous TDP-43 was slightly phosphorylated in cells treated with Sar-ppt seeds alone (Fig. 7), which may indicate that TDP-43 seeds can trigger not only
aggregation but also phosphorylation of the endogenous protein. This result also suggests that conformational changes of TDP-43 leading to aggregation may precede phosphorylation in the presence of TDP-43 seeds. Alternatively, soluble TDP-43 may be conformationally altered when it is associated with TDP-43 seeds in cells, and the resulting structurally changed TDP-43 may be the preferential target of phosphorylation by some kinase(s). In any case, further study will be needed to elucidate the molecular relationship between protein aggregation and phosphorylation.

There is increasing evidence of cell-cell transmission of aggregated proteins such as tau, α-synuclein, and TDP-43 in both cell culture and animal models (12, 26, 35–43). Therefore, it is a plausible hypothesis that prion-like propagation of aberrant protein aggregates is involved in the pathogenesis of most neurodegenerative diseases. In these models, recombinant protein aggregates or detergent-insoluble proteins prepared from diseased brains were used as seeds and introduced into cultured cells or brains of mice. Transduction of such exogenous seeds is indispensable for the formation of aggregates in these models. In the case of human diseased brains, abnormal protein aggregates are likely to be produced in some vulnerable neurons and then propagate between neuronal cells without such invasive treatment. However, it remains less well understood how the first aggregates to serve as seeds are formed in the cells. In this study, we found that detergent-insoluble phosphorylated TDP-43 prepared from cells expressing TDP-43 and CK1δ isolated as seeds for intracellular TDP-43 aggregation. Our results indicate that the insoluble hyperphosphorylated TDP-43 aggregates generated by abnormally hyperactivated CK1δ are not artifacts but have prion-like amyloid features and can propagate from cell to cell. Therefore, we suggest that aberrant activation of protein kinases can be a cause of TDP-43 proteinopathy.

In summary, our results show that hyperphosphorylation of TDP-43 by CK1δ isolated as seeds for intracellular TDP-43 aggregation. Our results indicate that the insoluble hyperphosphorylated TDP-43 aggregates generated by abnormally hyperactivated CK1δ are not artifacts but have prion-like amyloid features and can propagate from cell to cell. Therefore, we suggest that aberrant activation of protein kinases can be a cause of TDP-43 proteinopathy. In summary, our results show that hyperphosphorylation of TDP-43 by CK1δ isolated as seeds for intracellular TDP-43 aggregation. Our results indicate that the insoluble hyperphosphorylated TDP-43 aggregates generated by abnormally hyperactivated CK1δ are not artifacts but have prion-like amyloid features and can propagate from cell to cell. Therefore, we suggest that aberrant activation of protein kinases can be a cause of TDP-43 proteinopathy.

**Author Contributions**—T. N. and H. M. designed the research. T. N. conducted most of the biochemical and immunofluorescence experiments and wrote the manuscript with input from G. S., Y. T., and F. K. G. S. performed the yeast experiments. Y. T. carried out the real-time PCR analysis. F. K. conducted the mass spectrometric analysis. H. M. provided key reagents. T. N., G. S., Y. T., F. K., S. H., H. O., T. M., M. S., H. A., H. M., and M. H. analyzed the data.
Phosphorylated TDP-43 Aggregation Caused by Truncated CK1δ

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