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Regulation of Autophagy by Neuropathological Protein TDP-43*[S]

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TDP-43 is a DNA/RNA-binding protein with multicellular functions. As a pathosignature protein of a range of neurodegenerative diseases, TDP-43 is also the major component of the polyubiquitinated inclusions in the pathological cellular samples of these diseases. In normal cells, TDP-43 is processed and degraded by both autophagy and the ubiquitin-proteasome systems. We have found, by microarray hybridization and RT-PCR analyses, that the level of the mRNA encoding the major autophagy component *Atg7* is decreased upon depletion of TDP-43 by RNAi knockdown. This decrease of the *Atg7* mRNA level could be rescued by overexpression of an siRNA-resistant form of TDP-43, and it appears to be the result of destabilization of the *Atg7* mRNA, to which TDP-43 could bind through its RNA recognition motif 1 domain. Furthermore, depletion of TDP-43 with the consequent loss of the *Atg7* mRNA/ATG7 protein causes impairment of the autophagy and facilitates the accumulation of polyubiquitinated proteins as well as the autophagy/ubiquitin-proteasome system substrate p62 in the cells. These data demonstrate the function of TDP-43 as a maintenance factor of the autophagy system, and they suggest the existence of a feedback regulatory loop between TDP-43 and autophagy. A scenario in which loss of function of TDP-43 contributes to the development of TDP-43 proteinopathies is presented.

TDP-43 (TAR DNA-binding protein) (1) is a highly conserved and abundantly expressed protein among the mammals and invertebrates (2, 3). The protein is located mainly in the nucleus as punctuated structures designated T-bodies (4), but it is also distributed in the cytoplasm as discrete RNA granules (5). Multiple cellular functions have been implicated to be regulated by TDP-43, which consist of but are not restricted to transcriptional repression, RNA processing including splicing, biogenesis of miRNAs, and even translation (reviewed in Refs. 6–8). Some of these regulations have been shown to be mediated through the two RNA recognition motifs (RRM1 and RRM2) and the C-terminal glycine-rich, prion-like GQN domain (6, 7). Mutational studies have revealed that the RNA recognition motif 1 (RRM1)[3] domain contributes much of the nucleic acid binding activity, whereas the function of RRM2 is still unclear and appears to play a lesser role in RNA binding (7). Studies have also established that an intact GQN domain, which recruits additional heterogeneous ribonucleoprotein (hnRNPs), is also necessary for the splicing activity of TDP-43 (2, 9).

More recently, TDP-43 has been identified as the major pathological signature protein of patients with the frontotemporal lobar degeneration with ubiquitin-positive inclusions and amyotrophic lateral sclerosis (10, 11). In particular, the metabolism of TDP-43 in the diseased cells of frontotemporal lobar degeneration with ubiquitin-positive inclusions and amyotrophic lateral sclerosis are misregulated, resulting in the generation of polyubiquitinated, phosphorylated, and caspase-3-cleaved species concentrated in the ubiquitinated inclusions (UBIs) in the cytoplasm and, to a much lesser extent, in the nucleus. Often accompanied with formation of the cytoplasmic UBIs is depletion of the nuclear TDP-43 (10, 11) (reviewed in Ref. 12). A causative role of TDP-43 in the pathogenesis of these diseases is substantiated genetically by the identification of >30 different mutations in its C-terminal GQN domain (reviewed in Ref. 13). The molecular basis of misregulation of the TDP-43 metabolism remains to be examined. Also unclear are the exact roles of TDP-43 in the pathogenesis of diseases with TDP-43(+)UBIs. Both loss-of-function of TDP-43 and gain-of-cytotoxicity, *e.g.* as caused by the TDP-43(+)UBIs, have been suggested to facilitate the disease initiation as well as propagation (reviewed in Refs. 6, 7, 12, 14).

Regarding the scenario of pathogenesis by loss-of-function of TDP-43, it is interesting to note that knock-out of TDP-43 expression in mice and/or *Drosophila* could result in defects of early embryonic developments, impairment of the motor function, and decrease of learning ability (15–19). These studies, among others, have evidenced the importance of TDP-43 in development and/or neuronal function, but they do not provide clues to the role of this protein in TDP-43 proteinopathies on the molecular level. With respect to the metabolism of TDP-43, it is interesting to note that the homeostatic concentration of TDP-43 in normal cells is tightly regulated *in vivo*, as evidenced by the similar cellular amounts of TDP-43 in between wild type ES cells and mutant ES with knock-out of TDP-43 expression from one of the two alleles (15). Similar observation was also

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[3] The abbreviations used are: RRM1, RNA recognition motif 1; UBI, ubiquitinated inclusion; TDP-43[R], siRNA-resistant form of TDP-43; scRNA, scrambled RNA; ActD, actinomycin D; LC3, microtubule-associated protein light chain 3; UPS, ubiquitin-proteasome system; 3-MA, 3-methyladenine.

made in analysis of transgenic TDP-43 mice, in which the TDP-43 amounts were similar between the heterozygous and homozygous transgenic mice (20). Significantly, TDP-43 has been shown recently to bind and enhance the degradation of its own mRNA (21). This negative feedback loop provides one route through which the TDP-43 protein could auto-regulate its own cellular concentration. In this work, we present evidence that function of the autophagy system, which is responsible for processing and degradation of the TDP-43 protein, is maintained in part through TDP-43 binding to and stabilization of the mRNA encoding one of the essential components of autophagy.

EXPERIMENTAL PROCEDURES

Plasmids—Plasmids encoding FLAG-TDP-43 and FLAG-TDP-43 (ΔRRM1) have been described previously (22). Plasmids encoding an siRNA-resistant form of TDP-43, TDP-43^R, and its derivative lacking the RRM1 motif, TDP-43^R (ΔRRM1), were constructed by site-directed mutagenesis of the third bases of three individual codons in the siRNA-1 target sequence of the corresponding wild type cDNAs.

Cell Culture and Transfection—Neuro2A, a mouse neuroblastoma cell line, was cultured in minimum essential medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. NSC34, a motor neuron-like cell line (23), and NIH3T3, a fibroblast cell line (24), were maintained in Dulbecco's modified eagle medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Transfections of the plasmid DNA and RNA oligonucleotides were performed in six-well dishes with Lipofectamine 2000 (Invitrogen) as per the manufacturer's instructions.

The RNAi oligonucleotides targeting the *Tdp-43* mRNA (siRNA-1, 5′-GGAUCUGAAAGACUAUUUC-3′ (sense); siRNA-2, 5′-CCAAUGCUGAACCUAAGCA-3′ (sense) (25)) and *Atg7* mRNA (*Atg7*-siRNA (26)), and the scRNA oligonucleotide with scrambled sequence were all purchased from Ambion. (Sequences are available on request.) It should be noted here that ~50% of the Neuro2A cells transfected with the *Tdp-43* RNAi oligonucleotide, but not those transfected with the scRNA oligonucleotide, died at 72 h post-transfection. This toxic effect of *Tdp-43* siRNA oligonucleotides on the viability of Neuro2A cells was also noted and reported by Iguchi *et al.* (25).

For the rescue experiments, the different rescuing plasmids were transfected into Neuro2A cells after 24 h of the RNAi oligonucleotide transfection. Samples were collected at 36 h after the second transfection and analyzed. To induce autophagy, serum starvation of the cells was done for 15 h after RNAi oligonucleotide treatment for 48 h.

RNA Immunoprecipitation (RNA IP)—RNA IPs were performed as described previously (22).

Microarray Hybridization—Total RNAs were isolated using the TRIzol reagent (Invitrogen). The poly(A)$^+$ RNAs were used for labeling by biotin according to the standard GeneChip Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA). Each of the six sets of labeled cRNA probes was hybridized to a mouse genome 430 2.0 Array (Affymetrix). The data treatment and statistical analysis were carried out by using GeneSpring software (version 11.5, Agilent Technologies, Palo Alto, CA).

Antibodies—Antibodies used for immunoblotting and immunofluorescence staining included anti-FLAG (M2, Sigma), rabbit anti-TDP-43 (GeneTex), rabbit anti-ATG7 (Sigma), rabbit anti-LC3 (Sigma), guinea pig anti-p62 (Progen), and mouse anti-ubiquitin (Millipore).

Western Blot Analysis—Total proteins were resolved by SDS-PAGE, transferred onto PVDF membrane (Millipore, Billerica, MA), and hybridized with different antibodies in 5% nonfat milk. For ubiquitin and p62 blot analysis, the total proteins were extracted in the urea buffer as described previously (19) before gel electrophoresis.

Actinomycin D (ActD) Chasing—Neuro2A cells were transfected either with the scRNA oligonucleotide or siRNA-1 oligonucleotide for 48 h. Actinomycin D (Sigma) was then added into the cell culture medium (5 μg/ml), and cell samples were collected at 3-h intervals. Total RNAs were isolated and analyzed by semiquantitative RT-PCR. The PCR products were resolved on 1.5% agarose gels, and the intensity of the DNA bands were quantitated using an Alpha Imager 2200 (Alpha Innotech Corp.). The trend lines were drawn using Microsoft Excel software. The *p* values were calculated by Student's *t* test.

Immunofluorescence Staining—Neuro2A cells grown on coverslips were transfected with either the scRNA oligonucleotide or the siRNA-1 oligonucleotide for 48 h. MG132 was added into the culture medium and incubated for overnight. Separately, untransfected Neuro2A cells were also treated with 10 μM MG132 (Sigma) or 10 mM 3-MA (Sigma) for overnight. For staining, the cells were first washed with PBS, fixed with 4% paraformaldehyde, and subsequently permeabilized with 0.1% Triton X-100. The coverslips were blocked with 10% donkey serum for 1 h at room temperature and incubated with anti-TDP-43 (1:500; GeneTex) and anti-ubiquitin (1:500; Millipore) diluted in 1% donkey serum at 4° C overnight. The coverslips were washed in PBS three times and incubated with the secondary antibodies for 2 h. The slips were mounted using VectaShield (Vector Laboratories), and the images were scanned in a Zeiss LSM 510 Meta confocal microscope. The confocal data were quantitated using the MetaMorph software (Molecular Devices).

Electron Microscopy—For electron microscopy analysis, the cells were fixed by immersion in a mixture of 2.5% glutaraldehyde and 2.5% paraformaldehyde in 0.1 M sodium cacodylate buffer. Post fixation was performed in 1% osmium tetraoxide followed by an overnight immersion in 1% uranylacetate dissolved in 50 mM maleate buffer. Standard procedures for dehydration and embedding in spur resin were employed, and thin sections were examined in a TECNI Sprit TWIN FEI electron microscope.

RESULTS

Loss of TDP-43 Down-regulated ATG7—To investigate the function of TDP-43 in neuronal cells, we first carried out RNAi knockdown experiments in Neuro2A cells, which is a mouse neuroblastoma cell line (27). The Neuro2A cells were transfected either with scRNA oligonucleotide or with siRNA-1 oligonucleotide, and the total RNAs were isolated and subjected

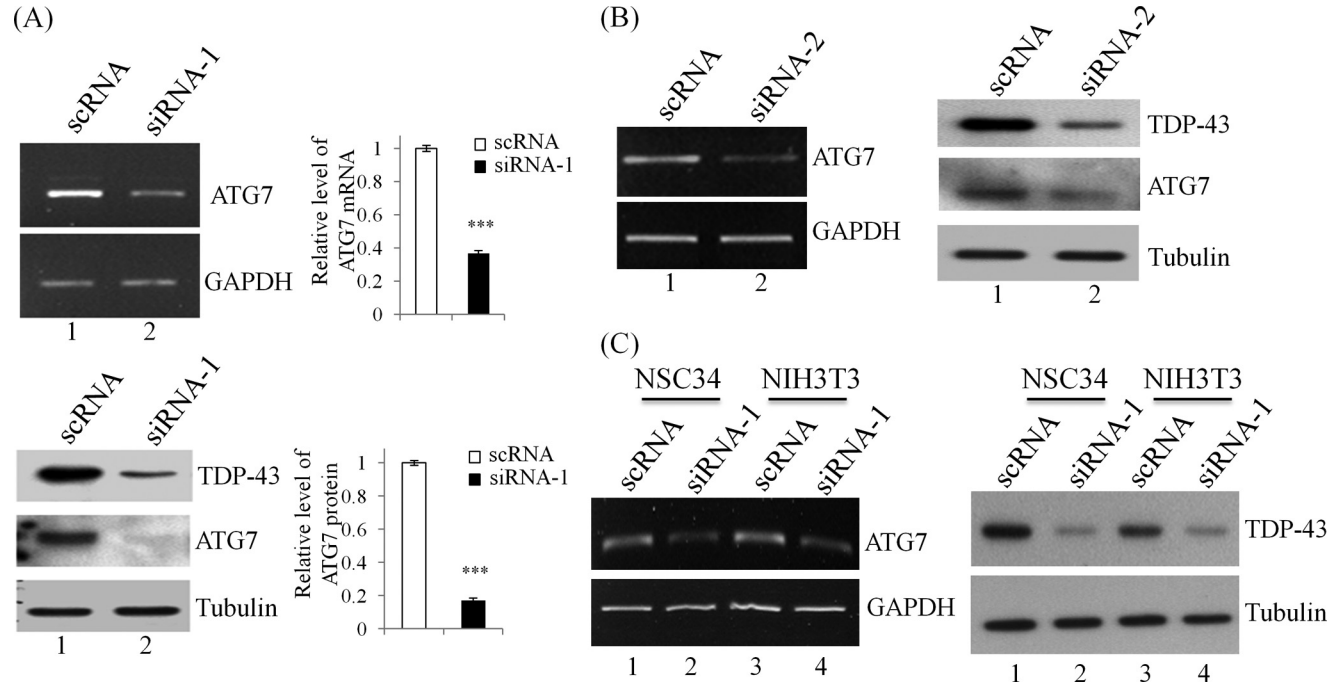

FIGURE 1. **Down-regulation of ATG7 by depletion of TDP-43.** *A*, *upper panels*, semi-quantitative RT-PCR analysis of the total RNAs from Neuro2A cells transfected with scRNA oligonucleotide (*lane 1*) or siRNA-1 oligonucleotide (*lane 2*). PCR primers specific for the *Atg7* mRNA and GAPDH mRNA were used in PCR. Note the down-regulation of the level of *Atg7* mRNA in siRNA-1 oligonucleotide-treated cells, as quantitatively presented in the histogram on the *right*. ***, p value < 0.001. *Lower panels*, the data of Western blotting analysis of total lysates from Neuro2A cells transfected with the scRNA oligonucleotide (*lane 1*) and siRNA-1 oligonucleotide (*lane 2*), respectively. Note the decreases of amounts of both the TDP-43 and ATG7 proteins in *lane 2*. ***, $p < 0.001$. The decrease of the ATG7 protein upon siRNA-1 treatment is also quantitatively presented in the histogram. *B*, repeat of the experiments in *A* above with use of the siRNA-2 oligonucleotide. Note the decreased amounts of both the *Atg7* mRNA (*left panel*, *lane 2*) and the ATG7 protein (*right panel*, *lane 2*). *C*, decrease of ATG7 expression in NSC34 and NIH3T3 cells upon TDP-43 knockdown by siRNA-1. The levels of *Atg7* mRNA in NSC34 and NIH3T3 cells were assayed by RT-PCR after transfection of the cells with siRNA-1 oligonucleotide for 48 h. Note the decrease of the amounts of *Atg7* mRNA (*left panel*, *lanes 2* and *4*) upon knockdown of the TDP-43 protein by si-RNA-1 (*right panel*, *lanes 2* and *4*).

to microarray analysis using the Affymetrix mouse genome 430 2.0 array. The TDP-43 knockdown efficiency was >90% in three independent experiments that we carried out. The raw data of the microarray hybridization were analyzed with the GeneSpring software (version 11.5), and a total of 221 differentially expressed genes were identified with a cut-off value of ~1.5-fold change (p value ≤ 0.05) in cells treated with the siRNA-1 oligonucleotide in comparison with cells treated with the scRNA oligonucleotide. Validation by semiquantitative RT-PCR analysis of 10 randomly chosen genes indicated that the credibility of the microarray array data stood at 60%. Interestingly, among the genes the expression of which were affected upon TDP-43 knockdown was *Atg7* (autophagy-related 7), a gene essential for autophagy and involved in the maintenance of cellular metabolism under physiological conditions (28). However, the mRNA levels of other ATG genes, including *Atg3*, *Atg5*, and *Atg12*, did not change in our microarray hybridization analysis.[4]

As shown by semi-quantitative RT-PCR in Fig. 1*A*, the *Atg7* mRNA level was significantly down-regulated, by ~3-fold, upon treatment of the cells with siRNA-1 oligonucleotide, as shown (Fig. 1*A*, compare *lanes 1* and *2*, *left upper panel*). The level of the *Atg7* mRNA in Neuro2A cells was also decreased upon transfection with the siRNA-2 oligonucleotide that was also capable of knocking down TDP-43 (compare *lanes 1* and *2*, *left panel* of Fig. 1*B*). The down-regulation of ATG7 was also confirmed at the protein level in TDP-43-depleted cells (compare *lanes 1* and *2* of Fig. 1*A*, *lower left panel*, and Fig. 1*B*, *right panel*).

To examine whether the decrease of the level of *Atg7* mRNA upon TDP-43 knockdown was a general phenomenon, the siRNA-1 was transfected into another two cell lines, NSC34 and NIH3T3, and the *Atg7* mRNA levels were analyzed by RT-PCR at 48 h post-transfection. As shown in Fig. 1*C*, the *Atg7* mRNA level was down-regulated in both types of cells treated with siRNA-1 oligonucleotide (Fig. 1*C*, compare *lanes 2* and *4* with *lanes 1* and *3*, respectively).

To further analyze the lowering of the level of ATG7 by RNAi knockdown of TDP-43, we carried out rescue experiment in which the siRNA-1 oligonucleotide was co-transfected with plasmids overexpressing the siRNA-1-resistant forms of TDP-43, TDP-43^R and TDP-43^R (ΔRRM1), which lacked the RNA-binding motif RRM1. As seen in Fig. 2, exogenous expression of the full-length TDP-43^R (Fig. 2, *lane 3*), but not TDP-43^R (ΔRRM1) (Fig. 2, *lane 4*), could rescue, albeit not complete, the siRNA-1-induced lowering of the level of either the level of ATG7 protein (Fig. 2*A*, *lane 2*) or the *Atg7* mRNA (Fig. 2*B*, *lane 2*). This data further excluded the possibility of an off-target effect by the siRNA-1 oligonucleotide.

The reduction of the amounts of both the *Atg7* mRNA and ATG7 protein upon knockdown of TDP-43 could be regulated at the level of transcription, RNA metabolism, or protein proc-

[4] J. K. Bose, unpublished data.

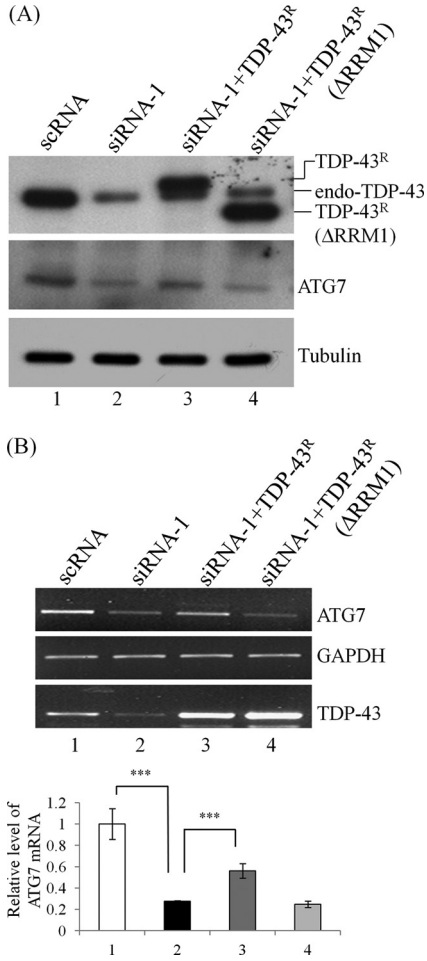

FIGURE 2. **Rescue of *Atg7* mRNA by TDP-43^R.** Neuro2A cells were transfected with scRNA (*lane 1*) or siRNA-1 (*lanes 2–4*). After 24 h, the cells were again transfected with plasmids overexpressing FLAG-tagged TDP-43^R (*lane 3*) or FLAG-tagged TDP-43^R (ΔRRM1) (*lane 4*), respectively. *A*, Western blot analysis of lysates of the transfected cells using anti-TDP-43, anti-ATG7, and anti-tubulin as the probes. Note the decrease of the amount of the endogenous TDP-43 (*endo-TDP-43*) upon siRNA-1 treatment (*lanes 2–4*). Also, note the elevation of the low level of ATG7 protein by the exogenous expression of the siRNA-1-resistant form TDP-43^R (*lane 3*) but not by TDP-43^R (ΔRRM1). *B*, RT-PCR analysis of RNAs from transfected cells. Note the decrease of *Atg7* mRNA by siRNA-1 treatment (*lane 2*) and its rescue by the exogenous TDP-43^R (*lane 3*) but not TDP-43^R (ΔRRM1). ***, p value < 0.001.

essing. In view of the existence of the two RRMs in the TDP-43 polypeptide, we first examined whether TDP-43 could bind to the *Atg7* mRNA. RNA IP experiments were performed using total lysates prepared from Neuro2A cells transfected with pEF-FLAG, pEF-FLAG-TDP-43, and pEF-FLAG-TDP-43 (ΔRRM1), respectively. The lysates were immunoprecipitated with anti-TDP-43 or anti-FLAG, and the RNAs contained within the immunoprecipitates were analyzed by RT-PCR. As shown in supplemental Fig. 1*A*, use of anti-TDP-43 enriched *Atg7* mRNA in the immunoprecipitate (supplemental Fig. 1*A*, top panel, compare *lanes 2* and *3*). In parallel, RNA IP showed that FLAG-TDP-43 could interact with *Atg7* mRNA but not FLAG-TDP-43 (ΔRRM1) (supplemental Fig. 1*B*, *top panel*, compare *lanes 3* and *4*). The data of Figs. 1 and 2 and supplemental Fig. 1 indicated that TDP-43 interacted with the *Atg7* mRNA and also maintained its steady-state level. Furthermore, the RRM1 motif was required for this interaction and the rescue effect.

TDP-43 Depletion Promoted Instability of Atg7 mRNA—Following the observation of binding of TDP-43 to *Atg7* mRNA, we first elucidated the time course of the decrease of the *Atg7* mRNA level by knockdown of TDP-43. For this, total RNAs were isolated from Neuro2A cells transfected with scRNA oligonucleotide or siRNA-1 oligonucleotide at 12, 18, 24, and 48 h post-transfection and subjected to semi-quantitative RT-PCR analysis. As shown in Fig. 3*A*, the down-regulation of *Atg7* mRNA became visible at 18 h, and it was most evident at 48 h post-transfection. We then compared the stability of the endogenous *Atg7* mRNAs in cells treated with the scRNA oligonucleotide and the siRNA-1 oligonucleotide, respectively. For this, the cells were treated with the oligonucleotides for 48 h and then incubated with 5 μg/ml of ActD. RNAs were isolated from the cells at different time intervals of ActD incubation and analyzed by semi-quantitative RT-PCR. As shown in Fig. 3*B*, TDP-43 depletion led to a decrease, by ~6 h, of the half-life of the endogenous *Atg7* mRNA. Thus, it appeared that under normal cellular conditions, the homeostatic concentration of *Atg7* was maintained in part through stabilization of its mRNA as mediated by TDP-43.

TDP-43 Knockdown Inhibited Autophagy—Because loss of ATG7 led to the impairment of autophagy (29, 30), we tested whether the TDP-43-mediated down-regulation of ATG7 also had a similar effect on autophagy. For this, we first probed the total lysates prepared from control and TDP-43 knockdown cells with antibody against LC3, or microtubule-associated protein light chain 3, the amounts of which reflected the relative autophagic activity in the cells (31). As shown in the Western blot of Fig. 4*A*, RNAi knockdown of TDP-43 in cells cultured in complete medium led to a significant decrease of the amount of LC3-II (Fig. 4*A*, compare *lanes 2* and *4* with *lanes 1* and *3*). A decrease of LC3-II upon TDP-43 knockdown was also observed when the serum-free medium was used to culture cells and induce autophagy (Fig. 4*A*, compare *lanes 6* and *8* with *lanes 5* and *7*). The changes in the patterns of LC3-I and LC3-II on the Western blots were in striking similarity to those occurred in cells with knockdown of ATG7 (Fig. 4*B*), suggesting that TDP-43 positively regulated the autophagy as mediated through ATG7.

We further used electron microscopy to check whether the autophagy system was indeed affected by TDP-43 knockdown. Neuro2A cells transfected with the scRNA and siRNA-1 oligonucleotide, respectively, were cultured in serum-free medium for 15 h, fixed, and examined under the electron microscope. As shown in the histogram of Fig. 4*C*, treatment of Neuro2A cells with siRNA-1 oligonucleotide resulted in a less number of the autophagosomes in their cytosol (exemplified in the bottom photos, Fig. 4*C*) when compared with cells transfected with the scRNA oligonucleotide (exemplified in the top photos, Fig. 4*C*).

Finally, we examined the status of ubiquitinated proteins in TDP-43-depleted Neuro2A cells. Ubiquitinated proteins were known to be degraded by both the autophagy and ubiquitin-proteasome system, or UPS (32–34). Furthermore, there were "cross-talks" between the autophagy and UPS (reviewed in Ref. 35), such as the inhibition of UPS by impairment of the

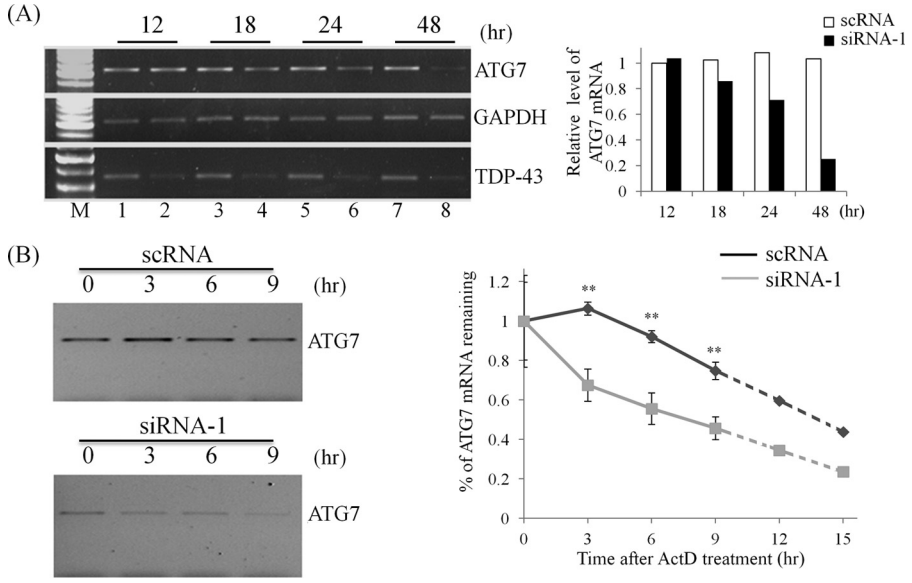

FIGURE 3. **Measurement of the stability of *Atg7* mRNA.** *A*, semi-quantitative RT-PCR analysis of the total RNAs from Neuro2A cells at different time of transfection with the scRNA oligonucleotide (*lanes 1*, *3*, *5*, and *7*) or siRNA-1 oligonucleotide (*lanes 2*, *4*, *6*, and *8*). Note that the down-regulation of the level of *Atg7* mRNA in siRNA-1 oligonucleotide-treated cells became visible at 18 h post-transfection, and it was most evident at 48 h post-transfection. The *right* histogram shows the quantitative comparison of the *Atg7* band intensities in the *left panel*. *B*, ActD chase experiments. The levels of *Atg7* mRNAs isolated from Neuro2A cells transfected with the scRNA or siRNA-1 oligonucleotides for 48 h and then treated with ActD for different time were analyzed by RT-PCR. The gel patterns are shown in the *two left panels*, and the relative levels of mRNAs are plotted against the time of ActD treatment on the *right*. **, *p* value < 0.01.

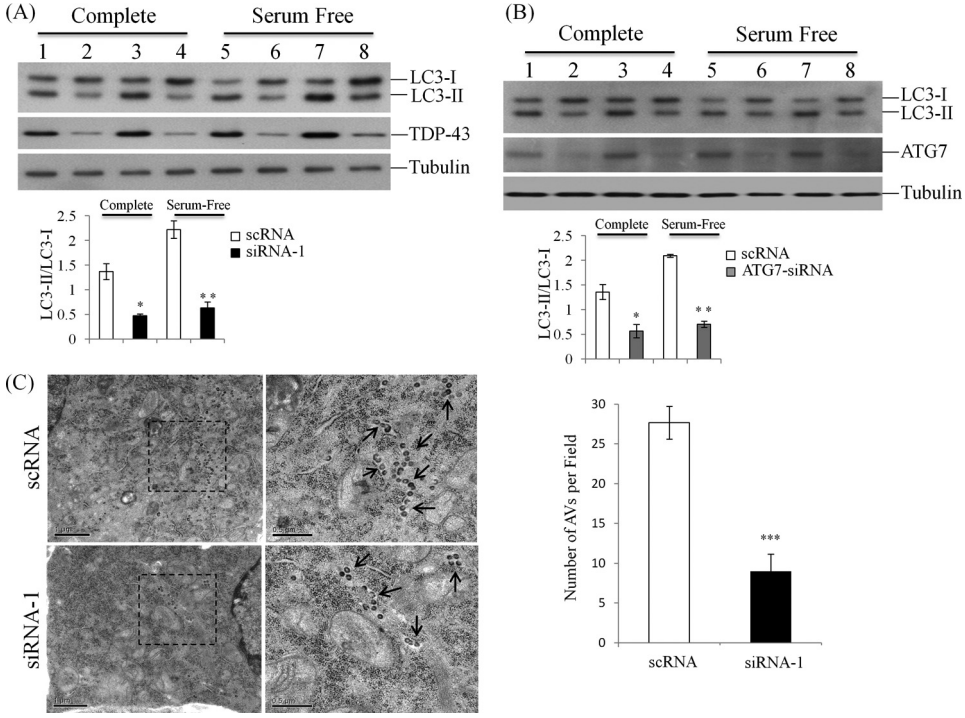

FIGURE 4. **Impairment of the autophagy in TDP-43-depleted cells.** *A*, Western blotting of cell lysates prepared from Neuro2A cells transfected with scRNA oligonucleotide (*lanes 1*, *3*, *5*, and *7*) or TDP-43 siRNA-1 oligonucleotide (*lanes 2*, *4*, *6*, and *8*). The cells were grown in either complete medium or serum-free medium. Data from duplicated experiments are shown. Impairment of the autophagy in TDP-43-depleted cells was evidenced by the switch of the relative abundance of LC3-II/LC3-I in *lanes 2*, *4*, *6*, and *8* relative to *lanes 1*, *3*, *5*, and *7*, respectively. *B*, Neuro2A cells were transfected with scRNA oligonucleotide (*lanes 1*, *3*, *5*, and *7*) or *Atg7* siRNA oligonucleotide (*lanes 2*, *4*, *6*, and *8*). Data from duplicated experiments are shown. Note the similar patterns of the relative abundance of LC3-II/LC3-I in *A* and *B*. The *bottom panels* in *A* and *B* show the quantitative analysis of the LC3-II/LC-I ratios. *, *p* value < 0.05; **, *p* value < 0.01. *C*, electron microscopic analysis of Neuro2A cells transfected with the scRNA oligonucleotide (*top panels*) or siRNA-1 oligonucleotide (*bottom panels*) for 48 h and serum-starved post-transfection. The marked areas in the *left two panels* were magnified to show the autophagosomes (*arrows*). Cells treated with siRNA-1 oligonucleotide exhibited significantly less number of autophagosomes when compared with cells treated with the scRNA oligonucleotide. The histogram shows the comparison of the mean numbers of autophagic vacuoles (*AVs*) per EM field in the two sets of samples ($n = 3$ for each). ***, *p* value < 0.001.

autophagy (36, 37). Longer inhibition of the autophagy even resulted in greater perturbation of UPS (36). Consequently, ubiquitinated proteins would accumulate and form intracellular aggregates if either the autophagy or UPS was impaired with the use of drugs such as 3-MA (autophagy inhibitor) and MG132 (proteasome inhibitor) (36). The formation of the

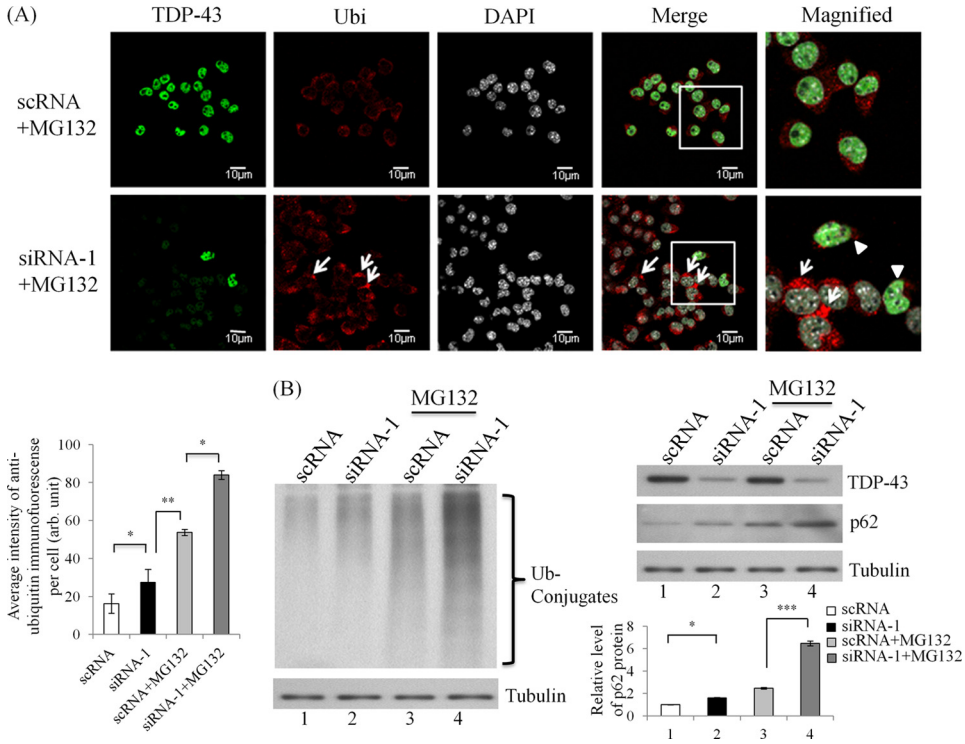

FIGURE 5. **Immunofluorescence staining and Western blotting analyses of Neuro2A cells treated with siRNA oligonucleotides and MG132.** Neuro2A cells were transfected with the scRNA oligonucleotide (*upper panels*) or siRNA-1 oligonucleotide (*lower panels*), treated with MG132 overnight, and then immunofluorescence co-stained with anti-TDP-43 (*green*) and anti-ubiquitin (*Ubi*; *red*). The nuclei were stained with DAPI. Note the much higher ubiquitin immunoreactivity in cells without TDP-43 due to knockdown by siRNA-1, as exemplified by the *arrow* in cells in the *bottom panels*. The *arrowheads* in the *far right panel* of the *bottom panels* point to those cells maintaining TDP-43 and with low ubiquitin immunoreactivity. The quantitative comparison of the average intensities of anti-ubiquitin/cell of Neuro2A cells treated with scRNA, siRNA-1, (scRNA+MG132), and (siRNA-1+MG132), respectively, is shown in the *lower left* histogram. *, p value < 0.05; **, p value < 0.01. *B*, Western blotting analysis of total proteins extracted in urea buffer from Neuro2A cells transfected with scRNA oligonucleotide (*lanes 1* and *3*) or siRNA-1 oligonucleotide (*lanes 2* and *4*) with or without MG132 treatment for overnight (*lanes 3* and *4*), with the use of anti-ubiquitin (*left panel*), anti-p62, anti-TDP-43, and anti-tubulin (*right panels*) as the probes. Note the higher ubiquitin (*Ub*) immunoreactivity (*left panel*) and higher amounts of p62 proteins (*right panel* and the histogram) in samples treated with siRNA-1 and/or MG132. The histogram shows quantitative data. *, p value < 0.05; ***, p value < 0.001. *arb. unit*; arbitrary unit.

intracellular aggregates was enhanced greatly upon inhibition of both the autophagy and UPS, *e.g.* with the combined use of *Atg6* siRNA and MG132 in cell culture (38). Notably, knock-out of the *Atg7* gene and the consequent impairment of the autophagy in mice also resulted in the accumulation of ubiquitinated protein aggregates in the cells (28, 30).

Following the above, we carried out immunofluorescence staining and Western blotting analyses of Neuro2A cells that had undergone different treatments inhibiting the autophagy and/or UPS. As exemplified in supplemental Fig. 2, Neuro2A cells transfected with siRNA-1, treated with MG-132, or treated with 3-MA all exhibited higher immunofluorescent anti-ubiquitin signals than the control Neuro2A cells with the scRNA oligonucleotide transfection (supplemental Fig. 2). Also, Neuro2A cells co-treated with siRNA-1 and MG132 accumulated more intracellular polyubiquitinated proteins than the cells with either treatment alone. As shown in Fig. 5*A*, Neuro2A cells transfected with the siRNA-1 oligonucleotide for 48 h and then treated with 10 μM of MG132 for overnight exhibited enhanced signals of anti-ubiquitin staining and increased number of ubiquitin-positive, cytoplasmic dots in cells with TDP-43 depletion (Fig. 5*A*, *bottom row*). Furthermore, the ubiquitin immunoreactivities of Neuro2A cells treated with siRNA-1 or (MG132+siRNA-1) were significantly higher than those treated with the scRNA or (MG132+scRNA), respectively (histogram of Fig. 5*A*). The immunofluorescence staining data were supported by the Western blotting analysis of Fig. 5*B*, in which the level of polyubiquitinated proteins in Neuro2A cells transfected with siRNA-1 was apparently higher than those transfected with scRNA (Fig. 5*B*, *left panel*, compare *lanes 4* and *2* with *lanes 3* and *1*, respectively). MG132 treatment also increased the level of polyubiquitinated proteins (Fig. 5*B*, *left panel*, compare *lanes 3* and *4* with *lanes 1* and *2*, respectively). Consistent with the scenario that depletion of TDP-43 lowered ATG7 expression, thus impairing autophagy, the pattern of increases of p62 in Neuro2A cells treated with the siRNA-1 oligonucleotide and/or MG132 (Fig. 5*B*, *right panel*) paralleled that of the increase of polyubiquitinated proteins shown in the *left panel* of Fig. 5*B*. The data of Figs. 4 and 5 together demonstrated that depletion of TDP-43 led to autophagy impairment and facilitated the accumulation of ubiquitin-positive aggregates in the cytoplasm.

DISCUSSION

As mentioned under the Introduction, TDP-43 is the major pathosignature protein of a wide range of neurological disorders referred to as the TDP-43 proteinopathies. In this study, we have discovered that depletion of TDP-43 in three different cell lines, including the mouse neuroblastoma Neuro2A cells in culture down-regulates the expression of *Atg7* at both the levels

of mRNAs and protein. More interestingly, we also have found that TDP-43 regulates ATG7 expression mainly through its binding to and stabilization of *Atg7* mRNA. The findings have pointed to a plausible feedback loop for the mutual regulation between TDP-43 and the cellular machineries, *i.e.* autophagy and UPS that degrade this DNA/RNA-binding protein. They also provide important insight into the mechanistic aspects of TDP-43 playing a role in TDP-43 proteinopathies.

The function of TDP-43 in the stabilization of *Atg7* mRNA becomes apparent from the ActD chase experiment of cells with or without RNAi knockdown of TDP-43 (Figs. 1–3). With its two RRM domains, TDP-43 potentially should be able to bind to a variety of RNA species *in vivo*. Indeed, a number of TDP-43-binding mRNA substrates have been identified previously, which include the mRNAs encoding the transmembrane conductance regulator, apolipoprotein A2(APOA2), low molecular weight NF (NFL), survival motor neuron, histone deacetylase 6, and TDP-43 itself, respectively(21, 22, 39–42). Recently, more RNAs have been shown to bind to TDP-43 *in vivo* (43, 44). In the case of TDP-43 interaction with the *Atg7* mRNA, its RRM1 domain very likely plays a major role (supplemental Fig. 1). Similarly, the RRM1 domain is also required for rescue of the *Atg7* mRNA level by TDP-43 (Fig. 2). It should be noted that TDP-43 has been shown before to also stabilize the hNFL mRNA by binding to the 3′-UTR (41). Also, TDP-43 binds to the 3′-UTR of its own mRNA, and this binding facilitates the degradation of *TDP-43* mRNA by the exosome machinery (21). This bilateral role of TDP-43 in regulating the stabilities of different RNAs is not without precedent. There are previous examples in which a protein, such as AUF1, could act both as a destabilizing factor of one mRNA and a stabilizing factor for another (45). The exact mechanism through which TDP-43 protects the *Atg7* mRNA from degradation remains to be elucidated.

Equally important, this work provides clear evidence for inhibition of the autophagy in TDP-43-depleted cells, as shown by the loss of the autophagy marker LC3-II protein (Fig. 4*A*), the decrease of the number of autophagosomes (Fig. 4*C*), the increase of the anti-ubiquitin immunoreactivity (Fig. 5*A* and supplemental Fig. 2), and the elevation of the amounts of polyubiquitinated proteins as well as p62 (Fig. 5*B*) upon TDP-43 knockdown. Furthermore, inhibition of autophagy by knockdown of TDP-43 is remarkably similar to the effect of RNAi knockdown of ATG7 (Fig. 4, *A* and *B*). Autophagy is an important process in the cells for intracellular protein quality control (46, 47). As mentioned previously, inhibition of the autophagy compromises the ubiquitin-proteasome pathway (36, 37). Evidence has been presented before that clearance of TDP-43 and its caspase-generated 35- and 25-kDa fragments is processed through both the autophagy (48–50) as well as the proteasome pathway (48, 49). For instance, use of the autophagy inhibitors such as 3-MA (49) and autophagy activators such as rapamycin (50) increases and reduces, respectively, the accumulation of TDP-43. Also, TDP-43 interacts with ubiquilin, a polyubiquitin-binding protein that is involved in both autophagy and UPS (51, 52). All these studies suggest the involvement of autophagy/UPS in the turnover of TDP-43 in normal cells. Thus, the scenario that TDP-43 participates in the maintenance of the function of autophagy provides an interesting example in which a substrate of autophagy, *e.g.* TDP-43, feeds back to positively regulate the autophagy.

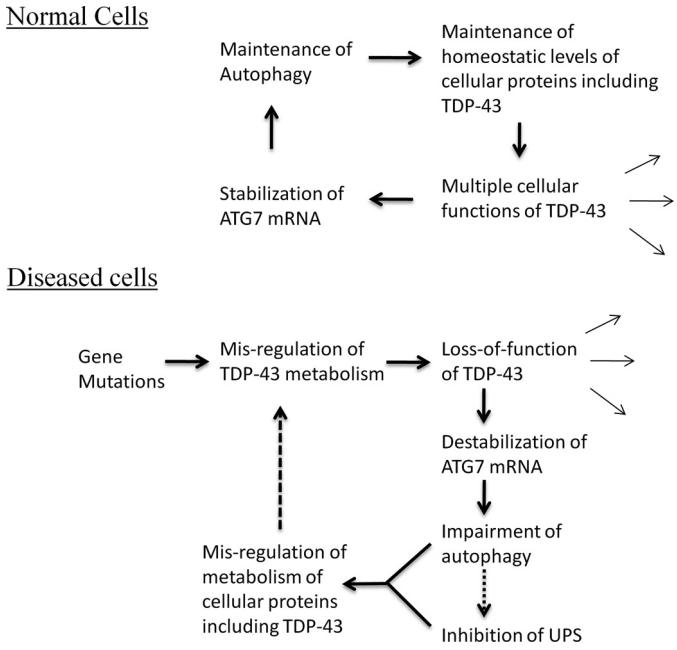

FIGURE 6. **Scenarios of the regulation of autophagy by TDP-43.** It is suggested that TDP-43 is required for the maintenance of autophagy. This function of TDP-43 is mediated through its binding to and stabilization of the *Atg7* mRNA. In the diseased cells with TDP-43 proteinopathies, misregulation leads to loss-of-function of TDP-43 and the consequent destabilization of the *Atg7* mRNA. The formation of the autophagosomes is then blocked due to the loss of the *Atg7* mRNA and protein. The impairment of the autophagy leads to mismetabolism of cellular proteins, including TDP-43.

Finally, the stabilization of *Atg7* mRNA and consequently the maintenance of a functional autophagy by TDP-43 support an important role of the loss of function of TDP-43 in the pathogenesis of neurodegenerative diseases signified with TDP-43(+)UBIs (Fig. 6). In particular, the removal of proteins with aberrant amounts and/or structures is essential for maintenance of the homeostasis of terminally differentiated cells such as the neurons (53). When the structure, cellular level, or subcellular location of TDP-43 is misregulated as the result of TDP-43 proteinopathy-associated gene mutations, the functions of TDP-43, including the maintenance of the autophagy, and consequently the UPS, would be lost (Fig. 6). Abnormal accumulation of proteins including TDP-43 itself would then occur in cells with the defective autophagy and UPS, thus leading to the formation of TDP-43(+)UBIs and the induction of neurocytotoxicity, etc. It is interesting to note here that TDP-43(+) inclusions in the cerebellar sections of patients with TDP-43 proteinopathies are p62-immunoreactive (54). The relative contribution of the scenario depicted in Fig. 6 to TDP-43(+) proteinopathies awaits to be examined in the future.

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REFERENCES

1. Ou, S. H., Wu, F., Harrich, D., García-Martínez, L. F., and Gaynor, R. B. (1995) *J. Virol.* **69,** 3584–3596
2. Wang, H. Y., Wang, I. F., Bose, J., and Shen, C. K. (2004) *Genomics* **83,** 130–139
3. Ayala, Y. M., Pantano, S., D'Ambrogio, A., Buratti, E., Brindisi, A., Marchetti, C., Romano, M., and Baralle, F. E. (2005) *J. Mol. Biol.* **348,** 575–588
4. Wang, I. F., Reddy, N. M., and Shen, C. K. (2002) *Proc. Natl. Acad. Sci. U.S.A.* **99,** 13583–13588
5. Wang, I. F., Wu, L. S., Chang, H. Y., and Shen, C. K. (2008) *J. Neurochem* **105,** 797–806
6. Wang, I. F., Wu, L. S., and Shen, C. K. (2008) *Trends Mol. Med.* **14,** 479–485
7. Buratti, E., and Baralle, F. E. (2009) *Adv. Genet.* **66,** 1–34
8. Buratti, E., and Baralle, F. E. (2010) *RNA Biol.* **7,** 420–429
9. D'Ambrogio, A., Buratti, E., Stuani, C., Guarnaccia, C., Romano, M., Ayala, Y. M., and Baralle, F. E. (2009) *Nucleic Acids Res.* **37,** 4116–4126
10. Arai, T., Hasegawa, M., Akiyama, H., Ikeda, K., Nonaka, T., Mori, H., Mann, D., Tsuchiya, K., Yoshida, M., Hashizume, Y., and Oda, T. (2006) *Biochem. Biophys. Res. Commun.* **351,** 602–611
11. Neumann, M., Sampathu, D. M., Kwong, L. K., Truax, A. C., Micsenyi, M. C., Chou, T. T., Bruce, J., Schuck, T., Grossman, M., Clark, C. M., McCluskey, L. F., Miller, B. L., Masliah, E., Mackenzie, I. R., Feldman, H., Feiden, W., Kretzschmar, H. A., Trojanowski, J. Q., and Lee, V. M. (2006) *Science* **314,** 130–133
12. Chen-Plotkin, A. S., Lee, V. M., and Trojanowski, J. Q. (2010) *Nat. Rev. Neurol* **6,** 211–220
13. Pesiridis, G. S., Lee, V. M., and Trojanowski, J. Q. (2009) *Hum. Mol. Genet.* **18,** R156–162
14. Lagier-Tourenne, C., Polymenidou, M., and Cleveland, D. W. (2010) *Hum. Mol. Genet.* **19,** R46–64
15. Wu, L. S., Cheng, W. C., Hou, S. C., Yan, Y. T., Jiang, S. T., and Shen, C. K. (2010) *Genesis* **48,** 56–62
16. Sephton, C. F., Good, S. K., Atkin, S., Dewey, C. M., Mayer, P., 3rd, Herz, J., and Yu, G. (2010) *J. Biol. Chem.* **285,** 6826–6834
17. Kraemer, B. C., Schuck, T., Wheeler, J. M., Robinson, L. C., Trojanowski, J. Q., Lee, V. M., and Schellenberg, G. D. (2010) *Acta Neuropathol.* **119,** 409–419
18. Feiguin, F., Godena, V. K., Romano, G., D'Ambrogio, A., Klima, R., and Baralle, F. E. (2009) *FEBS Lett.* **583,** 1586–1592
19. Lin, M. J., Cheng, C. W., and Shen, C. K. (2011) *PLoS One* **6,** e20371
20. Tsai, K. J., Yang, C. H., Fang, Y. H., Cho, K. H., Chien, W. L., Wang, W. T., Wu, T. W., Lin, C. P., Fu, W. M., and Shen, C. K. (2010) *J. Exp. Med.* **207,** 1661–1673
21. Ayala, Y. M., De Conti, L., Avendaño-Vázquez, S. E., Dhir, A., Romano, M., D'Ambrogio, A., Tollervey, J., Ule, J., Baralle, M., Buratti, E., and Baralle, F. E. (2011) *EMBO J.* **30,** 277–288
22. Bose, J. K., Wang, I. F., Hung, L., Tarn, W. Y., and Shen, C. K. (2008) *J. Biol. Chem.* **283,** 28852–28859
23. Cashman, N. R., Durham, H. D., Blusztajn, J. K., Oda, K., Tabira, T., Shaw, I. T., Dahrouge, S., and Antel, J. P. (1992) *Dev. Dyn.* **194,** 209–221
24. Todaro, G. J., and Green, H. (1963) *J. Cell Biol.* **17,** 299–313
25. Iguchi, Y., Katsuno, M., Niwa, J., Yamada, S., Sone, J., Waza, M., Adachi, H., Tanaka, F., Nagata, K., Arimura, N., Watanabe, T., Kaibuchi, K., and Sobue, G. (2009) *J. Biol. Chem.* **284,** 22059–22066
26. Kabuta, T., Suzuki, Y., and Wada, K. (2006) *J. Biol. Chem.* **281,** 30524–30533
27. Olmsted, J. B., Carlson, K., Klebe, R., Ruddle, F., and Rosenbaum, J. (1970) *Proc. Natl. Acad. Sci. U.S.A.* **65,** 129–136
28. Komatsu, M., Waguri, S., Ueno, T., Iwata, J., Murata, S., Tanida, I., Ezaki, J., Mizushima, N., Ohsumi, Y., Uchiyama, Y., Kominami, E., Tanaka, K., and Chiba, T. (2005) *J. Cell Biol.* **169,** 425–434
29. Levine, B., and Klionsky, D. J. (2004) *Dev. Cell* **6,** 463–477
30. Komatsu, M., Waguri, S., Chiba, T., Murata, S., Iwata, J., Tanida, I., Ueno, T., Koike, M., Uchiyama, Y., Kominami, E., and Tanaka, K. (2006) *Nature* **441,** 880–884
31. Kabeya, Y., Mizushima, N., Ueno, T., Yamamoto, A., Kirisako, T., Noda, T., Kominami, E., Ohsumi, Y., and Yoshimori, T. (2000) *EMBO J.* **19,** 5720–5728
32. Shin, J. (1998) *Arch Pharm. Res.* **21,** 629–633
33. Pankiv, S., Clausen, T. H., Lamark, T., Brech, A., Bruun, J. A., Outzen, H., Øvervatn, A., Bjørkøy, G., and Johansen, T. (2007) *J. Biol. Chem.* **282,** 24131–24145
34. Seibenhener, M. L., Geetha, T., and Wooten, M. W. (2007) *FEBS Lett.* **581,** 175–179
35. Koga, H., Kaushik, S., and Cuervo, A. M. (2011) *Ageing Res. Rev.* **10,** 205–215
36. Korolchuk, V. I., Mansilla, A., Menzies, F. M., and Rubinsztein, D. C. (2009) *Mol. Cell* **33,** 517–527
37. Ravikumar, B., Sarkar, S., Davies, J. E., Futter, M., Garcia-Arencibia, M., Green-Thompson, Z. W., Jimenez-Sanchez, M., Korolchuk, V. I., Lichtenberg, M., Luo, S., Massey, D. C., Menzies, F. M., Moreau, K., Narayanan, U., Renna, M., Siddiqi, F. H., Underwood, B. R., Winslow, A. R., and Rubinsztein, D. C. (2010) *Physiol. Rev.* **90,** 1383–1435
38. Ding, W. X., Ni, H. M., Gao, W., Yoshimori, T., Stolz, D. B., Ron, D., and Yin, X. M. (2007) *Am. J. Pathol.* **171,** 513–524
39. Buratti, E., Dörk, T., Zuccato, E., Pagani, F., Romano, M., and Baralle, F. E. (2001) *EMBO J.* **20,** 1774–1784
40. Mercado, P. A., Ayala, Y. M., Romano, M., Buratti, E., and Baralle, F. E. (2005) *Nucleic Acids Res.* **33,** 6000–6010
41. Strong, M. J., Volkening, K., Hammond, R., Yang, W., Strong, W., Leystra-Lantz, C., and Shoesmith, C. (2007) *Mol. Cell Neurosci.* **35,** 320–327
42. Fiesel, F. C., Voigt, A., Weber, S. S., Van den Haute, C., Waldenmaier, A., Görner, K., Walter, M., Anderson, M. L., Kern, J. V., Rasse, T. M., Schmidt, T., Springer, W., Kirchner, R., Bonin, M., Neumann, M., Baekelandt, V., Alunni-Fabbroni, M., Schulz, J. B., and Kahle, P. J. (2010) *EMBO J.* **29,** 209–221
43. Tollervey, J. R., Curk, T., Rogelj, B., Briese, M., Cereda, M., Kayikci, M., König, J., Hortobágyi, T., Nishimura, A. L., Zupunski, V., Patani, R., Chandran, S., Rot, G., Zupan, B., Shaw, C. E., and Ule, J. (2011) *Nat. Neurosci.* **14,** 452–458
44. Polymenidou, M., Lagier-Tourenne, C., Hutt, K. R., Huelga, S. C., Moran, J., Liang, T. Y., Ling, S. C., Sun, E., Wancewicz, E., Mazur, C., Kordasiewicz, H., Sedaghat, Y., Donohue, J. P., Shiue, L., Bennett, C. F., Yeo, G. W., and Cleveland, D. W. (2011) *Nat. Neurosci.* **14,** 459–468
45. Barreau, C., Paillard, L., and Osborne, H. B. (2005) *Nucleic Acids Res.* **33,** 7138–7150
46. Goldberg, A. L. (2003) *Nature* **426,** 895–899
47. Shintani, T., and Klionsky, D. J. (2004) *Science* **306,** 990–995
48. Wang, X., Fan, H., Ying, Z., Li, B., Wang, H., and Wang, G. (2010) *Neurosci. Lett.* **469,** 112–116
49. Urushitani, M., Sato, T., Bamba, H., Hisa, Y., and Tooyama, I. (2010) *J. Neurosci. Res.* **88,** 784–797
50. Caccamo, A., Majumder, S., Deng, J. J., Bai, Y., Thornton, F. B., and Oddo, S. (2009) *J. Biol. Chem.* **284,** 27416–27424
51. Kim, S. H., Shi, Y., Hanson, K. A., Williams, L. M., Sakasai, R., Bowler, M. J., and Tibbetts, R. S. (2009) *J. Biol. Chem.* **284,** 8083–8092
52. Rothenberg, C., Srinivasan, D., Mah, L., Kaushik, S., Peterhoff, C. M., Ugolino, J., Fang, S., Cuervo, A. M., Nixon, R. A., and Monteiro, M. J. (2010) *Hum. Mol. Genet.* **19,** 3219–3232
53. Hara, T., Nakamura, K., Matsui, M., Yamamoto, A., Nakahara, Y., Suzuki-Migishima, R., Yokoyama, M., Mishima, K., Saito, I., Okano, H., and Mizushima, N. (2006) *Nature* **441,** 885–889
54. King, A., Maekawa, S., Bodi, I., Troakes, C., and Al-Sarraj, S. (2011) *Neuropathology* **31,** 239–249