A “Two-hit” Hypothesis for Inclusion Formation by Carboxyl-terminal Fragments of TDP-43 Protein Linked to RNA Depletion and Impaired Microtubule-dependent Transport

Carboxyl-terminal fragments (CTFs) of TDP-43 aggregate to form the diagnostic signature inclusions of frontotemporal lobar degeneration and amyotrophic lateral sclerosis, but the biological significance of these CTFs and how they are generated remain enigmatic. To address these issues, we engineered mammalian cells with an inducible tobacco etch virus (TEV) protease that cleaves TDP-43 containing a TEV cleavage site. Regions of TDP-43 flanking the second RNA recognition motif (RRM2) are efficiently cleaved by TEV, whereas sites within this domain are more resistant to cleavage. CTFs containing RRM2 generated from de novo cleavage of nuclear TDP-43 are transported to the cytoplasm and efficiently cleared, indicating that cleavage alone is not sufficient to initiate CTF aggregation. However, CTFs rapidly aggregated into stable cytoplasmic inclusions following de novo cleavage when dynein-mediated microtubule transport was disrupted, RNA was depleted, or natively misfolded CTFs were introduced into these cells. Our data support a “two-hit” mechanism of CTF aggregation dependent on TDP-43 cleavage.

The heterogeneous nuclear ribonucleoprotein (hnRNP) TDP-43 (TAR DNA-binding protein of 43 kDa) forms pathological inclusions that are diagnostic hallmarks of amyotrophic lateral sclerosis (ALS) and the major form of frontotemporal lobar degeneration (FTLD-TDP) (1). Missense mutations in TDP-43 provide a genetic link between ALS and FTLD-TDP, a loss of nuclear TDP-43 coincides with cytoplasmic TDP-43 inclusions (1). These inclusions contain full-length TDP-43 and TDP-43 C-terminal fragments (CTFs) that are hyperphosphorylated and ubiquitinated. However, it is unclear how each of these species of TDP-43 contributes to inclusion formation or disease pathogenesis.

TDP-43 is a typical 2XRRM-gly hnRNP composed of two tandem RNA recognition motifs (RRM1 and RRM2) followed by a glycine-rich carboxyl terminus (Fig. 1) (7). Both RRMs retain nucleic acid binding properties, yet only RRM1 appears essential for RNA splicing (8). The glycine-rich domain is proposed to interact with other hnRNPs to synergistically affect alternative splicing of specific RNA transcripts (9, 10). A common feature of shuttling hnRNPs involved in RNA splicing is that their nuclear export is coupled to the export and maturation of mRNA in distinct ribonucleoprotein (RNP) complexes (11, 12). In this respect, the nucleocytoplasmic shuttling of TDP-43 is dependent on its bipartite importin-α nuclear localization signal, a chromosome maintenance region 1 (CRM-1) nuclear export signal in RRM2, and undefined aspects of the carboxyl-terminal glycine-rich domain (13, 14).

CTFs are signatures of disease exclusively associated with TDP-43 pathology, and CTFs cleaved in the middle of RRM2 extending from amino acid 208 to the carboxyl terminus of TDP-43 have been recovered from FTLD-TDP brains (15). Overexpression of CTFs of varying lengths in cultured cells produces cytoplasmic aggregates that are ubiquitinated and phosphorylated, recapitulating biochemical properties of authentic TDP-43 inclusions (15-18). Experiments performed in vitro demonstrate that CTFs readily aggregate and form fibrillar structures (19, 20). However, CTF expression in transgenic flies is well tolerated without neurotoxicity or motor phenotypes (21, 22). This raises the question of whether TDP-43 CTFs initiate the formation of TDP-43 inclusions and play a role in neurotoxicity.

To elucidate the role of de novo generated TDP-43 CTFs in TDP-43 proteinopathies, we established a mammalian cell system that stably expresses an inducible form of the TEV protease to enable temporally regulatable site-specific cleavage of TDP-43. Cleavage of nuclear TDP-43 produces CTFs that are soluble, transported to the cytoplasm, and efficiently cleared from cells. However, de novo generated CTFs, aggregated to

This is an Open Access article under the CC BY license.
form inclusions in cells when they also expressed insoluble cytoplasmic CTF seeds, were depleted of RNA, or their dynein-mediated MT transport was disrupted. These novel findings suggest a “two-hit” hypothesis for the formation of CTF-rich TDP-43 inclusions in FTLD-TDP and ALS wherein a second deleterious event or “second hit” is required to form inclusions following the generation of CTFs.

**EXPERIMENTAL PROCEDURES**

**Generation of TEV Cell Lines and Plasmids**—The TEV protease coding sequence was cloned into pCDNA5/TO plasmid (Invitrogen) with an N-terminal hemagglutinin tag (HA-TEV; TEV<sub>nuc</sub>) for cytoplasmic localization or a triple SV40 nuclear localization signal (NLS) for nuclear localization (NLS<sub>SV40</sub>-HA-TEV-NLS<sub>SV40</sub>-NLS<sub>SV40</sub>) (supplemental Table 1) similar to Pauli et al. (23). TREX-293 (Invitrogen) cells transfected with the TEV<sub>cyt</sub> and TEV<sub>nuc</sub> plasmids using Lipofectamine 2000 (Invitrogen) were grown in selection media (DMEM, 10% tetracycline-screened FBS, or glutamine, penicillin/streptomycin, 0.5 μg/ml blasticidin, 200 μg/ml hygromycin) at 37 °C followed by subcloning for individual clones expressing TEV<sub>nuc</sub> and TEV<sub>cyt</sub>. Stable clones were screened for HA-tagged TEV<sub>nuc</sub> and TEV<sub>cyt</sub> expression following an overnight incubation in the presence of 1 μg/ml tetracycline.

The wild type coding sequence of TDP-43 was amplified by primer extension from a cDNA clone (14) using primers that encode an N-terminal FLAG tag and a C-terminal Myc tag, followed by insertion into HindIII/XhoI of pCDNA 3.1 (+) (Invitrogen). The TEV protease cleavage site (ENLYFQG) was inserted at amino acid positions Gln<sup>182</sup>, Gln<sup>204</sup>, Arg<sup>208</sup>, Gly<sup>215</sup>, Lys<sup>224</sup>, Ala<sup>260</sup>, and Arg<sup>272</sup> of TDP-43 by Exsite mutagenesis (Stratagene) (supplemental Table 1). GFP-mRuby plasmid for mammalian cell expression was generated by primer extension from a cDNA clone (14) using primers that encode an N-terminal FLAG tag and a C-terminal Myc tag, followed by insertion into HindIII and BamHI of the mRuby expression vector (24). TDP-43(182tev) was subcloned from the pCDNA 3.1(+) plasmid into GFP-mRuby using HindIII and BamHI.

The Gal4DBD-TEV-VP16 test substrate was made by primer extension of VP16 from pAct (Promega) and inserted into the NotI and KpnI restriction sites of the pBind vector (Promega) to make pBind-Act. Oligonucleotides encoding the TEV protease site were annealed and inserted into BamHI and MluI restriction sites of the pBind-Act vector. Myc-tagged coiled coil 1 (CC1) in pCDNA 3.1 was a gift from Dr. Holzbaur (University of Pennsylvania).

**De Novo TDP-43 Cleavage Assays**—De novo cleavage of TDP-43 was performed in TEV<sub>nuc</sub> or TEV<sub>cyt</sub> cells by transfection of a cell monolayer with the appropriate FLAG-TDP-43(tev)-Myc or GFP-TDP-43(tev)-mRuby plasmids using Fugene HD (Roche Applied Science). After 16 h of TDP-43 maturation, TEV<sub>nuc</sub> or TEV<sub>cyt</sub> expression was induced with 1 μg/ml tetracycline for indicated time periods. To determine whether GFP-TDP-43(208–414) inclusions sequester de novo cleaved TDP-43, TEV<sub>nuc</sub> cells were cotransfected with FLAG-TDP-43(182tev)-Myc and GFP-TDP-43(208–414) (in pcDNA5/TO) overnight, followed by induction of TEV<sub>nuc</sub> and GFP-TDP-43(208–414) for the simultaneous expression of GFP-TDP-43(208–414) and cleavage of FLAG-TDP-43(182tev)-Myc.

**Biochemical Extractions**—Cell lysates were prepared according to the method of Igaz et al. (15) except a less stringent lysis buffer, RSB-150 (20 mM Tris, pH 7.4, 0.15 mM NaCl, 2.5 mM MgCl<sub>2</sub>) with protease inhibitors, 0.5 mM phenylmethylsulfonyl fluoride, leupeptin, N-p-tosyl-L-phenylalanine chloromethyl ketone, N<sup>ε</sup>-tosyl-L-lysine chloromethyl ketone, trypsin inhibitor, and phosphatase inhibitors 2 mM imidazole, 1 mM NaF, and 1 mM sodium orthovanadate (Sigma), was used, followed by extraction of the insoluble material with urea buffer (15). Approximately 25–30 μg of the soluble RSB-150 fraction and ~75 μg of the insoluble urea fraction were separated on SDS-polyacrylamide gels and transferred to 0.2-μm nitrocellulose for immunoblot with the specified antibodies.

The relative solubility of CTFs extracted from cells that produce CTFs by TEV<sub>nuc</sub> cleavage was compared with aggregate-prone CTFs and CTFs extracted from FTLD-TDP brain tissue. For de novo cleaved CTFs, cells were transfected with FLAG-TDP-43(182tev) without a C-terminal Myc tag, followed by TEV<sub>nuc</sub> cleavage and sequential extraction in RSB-150, followed by urea buffer extraction. Aggregate-prone CTFs were generated by transfection of cDNA coding for amino acids 182–414 (see supplemental Table 1) followed by sequential extraction. CTFs were extracted from FTLD-TDP brain tissue using ~5 g of brain tissue (temporal lobe) as described previously (1) except the tissue was extracted in RSB-150 (5 ml/g of tissue), followed by urea buffer (0.1 ml/g of tissue).

**Antibodies and Immunofluorescence**—For immunoblot analysis, N-terminal fragments (NTFs) and CTFs along with full-length TDP-43 were visualized using a monoclonal antibody (mAb) anti-FLAG (Sigma), anti-eGFP (Millipore), and previously described polyclonal antibodies that recognize the N terminus (TDP-43n) and C terminus of TDP-43 (TDP-43c) (25). Phosphorylated TDP-43 was visualized using a rat mAb that recognizes phosphorylated TDP-43 at serines 409 and 410 (1D3) (26). HA-tagged TEV and GAPDH were visualized with a rat mAb anti-HA (3F10, Roche Applied Science) and mouse anti-GAPDH (Advanced Immunochemical, respectively).

For immunofluorescence, cells were fixed in 4% paraformaldehyde, followed by lysis in 0.2% Triton X-100 (Sigma) and blocking in BSA buffer as described previously (15). To detect FLAG and Myc-tagged TDP-43, cells were incubated overnight at 4 °C in a mixture of 1:2,000 mouse anti-FLAG (Sigma) and 1:3,000 rabbit anti-Myc (Sigma), followed by anti-mouse and anti-rabbit secondary antibodies conjugated with Alexa Fluor 488 and Alexa Fluor 594 (Vector Laboratories). In experiments using GFP-TDP-43(208–414), a 1:200 dilution of an anti-mouse antibody conjugated to aminomethylcoumarin acetate was used to detect the anti-FLAG antibody (Vector Laboratories). Triple fluorescence of GFP and mRuby-tagged TDP-43 with C1C-Myc was detected using 1:2,000 rabbit anti-Myc (Sigma) and 1:250 goat anti-mouse conjugated with aminomethylcoumarin acetate (Vector Laboratories). Cell nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI) and mounted onto glass slides using Fluoromount G (SouthernBiotech).
Microscopy data were collected on an Olympus BX51 microscope equipped with an Olympus PD71 camera. Where appropriate, the number of cells showing cytoplasmic CTFs, nuclear NTFs, and uncleaved TDP-43(tev) substrates were counted using ImagePro Plus (Media Cybernetics) and expressed as a percentage of the number of transfected cells.

Pulse-Chase Experiments—Pulse-chase experiments were performed as described by Jansens and Braakman (27). TEV\textsubscript{nu}c cells transfected with flag-TDP-43(182tev)-Myc were incubated overnight at 37 °C. After a 15-min incubation in depletion medium, cells were pulsed for 10 min in 2 ml of labeling medium (DMEM (−Met−Cys)) + 100 µCi/ml [\textsuperscript{35}S]methionine) followed by different chase periods in full medium containing excess methionine and cysteine (DMEM, 10% tetracycline screened fetal bovine serum, glutamine, penicillin/streptomycin, 5 mM methionine, 5 mM cysteine) supplemented with 1 µg/ml tetracycline for induction of TEV\textsubscript{nu}c expression. Cells were harvested at each time point by scraping with a rubber policeman in 500 µl of radioimmune precipitation assay buffer followed by sonicating and centrifugation at 16,000 × g for 15 min at 4 °C. Soluble lysates were incubated with protein A/G beads conjugated with either 5 µg of anti-FLAG (Sigma) or 5 µg of a mouse mAb that recognizes the C terminus of TDP-43 (25). Immunoprecipitations were incubated for 2 h at 4 °C, followed by four 1-ml washes in radioimmune precipitation assay buffer. Proteins were eluted with 2× SDS-gel loading buffer, separated by SDS-PAGE, and transferred to a 0.2-µm nitrocellulose membrane. The amount of radioactivity in each protein band was assessed by autoradiography using a high density phosphor screen and a PhosphorImager, followed by quantification using ImageQuant (Amersham Biosciences). The total level of each TDP-43 species was assessed by immunoblotting using anti-TDPc and anti-FLAG. The clearance of each TDP-43 species was assessed by immunoblotting using anti-TDPc and anti-FLAG. The clearance of each TDP-43 species was fit using a first order decay model of the form [A] = [A]₀e⁻^(-t), where [A] is the normalized concentration of TDP-43 at time t, and ½ is the mean lifetime. The halflife was calculated using the relationship, t₁/₂ = ln2.

Inhibition of the Proteasome- and Dynemin-mediated Transport—To inhibit the proteasome, TEV\textsubscript{nu}c cells transfected with flag-TDP-43(182tev)-Myc were treated with 10 µM clasto-lactacycin-β-lactone (EMD Biosciences) for 5 h before the addition of 1 µg/ml tetracycline for an additional 16 h. The change in CTF level was calculated from the CTF band intensities obtained from immunoblot of five replicate experiments using the Multigauge (Fuji) image analysis software package.

Dynemin-mediated MT transport was inhibited using erythro-9-(2-hydroxy-3-nonyl)adenine (EHNA) (Sigma) or by coexpression of the CC1 domain from the p150(glued) subunit of dynactin (28). Dynemin function before and after inhibition with EHNA and CC1-Myc was assessed by analysis of Golgi morphology by immunostaining with the Golgi marker golgin (GM-130) (BD Transduction Laboratories). Cells transfected with FLAG-TDP-43(182tev)-Myc overnight were treated with 0.1 mM EHNA for 5 h followed by 16 h of TEV\textsubscript{nu}c cleavage. The effect of CC1-mediated dynemin inhibition on CTF localization was performed by cotransfection of cells with GFP-TDP-43(182tev)-mRuby and Myc-tagged CC1 for 24 h, followed by cleavage with TEV\textsubscript{nu}c for 16 h.

RNase Treatment—RNase treatment of cells was performed as described previously (29, 30). Briefly, TEV\textsubscript{nu}c cells transfected with GFP-TDP-43(182tev)-mRuby were incubated in the presence and absence of 1 µg/ml tetracycline for 16 h of TEV\textsubscript{nu}c expression to generate CTFs. Cells were washed with cytoskeleton buffer (CSK buffer; 0.1 M NaCl, 0.3 M sucrose, 3 mM MgCl₂, 10 mM PIPES, pH 6.8), followed by incubation with CSK buffer plus 0.05% Triton X-100 for 3 min on ice. Cells washed twice with CSK buffer were incubated with 1 mg/ml RNase A (Sigma) in CSK buffer for 15 min followed by fixation in 4% paraformaldehyde and DAPI staining. For sequential extractions, cells were lysed by sonication in RSB-150, 1 mg/ml RNase A, followed by centrifugation and extraction of the soluble and insoluble fractions as described (25).

RESULTS

Generation of TEV Protease Cell Lines—The protease that cleaves TDP-43 in FTLD-TDP and ALS to produce a CTF of ∼25 kDa that is phosphorylated and aggregated is unknown. Our approach to study TDP-43 cleavage and downstream events from CTF production utilized the highly specific TEV protease. The engineered TDP-43 proteolysis system was designed by inserting the TEV cleavage site (ENLYF(Q↓G)) at specific positions in TDP-43 possessing a FLAG tagged at the N terminus and a Myc tag at the C terminus (i.e. FLAG-TDP-43-Myc) (Fig. 1A). Cells expressing a tetracycline-inducible TEV protease generated FLAG-tagged NTFs and Myc-tagged CTFs that were further tracked by immunofluorescence and immunoblot of cell extract (Fig. 1A). These novel tools allowed us to elucidate the cell biology and fate of NTFs and CTFs through de novo cleavage of TDP-43.

Two stable cell lines of the tetracycline-on TREx-293 cell system were generated that contain a tetracycline-inducible TEV protease that is either targeted to the nucleus (TEV\textsubscript{nu}c) or remains in the cytoplasm (TEV\textsubscript{cy}t). As previously shown (29), expression of TEV\textsubscript{nu}c and TEV\textsubscript{cy}t were properly localized, we examined the distribution of the hemagglutinin-tagged TEV proteases by immunofluorescence. TEV\textsubscript{nu}c appeared to co-localize with nuclear TDP-43, whereas TEV\textsubscript{cy}t appeared predominantly cytoplasmic (Fig. 1B). As previously shown (23), expression of the TEV protease does not alter the cell morphology, nor is it toxic (data not shown). TEV expression was observed after 6 h of induction with tetracycline and maintained expression throughout the experiment (Fig. 1C).

To verify that the TEV protease is biologically active in cells, we utilized a UAS\textsubscript{GAL4} promoter-driven luciferase reporter that is sensitive to a TEV-cleavable GAL4-VP16 transcription factor (Fig. 1D). In the absence of tetracycline, no TEV\textsubscript{nu}c or TEV\textsubscript{cy}t was expressed (Fig. 1C), and the intact GAL4-ENLYFQ/G-VP16 activated transcription of the luciferase gene in both cell lines (Fig. 1D). Induction of TEV\textsubscript{nu}c and TEV\textsubscript{cy}t with tetracycline activated cleavage of the GAL4-ENLYFQ/G-VP16 test substrate, resulting in the loss of luciferase activity (Fig. 1D). This demonstrates that expression of TEV\textsubscript{cy}t and TEV\textsubscript{nu}c is tightly regulated by the tetracycline repressor and is functionally active in mammalian cells.
De Novo Cleavage of TDP-43

**TEV Site Scanning in TDP-43 Defines CTF Stability**—To probe protease sites in TDP-43 that are structurally accessible and susceptible to TEV cleavage, we inserted the TEV recognition sequence (ENLYFQ) at specific amino acid positions in TDP-43 (Fig. 2A). A TEV recognition site inserted at Glu182 tests cleavage within the unstructured linker between RRM1 and RRM2, generating a CTF with RRM2 and the entire C-terminal domain (Fig. 2, A and B). TEV cleavage within the highly structured RRM2 at Arg272 (Fig. 2B) was generated to mimic a putative cleavage position identified by sequencing CTFs purified from FTLD-TDP brain tissue (15). TEV cleavage at Ala260 and Arg272 was tested to probe the fate of CTFs and NTFs generated by cleavage in the glycine-rich C-terminal domain (Fig. 2A). Cells transfected with each construct followed by TEV nuc cleavage were assessed by immunofluorescence and immunoblot to detect localization and levels of full-length TDP-43 and cleavage products (Fig. 2, C and D).

Cleavage of TDP-43 at Glu182 produced CTFs with a prominent cytoplasmic localization pattern, following cleavage by TEV nuc (Fig. 2C, arrows). However, some cells showed complete cleavage of all CTFs while retaining abundant nuclear NTFs (Fig. 2C, arrowheads). Immunoblots of extracts from cells transfected with Glu182 cleavage showed NTFs and CTFs that migrate with the expected Mr of each fragment (Fig. 2D). In contrast, there were no TEV nuc cleavage products in the absence of a TEV cleavage site (Fig. 2D). This illustrates that cleavage of TDP-43 at a position that includes RRM2 and the C-terminal domain generates stable CTFs that translocate to the cytoplasm, whereas the corresponding NTFs are retained in the nucleus.

Cleavage of TDP-43 at Arg208 does not produce significant levels of the expected NTFs and CTFs (Fig. 2D). This is most likely due to the inaccessibility of Arg208 to TEV nuc cleavage (Fig. 2B) (31). We further probed the susceptibility of cleavage within RRM2 by inserting additional cleavage sites in four loops that connect elements of secondary structure in RRM2 (Fig. 2, A and B). Although these loops would be predicted to be more accessible to cleavage than Arg208, these sites (including glutamic acid 204 in loop 1, glycine 215 in loop 2, lysine 224 in loop 3, and glycine 252 in loop 5) were also resistant to TEV cleavage (data not shown). Thus, for reasons that are not entirely clear, cleavage positions in RRM2 are resistant to cleavage.

**De novo** cleaved CTFs that lack RRM2 are rapidly cleared from the cell. Cleavage of TDP-43 at Ala260 produces unstable CTFs, with only a small number of cells showing weak cytoplasmic CTF staining and virtually no detectable CTFs by immunoblot (Fig. 2, C and D). This observation is even more pronounced when TDP-43 cleavage occurs in the middle of the most glycine-rich sequence at Arg272. This site is homologous with a physiological cleavage site in hnRNP-A1 that is conserved in TDP-43 (32). In comparison, NTFs generated by both Ala260 and Arg272 cleavage are not readily cleared and are retained in the nucleus (Fig. 2, C and D).

The TEV site scanning data demonstrate three distinct types of TDP-43 cleavage: 1) efficient cleavage to generate stable CTFs that translocate to the cytoplasm, 2) inaccessible cleavage sites in RRM2 that are resistant to cleavage, and 3) efficient cleavage sites in the C-terminal domain that generate unstable rapidly degraded CTFs. Because **de novo** cleavage of TDP-43 at Glu182 efficiently produces stable CTFs that translocate to the cytoplasm, we studied these CTFs in more detail to understand if and how secondary perturbations might induce them to form abnormal CTF aggregates similar to those seen in neurodegenerative TDP-43 proteinopathies, such as ALS and FTLD-TDP.
Because nuclear cleavage of TDP-43 produces cytoplasmic CTFs without inclusions, we tested whether cytoplasmic cleavage of TDP-43 generates a similar distribution of CTF and NTF localization. Here, TEV<sub>cyt</sub> cleavage is expected to target the small pool of TDP-43 that shuttles between the nucleus and cytoplasm (13, 14). TEV<sub>cyt</sub> cleavage of TDP-43 at Gln<sup>182</sup> produced a similar distribution of cytoplasmic CTFs, whereas NTFs generated in the cytoplasm translocated back into the nucleus without appreciable cytoplasmic localization (supplemental Fig. 1). Therefore, CTFs generated by cytoplasmic cleavage of TDP-43 show similar diffuse cytoplasmic distribution as those generated by nuclear cleavage of TDP-43.

**Nuclear Cleavage of TDP-43 Causes Rapid Nuclear Export and CTF Clearance**—Nuclear cleavage of TDP-43 produced CTFs that shuttled out of the nucleus, where they are redistributed throughout the cytoplasm (Fig. 3A). This phenomenon is reproducible in multiple cell types including the motor neuron-like NSC-34 cells (supplemental Fig. 2). In NSC-34 cells, de novo cleaved CTF localization extends out to the periphery of the cell and throughout the neurite-like projections. This suggests that cellular trafficking may play a role in the cytoplasmic CTF redistribution. However, rather than aggregating as in previous models of CTF expression (15–18), de novo cleaved CTFs were rapidly cleared in virtually all cells within 48 h of TEV<sub>nuc</sub> cleavage.
expression (Fig. 3A). Cells that degrade and clear CTFs are indicated by the nuclear localization of the remaining NTF (Fig. 3A). This illustrates that CTFs and NTFs have significantly different rates of clearance following TDP-43 cleavage.

To further characterize the relative clearance rates of NTFs and CTFs, we labeled total protein in cells transfected with FLAG-TDP-43(182tev)-Myc with [35S]methionine, followed by different chase periods in media that induce TEVnuc cleavage (Fig. 3B). The turnover of the full-length protein in cells without TEVnuc expression is consistent with the turnover rate of endogenous TDP-43, with a half-life of ~34–40 h (Fig. 3B). In the presence of TEVnuc, FLAG-TDP-43(182tev)-Myc rapidly decays with a half-life of 1.7 h, reflecting the kinetics of TEVnuc catalysis in cells (Fig. 3B). Levels of CTFs and NTFs increase with similar kinetics as the loss of the full-length substrate (Fig. 3B, inset). The NTF decay is similar to decay of full-length TDP-43, with a half-life of ~34 h (Fig. 3B), whereas the CTF half-life is only 4.2 h (Fig. 3B). This reflects the combined rate of efficient nuclear export, cytoplasmic transport, and degradation of CTFs.

Next, we determined whether the efficient nuclear export of CTFs was a result of passive diffusion through the nuclear pore. It is generally accepted that proteins larger than ~40 kDa are not efficiently transported across the nuclear pore by diffusion. Because CTFs with a C-terminal Myc tag are only 26 kDa, we attached the 30-kDa mRuby protein to the carboxyl terminus of TDP-43(182tev) to generate a ~56-kDa CTF following TEVnuc cleavage. Cells transfected with a GFP-TDP-43(182tev)-mRuby showed a typical nuclear localization pattern in the absence of cleavage (Fig. 3C). If CTFs simply diffuse out of the nucleus, the nuclear export of the ~56-kDa CTF would be expected to be blocked or impaired after TEVnuc cleavage. However, following cleavage with TEVnuc, the expected ~56-kDa CTFs appeared in the cytoplasm with a similar distribution and frequency compared with Myc-tagged CTFs (Fig. 3C). This suggests that CTFs do not simply diffuse through the nuclear pore. The CTF-mRuby fragment also exhibited similar clearance rates, suggesting that the larger mRuby fusion does not dramatically affect the rate of nuclear export, cytoplasmic distribution, and CTF clearance (Fig. 3C).

Reports of CTF and full-length TDP-43 degradation suggest that the proteasome is primarily responsible for the degradation of both CTFs and full-length TDP-43 (14, 15, 34). To verify that de novo cleaved CTFs are degraded by the proteasome, cells transfected with FLAG-TDP-43(182tev)-Myc were treated with the proteasome inhibitor clasto-lactacystin-β-lactone during CTF production with TEVnuc. Protein extracted from clasto-lactacystin-β-lactone-treated cells show a 3-fold increase in CTF levels compared with untreated controls (supplemental Fig. 3). This confirms that de novo CTFs are degraded by the proteasome. Interestingly, a 3-fold increase in the level of cytoplasmic CTFs did not change the cytoplasmic localization pattern or solubility of the CTF (data not shown). These observations show that CTF clearance includes transport of CTFs to the cytoplasm, followed by efficient degradation by the proteasome.

**Comparison of Aggregate-prone and de Novo Generated CTFs**

The biochemical properties of CTFs generated from de novo cleavage with TEV were compared with CTFs extracted from FTLD-TDP brain (1, 25) and in cells transiently transfected with a cDNA encoding a CTF containing amino acids 182–414. De novo cleavage of TDP-43 at Gln182 resulted in CTFs and NTFs that were extracted in the soluble fraction (Fig. 4A). In contrast, overexpression of the same CTF by transfection shows that it is predominantly extracted in the insoluble fraction and is hyperphosphorylated at serines 409 and 410, a robust marker of TDP-43 pathology (Fig. 4A). The pathological CTFs extracted from FTLD-TDP brain tissue are heavily phosphorylated and present exclusively in the insoluble fraction (26, 35) (Fig. 4A). Therefore, CTFs generated by de novo cleavage of TDP-43 possess a distinct set of biochemical properties compared with overexpression of truncated TDP-43 constructs. This indicates that a second event or perturbation of these CTFs may be required to convert CTFs generated by de novo cleavage into insoluble aggregates. Therefore, we tested three plausible conditions that are linked to mechanisms of motor neuron disease to determine if one or more of these perturbations would initiate aggregation of de novo cleaved CTFs: 1) seeding cells with aggregate-prone CTFs, 2) depleting RNA, and 3) disrupting cellular trafficking.

To determine if aggregate-prone CTF seeds can sequester soluble de novo cleaved CTFs, we performed a TEVnuc cleavage
assay in cells cotransfected with FLAG-TDP-43(182tev)-Myc and a GFP-tagged CTF. Here, a tetracycline-inducible construct of GFP-TDP-43(208–414) and a CTF show that expression of the aggregate-prone CTF prevents their colocalization and coaggregation (17). This also confirms previous observations that overexpression of CTFs in cells leads to the formation of cytoplasmic inclusions but does not sequester nuclear TDP-43 (15, 18). However, when cells were transfected with FLAG-TDP-43(182tev)-Myc and GFP-TDP-43(208–414), followed by TEV\textsuperscript{nuc} cleavage, we observed striking colocalization of the \textit{de novo} cleaved CTFs with the GFP-TDP-43(208–414) inclusions (Fig. 4E). Although only ~10% of all transfected cells contained both GFP-TDP-43(208–414) inclusions and \textit{de novo} cleaved CTFs, nearly all of these cells displayed colocalization of GFP-TDP-43(208–414) CTF and the \textit{de novo} cleaved CTFs. Therefore, the introduction of aggregate-prone CTF seeds can recruit soluble and otherwise non-aggregate-prone CTFs into inclusions formed by the CTF seeds.

**RNA Dependence of CTF Aggregation**—In contrast to direct expression of CTFs that are not trafficked to the nucleus and localized to the cytoplasm (15), the generation of CTFs by \textit{de novo} cleavage starts with full-length TDP-43 in the nucleus and presumably retains its RNA associated functions. Indeed, cotransfection of cells with the TEV-cleavable FLAG-TDP-43(182tev)-Myc substrate and the CFTR RNA-splicing reporter plasmid illustrates that this protein retains its functional activity for CFTR splicing (supplemental Fig. 4). Therefore, the uncleaved FLAG-TDP-43(182tev)-Myc used in this study associates with nuclear RNA and retains its function in RNA splicing. Because TDP-43 interacts with hnRNPs and RNAs that are efficiently exported to the cytoplasm (9, 11, 36, 37), we determined the effect of RNA on CTF aggregation.

To determine whether RNA alters CTF solubility or localization, we removed the cellular pool of RNA by treating cells containing cytoplasmic CTFs with RNase. For these experiments, cells transfected with GFP-TDP-43(182tev)-mRuby were incubated overnight in the presence and absence of TEV\textsuperscript{nuc} followed by a 15-min RNase treatment before fixation. In the presence of RNase, uncleaved TEP-D43 displays a speckled nuclear pattern consistent with the RNA binding-deficient localization phenotype for TDP-43 (Fig. 5A) (8, 13). RNase-treated cells containing cytoplasmic CTFs showed a remarkable redistribution of CTFs from the diffuse cytoplasmic pattern to inclusion-like foci (Fig. 5B). This striking alteration in the localization of CTFs suggests that the distribution of CTFs can be altered by removal of cellular RNA and that direct or indirect interactions of TDP-43 CTFs with RNA may prevent them from aggregating. Biochemical extraction of cells possessing cytoplasmic CTFs showed a corresponding shift in CTF solubility in cells treated with RNase (Fig. 5C), further supporting the possibility that soluble CTFs are stabilized by interactions with RNA.

**Cytoplasmic CTF Distribution Is Dependent on Dynein Transport**—Because \textit{de novo} cleaved nuclear CTFs are trafficked to the cytoplasm, we asked if disruption of cellular transport mechanisms would affect the distribution of CTFs follow-
De Novo Cleavage of TDP-43

FIGURE 5. RNA dependence of de novo cleaved CTF localization. A and B, RNase pretreatment causes a redistribution of full-length TDP-43 and CTFs. TEVnuc cells transfected with GFP-TDP-43(182tev)-mRuby were incubated overnight in the absence (A) and presence (B) of TEVnuc followed by a 15-min pretreatment in CSK buffer containing 1 mg/ml RNase A (see “Experimental Procedures”). CTFs appear redistributed as cytoplasmic inclusions (B, arrows). Scale bar, 5 μm. C, sequential extraction of cells transfected with FLAG-TDP-43(182tev)-Myc, followed by cleavage and RNase treatment, shows a shift in de novo cleaved CTF to the insoluble fraction. Symbols mark the migration of FLAG-TDP-43(182tev)-Myc (***) and endogenous TDP-43 (*).

We show that cleavage of TDP-43 coupled with loss of association with dynein and dynein motor function promotes the dynamic transport of CTFs. To further demonstrate the role of dynein in CTF transport and clearance, we cotransfected cells with GFP-TDP-43(182tev)-mRuby and CC1-Myc, followed by cleavage with TEVnuc. CC1 is a domain in the p150(glu) subunit of dynactin that interacts with dynein and inhibits dynein motor function (28). Immunoblot of biochemical extract from cells cotransfected with CC1-Myc and GFP-TDP-43(182tev)-mRuby show an increased level of CTFs in cells containing the dynin inhibitor CC1-Myc. This supports the hypothesis that CTF clearance is dependent on dynein transport (Fig. 6F). Notably, we observed four distinct phenotypes of cytoplasmic CTFs in cells cotransfected with the dynein inhibitor CC1-Myc. Consistent with the EHNA treatment of uncleaved TDP-43, overexpression of CC1-Myc did not affect the nuclear localization of the uncleaved GFP-TDP-43(182tev)-mRuby protein (Fig. 6G). In the presence of TEVnuc, the majority of cotransfected cells displayed cytoplasmic CTFs with a diffuse cytoplasmic localization pattern similar to cells treated with EHNA (Fig. 6, compare D with H). However, ~15% of cotransfected cells harboring cytoplasmic CTFs showed a clear redistribution of CTFs in the form of discrete CTF inclusions (Fig. 6I). Surprisingly, ~5% of cells with CTFs also showed cytoplasmic inclusions formed by full-length TDP-43 (Fig. 6I). Because full-length TDP-43 inclusions were not observed in the CC1-Myc transfected cells without cleavage (Fig. 6G), this implies that formation of inclusions formed by full-length TDP-43 is dependent on TDP-43 cleavage. Taken together, these observations define a novel role for dynein-mediated transport in modulating the distribution of CTFs, and they also imply that defects in axonal transport observed in mouse models of motor neuron disease (39) may contribute to the aggregation of CTFs with full-length TDP-43 inclusions.

DISCUSSION

CTFs are major components of TDP-43 inclusions in TDP-43 proteinopathies. Therefore, understanding TDP-43 proteolytic processing is likely to be as fundamental in providing insights into the mechanism(s) of TDP-43 pathogenesis as elucidating mechanisms of Aβ generation has been for understanding the pathobiology of Alzheimer disease (40). Because the protease that generates TDP-43 CTFs in FTLD-TDP and ALS remains unknown, we established the TEV protease system in mammalian cells as a novel model system to study nuclear TDP-43 cleavage and fate of N-terminal and C-terminal fragments of TDP-43. Our findings show that RRM2 in TDP-43 is relatively resistant to cleavage and that the stability of de novo cleaved CTFs is dependent on RRM2 and the C-terminal glycine-rich domain. Unlike previous studies demonstrating that acute expression of de novo cleaved CTFs results in the formation of cytoplasmic aggregates (15-18), CTFs generated from de novo TDP-43 cleavage at Gln\textsuperscript{182} are efficiently exported from the nucleus and degraded by the proteasome with a relatively short half-life. Thus, other factors must contribute to CTF aggregation. We show that cleavage of TDP-43 coupled with loss of asso-
cytoplasmic CTFs that stimulate the formation of cytoplasmic CTF inclusions. Alternatively, it is possible that smaller fragments, such as Gln \textsuperscript{182} CTF, are generated from larger CTFs similar to the full-length CTF studied here. Consistent with this possibility, CTFs that lack RRM2, it is likely that RRM2 is responsible for the increased stability of Gln \textsuperscript{182} CTFs. Alternatively, it is possible that the loss of the nuclear export sequence in RRM2 prevents export of these shorter CTFs and promotes their nuclear degradation. In contrast, cleavage at Gln \textsuperscript{182}, Ala \textsuperscript{260}, and Arg \textsuperscript{272} produces NTFs that are retained in the nucleus with a half-life that is similar to the full-length protein. This suggests that NTFs may be degraded in a similar manner as nuclear TDP-43. Because NTFs are generally not observed in post-mortem FTLD-TDP or ALS brains (25), it is possible that TDP-43 may become modified before cleavage to generate an NTF that is more rapidly cleared.

Studies of TDP-43 complexes with protein and RNA are beginning to provide a clearer understanding of TDP-43 as a component of large RNP complexes (9, 41, 42). Our observations that CTF transport are dependent on interactions of TDP-43 with RNA and on dynein-mediated MT transport are consistent with its classification as a shuttling RNP protein (12). However, we speculate that a chain of events following \textit{de novo} cleavage of TDP-43 must occur to alter CTF solubility and clearance, as outlined in Fig. 7. TDP-43 is a nuclear hnRNP that is likely to exist in both free and RNA-associated states. Model cleavage of TDP-43 by TEV protease at Gln \textsuperscript{182} generates NTFs and CTFs that stimulate the nuclear export of CTFs and nuclear retention of NTs. CTFs do not passively diffuse across the nuclear pore, suggesting that nuclear export of the CTFs is associated with a specific transport mechanism (43). Therefore, we propose that CTFs are bound to or "piggy-backed" onto RNA as they exit the nucleus and undergo transport throughout the cytoplasm. This is consistent with established models of hnRNP nuclear transport mechanisms.
De Novo Cleavage of TDP-43

![Diagram of CTF cleavage and transport]

FIGURE 7. Model of CTF cleavage and transport. TDP-43 in the unbound or RNA-associated state is a substrate for TEVnuc-mediated cleavage. Nuclear retention of NTFs may contribute to their long-lived stability and may potentially generate alternative RNA targets in the absence of RRM2 and the glycine-rich C terminus. Nuclear CTFs are exported to the cytoplasm potentially "piggy-backed" with RNA as an RNP transport particle. Cytoplasmic RNPs are transported throughout the cell via cytoskeleton networks, including MTs. Loss of RNA or the RNP transport mechanisms alters CTF distribution and clearance and potentially contributes to CTF aggregation.

export as a mRNA–RNP complex that is dependent on RRM2 (11, 12). However, a second event, such as the loss of direct or indirect interactions with RNA or failed MT transport, could initiate the aggregation process. Once initiated, the aggregated CTFs can serve as a nidus to recruit additional de novo generated CTFs forming large cytoplasmic inclusions, as was seen when the aggregate-prone GFP-TDP-43(208–414) seed was expressed in cells.

In summary, we established a unique cell model to investigate the proteolytic cleavage of TDP-43. This system is adaptable to biological processes that involve proteolysis of a specific protein in cells. For TDP-43, we defined three specific types of cleavage that provide tangible clues to a mechanism of TDP-43 pathogenesis that includes proteolysis. Significantly, our data suggest that physical changes in protein structure must occur before cleavage sites within RRM2 are exposed and that CTF aggregation is a "two-hit" or two-step process that includes TDP-43 cleavage coupled to additional subsequent cellular stresses that may serve as "triggers" for TDP-43 aggregation and the onset of TDP-43 proteinopathies, as exemplified by ALS and FTLD-TDP.

Acknowledgments—We thank Dr. Greg Van Duyne for the TEV protease construct, Dr. Erika Holzbaur for the CCI-Myc construct, Andrew Huang and Chi Li for technical assistance, and Drs. Todd Cohen, Linda Kwong and Eddie Lee for critical reading of the manuscript.

REFERENCES
1. 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.-Y. (2006) Science 314, 130–133
2. Lagier-Tourenne, C. and Cleveland, D. W. (2009) Cell 136, 1001–1004
3. Pesiridis, G. S., Lee, V. M.-Y., and Trojanowski, J. Q. (2009) Hum. Mol. Genet. 18, R156–R162
4. Gregory, R. I., Yan, K. P., Amuthan, G., Chen-Plotkin, A., Winton, M. J., Unger, T. L., Xu, Y., Neumann, M., Trojanowski, J. Q., and Lee, V. M.-Y. (2009) J. Biol. Chem. 283, 13302–13309
5. Igaz, L. M., Kwong, L. K., Xiong, Y., antibody, E., Dickson, D. W., and Petrucelli, L. (2009) J. Biol. Chem. 284, 8516–8524
6. Dormann, D., Capell, A., Carlson, A. M., Shankaran, S. S., Rodde, R., Neumann, M., Kremmer, E., Matsuura, T., Yamanouchi, K., Nishihara, M., and Haass, C. (2008) J. Neurochem. 101, 1082–1094
7. Nonaka, T., Kametani, F., Arai, T., Akiyama, H., and Hasegawa, M. (2009) Hum. Mol. Genet. 18, 3355–3364
8. Zhang, Y. J., Xu, Y. F., Cook, C., Gendron, T. F., Roettges, P., Link, C. D., Lin, W. L., Tong, J., Castanedes-Casey, M., Ash, P., Gass, J., Rangachari, V., Buratti, E., Baralle, F. E. (2000) J. Cell Biol. 121, 3778–3785
9. Winton, M. J., Igaz, L. M., Wong, M. M., Kwong, L. K., Trojanowski, J. Q., and Lee, V. M.-Y. (2008) J. Biol. Chem. 283, 13302–13309
10. Igaz, L. M., Kwong, L. K., Chen-Plotkin, A., Winton, M. J., Unger, T. L., Xu, Y., Neumann, M., Trojanowski, J. Q., and Lee, V. M.-Y. (2009) J. Biol. Chem. 284, 8516–8524
11. Johnson, B. S., Snead, D., Lee, J. M., McCaffery, J. M., Shorter, J., and Gitler, A. D. (2009) J. Biol. Chem. 284, 20329–20339
12. Li, Y., Ray, P., Rao, E. J., Shi, C., Guo, W., Chen, X., Woodruff, E. A., 3rd, Fushimi, K., and Wu, J. Y. (2010) Proc. Natl. Acad. Sci. U.S.A. 107, 3169–3174
13. Lu, Y., Ferris, J., and Gao, F. B. (2009) Mol. Brain 2, 30
14. Pauli, A., Althoff, F., Oliveira, R. A., Heidmann, S., Schuldiner, O., Lehner, C. F., Dickson, B. J., and Nasmuth, K. (2008) Dev. Cell 14, 239–251
15. Kredel, S., Oswald, F., Nienhaus, K., Deuschle, K., Röcker, C., Wolff, M., Heilker, R., Nienhaus, G. U., and Wiedenmann, J. (2009) PLoS One 4, e4391
16. Igaz, L. M., Kwong, L. K., Xu, Y., Truax, A. C., Uryu, K., Neumann, M., Clark, C. M., Elman, L. B., Miller, B. L., Grossman, M., McCluskey, L. F., Trojanowski, J. Q., and Lee, V. M.-Y. (2008) Am. J. Pathol. 173, 182–194
17. Neumann, M., Kwong, L. K., Lee, E. B., Kremmer, E., Flatley, A., Xu, Y., Forman, M. S., Troost, D., Kretzschmar, H. A., Trojanowski, J. Q., and Lee, V. M.-Y. (2009) Acta Neuropathol. 117, 137–149
18. Jansens, A., and Braakman, I. (2003) Methods Mol. Biol. 232, 133–145
19. Prasanth, K. V., Sacco-Bubulay, P. A., Prasanth, S. G., and Spector, D. L. (2003) Mol. Biol. Cell 14, 1043–1057
20. Prasanth, K. V., Prasanth, S. G., Xuan, Z., Hearn, S., Freier, S. M., Bennett,
C. F., Zhang, M. Q., and Spector, D. L. (2005) Cell 123, 249–263
31. Kuo, P. H., Doudeva, L. G., Wang, Y. T., Shen, C. K., and Yuan, H. S. (2009) Nucleic Acids Res. 37, 1799–1808
32. Williams, K. R., Stone, K. L., LoPresti, M. B., Merrill, B. M., and Planck, S. R. (1985) Proc. Natl. Acad. Sci. U.S.A. 82, 5666–5670
33. Bonner, W. M. (1975) J. Cell Biol. 64, 421–430
34. Zhang, Y. J., Gendron, T. F., Xu, Y. F., Ko, L. W., Yen, S. H., and Petrucelli, L. (2010) Mol. Neurodegener. 5, 33
35. Hasegawa, M., Arai, T., Nonaka, T., Kametani, F., Yoshida, M., Hashizume, Y., Beach, T. G., Buratti, E., Baralle, F., Morita, M., Nakano, I., Oda, T., Tsuchiya, K., and Akiyama, H. (2008) Ann. Neurol. 64, 60–70
36. Milly, S., Shu, H. I., Zhao, Y., and Píñol-Roma, S. (2001) Mol. Cell. Biol. 21, 7307–7319
37. Ling, S. C., Albuquerque, C. P., Han, J. S., Lagier-Tourenne, C., Tokunaga, S., Zhou, H., and Cleveland, D. W. (2010) Proc. Natl. Acad. Sci. U.S.A. 107, 13318–13323
38. Cortésy-Theulaz, I., Pauloin, A., and Pfeffer, S. R. (1992) J. Cell Biol. 118, 1333–1345
39. Zhang, B., Tu, P., Abtahian, F., Trojanowski, J. Q., and Lee, V. M.-Y. (1997) J. Cell Biol. 139, 1307–1315
40. De Strooper, B., and Annaert, W. (2010) Annu. Rev. Cell Dev. Biol. 26, 235–260
41. Kim, S. H., Shanware, N. P., Bowler, M. J., and Tibbetts, R. S. (2010) J. Biol. Chem. 285, 34097–34105
42. Sephton, C. F., Cenik, C., Kucukural, A., Dammer, E. B., Cenik, B., Han, Y., Dewey, C. M., Roth, F. P., Herz, J., Peng, J., Moore, M. J., and Yu, G. (2011) J. Biol. Chem. 286, 1204–1215
43. Nishimura, A. L., Zupunski, V., Troakes, C., Kathe, C., Fratta, P., Howell, M., Gallo, J. M., Hortobágyi, T., Shaw, C. E., and Rogelj, B. (2010) Brain 133, 1763–1771