# Phosphorylation regulates proteasomal-mediated degradation and solubility of TAR DNA binding protein-43 C-terminal fragments

Yong-Jie Zhang*, Tania Gendron*, Ya-Fei Xu, Li-Wen Ko, Shu-Hui Yen, and Leonard Petrucelli

* Y-J Zhang and T Gendron contributed equally to this work

**Department of Neuroscience, Mayo Clinic College of Medicine, Jacksonville, FL**

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## ABSTRACT

Inclusions of TAR DNA binding protein-43 (TDP-43) are the defining pathological feature of several neurodegenerative diseases collectively referred to as TDP-43 proteinopathies. These diseases are characterized by the presence of cellular aggregates composed of abnormally phosphorylated, N-terminally truncated and ubiquitinated TDP-43 in the spinal cord and/or brain. Recent studies indicate that C-terminal fragments of TDP-43 are aggregation-prone and induce cytotoxicity. However, little is known regarding the pathways responsible for the degradation of these fragments and how their phosphorylation contributes to the pathogenesis of disease. Herein, we established a human neuroblastoma cell line (M17D3) that conditionally expresses an enhanced green fluorescent protein (GFP)-tagged caspase-cleaved C-terminal TDP-43 fragment (GFP-$TDP_{220-414}$). We report that expression of this fragment within cells leads to a time-dependent formation of inclusions that are immunoreactive for both ubiquitin and phosphorylated TDP-43, thus recapitulating pathological hallmarks of TDP-43 proteinopathies. Phosphorylation of GFP-$TDP_{220-414}$ renders it resistant to degradation and enhances its accumulation into insoluble aggregates. Nonetheless, GFP-$TDP_{220-414}$ inclusions are reversible and can be cleared through the ubiquitin proteasome system. Moreover, both Hsp70 and Hsp90 bind to GFP-$TDP_{220-414}$ and regulate its degradation.

## RESULTS

### TDP-43 C-terminal fragments share similar pathological properties

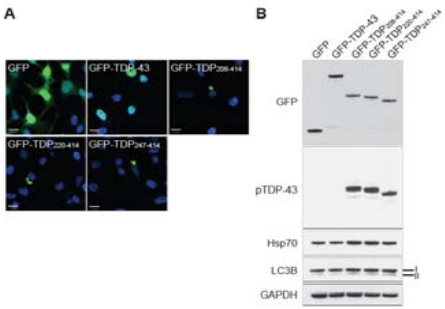


**Figure 1. TDP-43 C-terminal fragments share similar pathological properties.** M17 cells were transiently transfected to express GFP, GFP-TDP-43 or various GFP-TDP-43 C-terminal fragments for 2 days. (**A**) Fluorescent confocal microscopy demonstrated diffuse cytoplasmic and nuclear distribution of GFP and a predominantly nuclear localization of full-length GFP-TDP-43. In contrast, GFP-TDP-43 C-terminal fragments formed cytoplasmic inclusions. Scale bar, 10 μM (**B**) Western blotting of cell lysates using a phosphorylation-dependent (pS409/pS410) TDP-43 antibody (termed pTDP-43) showed marked immunoreactivity for C-terminal fragments of TDP-43; full-length GFP-TDP-43, however, was not phosphorylated. Of note, the expression of TDP-43 C-terminal fragments, but neither that of full-length TDP-43 nor GFP alone, resulted in the induction of Hsp70.

### Time-dependent expression and aggregation of GFP-$TDP_{220-414}$ in M17D3 cells

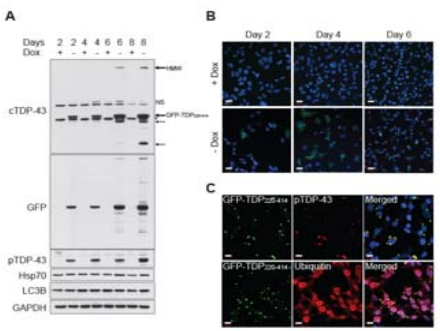


**Figure 2. Time-dependent expression and aggregation of GFP-$TDP_{220-414}$ in M17D3 cells.** M17D3 cells were cultured in media lacking or containing Dox to induce or suppress the expression of GFP-$TDP_{220-414}$, respectively. (**A**) The levels of both total and phosphorylated-GFP-$TDP_{220-414}$ (indicated by arrow) increased in a time-dependent manner and high-molecular weight (HMW) GFP-$TDP_{220-414}$ products were observed by 6 days of induction. In contrast, levels of endogenous TDP-43 (indicated by *) remained constant. TDP-43 fragments were observed (dashed arrows) at days 6 and 8, which likely resulted from the truncation of GFP-$TDP_{220-414}$. Dox=doxycycline, NS= non-specific band. (**B**) Immunofluorescence studies showed that no GFP signal was present in cells treated with doxycycline, confirming that GFP-$TDP_{220-414}$ expression was tightly regulated. In the absence of doxycycline, GFP-$TDP_{220-414}$ inclusions were observed after only 2 days of induction and they markedly increased in number by day 4. At day 6, larger, but fewer, inclusions were present per cell. Scale bar, 20 μM. (**C**) The cytoplasmic inclusions composed of GFP-$TDP_{220-414}$ were ubiquitinated and phosphorylated. Scale bar, 10 μM.

### GFP-$TDP_{220-414}$ is preferentially degraded through the ubiquitin-proteasome pathway

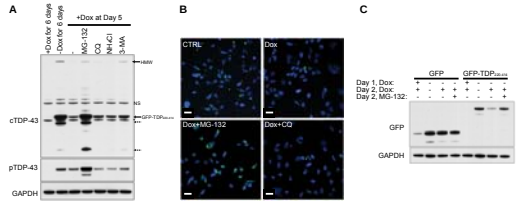


**Figure 3. The TDP-43 C-terminal fragment is preferentially degraded through the ubiquitin-proteasome pathway.** To monitor the degradation of GFP-$TDP_{220-414}$, M17D3 were grown in Dox-free media for 5 days to induce GFP-$TDP_{220-414}$ expression. GFP-$TDP_{220-414}$ expression was then inhibited for 24 h by Dox +/- the proteasome inhibitor, MG-132, the lysosome inhibitors, chloroquine or $NH_4Cl$, or the autophagy inhibitor, 3-MA. (**A**) GFP-$TDP_{220-414}$ (indicated by arrow) accumulation was significantly decreased 24 h after Dox treatment. Of interest, the degradation of GFP-$TDP_{220-414}$ was completely suppressed by the MG-132, but only slightly suppressed by lysosomal and autophagy inhibitors. (**B**) Numerous inclusions were present in cells after 6 d of GFP-$TDP_{220-414}$ induction in Dox-free media (CTRL). However, if cells were treated with Dox during the last 24 h of the 6-day experiment, few inclusions remained (Dox). This clearance of inclusions was inhibited by MG-132 (Dox+MG-132) but not chloroquine (Dox+CQ). Scale bar, 20 μM. (**C**) To determine if the GFP tag is responsible for targeting GFP-$TDP_{220-414}$ to the proteasome, M17D cells were transiently transfected with pTRE-GFP or pTRE-GFP-$TDP_{220-414}$ for 2 days. Cultures were treated with or without DOX and MG-132, as indicated. As expected, proteasome inhibition suppressed the degradation of GFP-$TDP_{220-414}$. In contrast, GFP levels remained unchanged even if GFP expression was blocked by Dox.

### Knockdown of heat shock proteins leads to the accumulation of TDP-43 C-terminal fragments

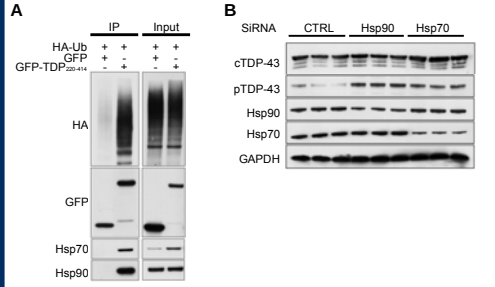


**Figure 4. Knockdown of heat shock proteins leads to the accumulation of TDP-43 C-terminal fragments.** (**A**) Co-immunoprecipitation studies showed that GFP-$TDP_{220-414}$, but not GFP, binds to Hsp70 and Hsp90. (**B-C**) To explore the role of Hsp70 and Hsp90 in the degradation of GFP-$TDP_{220-414}$, M17D3 cells were grown in Dox-free medium for 4 days then treated with siRNA targeted to Hsp90 or Hsp70 or with a validated negative siRNA control. Compared to control cells, the protein levels of Hsp90 or Hsp70 were decreased to 66% and 40%, respectively, after the 2 day treatment with siRNA. Knockdown of Hsp70 or Hsp90 resulted in the accumulation of total GFP-$TDP_{220-414}$ and, to a greater extent, of p-GFP-$TDP_{220-414}$. Data was collected from 3 separate experiments and shown as the mean ±SEM. ** represents $P<0.001$.

### Phosphorylated TDP-43 C-terminal fragment is resistant to proteasomal degradation

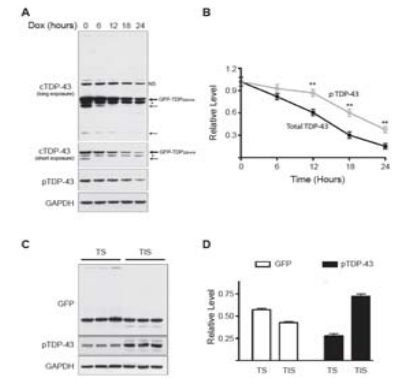


**Figure 5. Phosphorylated TDP-43 C-terminal fragment is resistant to proteasomal degradation.** To calculate the half-life of GFP-$TDP_{220-414}$, M17D3 cells were maintained in Dox-free media for 5 d to induce GFP-$TDP_{220-414}$ expression. Dox was then added to the media to arrest transcription of GFP-$TDP_{220-414}$. (**A**) Cell lysates were prepared at the indicated times post-Dox for Western blot analysis. Note the time-dependent clearance of total and phosphorylated GFP-$TDP_{220-414}$ (arrow). (**B**) Densitometric quantification of GFP-$TDP_{220-414}$ remaining in cells revealed that phosphorylated-GFP-$TDP_{220-414}$ was degraded more slowly than total GFP-$TDP_{220-414}$. The half life ($T_{½}$) of total GFPTDP$_{220-414}$ was 14.2 hours, whereas $T_{½}$ for phosphorylated-GFP-$TDP_{220-414}$ was 22.1 hours. Data was collected from 3 separate experiments and is shown as the mean ±SEM.** represents $P<0.001$, as assessed by 1-way ANOVA, followed by Tukey's posthoc analysis. (**C**) To examine the solubility of total and phosphorylated-GFP-$TDP_{220-414}$, cell lysates were separated in Triton X-100-soluble and insoluble fractions. Although a considerable amount of total GFP-$TDP_{220-414}$ was detected in the detergent soluble fraction, the majority of phosphorylated-GFP-$TDP_{220-414}$ accumulated in the detergent insoluble fraction. Data was collected from 3 separate experiments and is shown as the mean ±SEM.

## CONCLUSIONS

Our data indicates that inclusions formed from TDP-43 C-terminal fragments are reversible. Given that TDP-43 inclusions have been shown to confer toxicity, our findings have important therapeutic implications and suggest that modulating the phosphorylation state of TDP-43 C-terminal fragments may be a promising therapeutic strategy to clear TDP-43 inclusions.

## ACKNOWLEDGEMENTS

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