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Dual-specificity Phosphatase 8 Promotes the Degradation of the Polyglutamine Protein Ataxin-1

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Dual-specificity phosphatases (DUSPs), which constitute type I cysteine-based protein tyrosine phosphatases, are a subfamily of protein phosphatases that can dephosphorylate both tyrosine and serine/threonine residues within the same substrate.[1] To date, at least 16 mammalian DUSPs showing dephosphorylation activity towards MAP kinases (MAPKs) have been identified.[2] Although the actual physiological roles of many DUSP proteins are still largely unknown, growing information have revealed their correlation with numbers of human diseases *e.g.*, multiple types of cancer, cerebral ischemia and neurodegenerative diseases.[3-6] It is generally assumed that DUSP proteins have distinct roles in diverse cellular processes; however, the understanding of specific functions of individual DUSP protein has been somewhat difficult partly due to the overlapping substrate specificity.[7] A member of DUSP subfamily, DUSP8 (also known as M3/6 or hVH5) is reported to selectively regulate JNK pathway and, to a lesser extent, p38 pathway.[7,8] An interesting characteristic of DUSP8 is that protein damaging conditions can inactivate this phosphatase by increasing insolubility or decreasing stability.[9,10]

In a group of neurodegenerative diseases collectively known as polyglutamine (polyQ) diseases, the expansion of CAG (glutamine) repeats leads to misfolding and aggregation of causative proteins.[11] Similar to protein damaging conditions (*e.g.*, heat shock and oxidative stress), polyQ-induced toxicity involves the aggregation of aberrant proteins and the concomitant activation of MAP kinase through the inhibition of MAPK phosphatases. It was shown that the solubility of DUSP8 is impaired by the expansion of polyQ-repeats.[6] In the previous study, we demonstrated that polyglutamine-repeat protein ataxin-1 (ATXN1; the causative protein of spinocerebellar ataxia type 1) interacts with a number of DUSP proteins including DUSP8 and the cellular state of oxidative stress influences the association between ataxin-1 and DUSP8.[12] During the course of studies, we repeatedly observed that the cellular levels of ataxin-1 were diminished by DUSP8 over-expression, implicating that it may facilitate the down-regulation of ataxin-1. Since it was plausible that DUSP8 decreases the solubility of ataxin-1 rather than directly promoting protein degradation and thereby causes its accumulation in insoluble fraction, we first measured the effects of DUSP8 on the relative amounts of ataxin-1 in soluble and insoluble fractions. To this end, we fractionated the lysate of HEK293T cells co-expressing ataxin-1 and DUSP8 and then examined whether DUSP8 causes increased degradation of ataxin-1 or enhanced partitioning of polyQ proteins into insoluble fraction.

Over-expression of DUSP8 significantly reduced the steady-state level of ataxin-1 both in the soluble and insoluble fractions supporting the notion that the down-regulation of this polyQ protein is indeed caused by the increased degradation (Fig. 1(a)). In fact, DUSP8 over-expression almost completely abolished the partitioning of ataxin-1 into the insoluble fraction as well as the formation of large aggregates containing polyQ-expanded ataxin-1[82Q], measured by filter retardation assay (Fig. 1(b)). To determine if ubiquitin-proteasome system is responsible for the DUSP8-induced degradation of ataxin-1, we treated cells with the proteasome inhibitor MG132 (5 μM). Surprisingly, MG132 treatment only partially blocked the degradation of ataxin-1 implicating that other cellular proteolytic pathways (*e.g.*, autophagy-lysosome system) also participate in DUSP8-induced down-regulation of ataxin-1 (Fig. 1(a)). Besides its effects on the stability of ataxin-1, MG132 treatment also stabilized DUSP8 proteins and increased its partitioning into insoluble fraction which is consistent with the results shown in a previous study.[13]

Next, we examined if another DUSP proteins interacting with ataxin-1 (*i.e.*, DUSP2, 4, 6, 11, 13 and 24)[12] are also capable of down-regulating ataxin-1. As shown in Figure 2, over-expression of DUSP24 had little or no effect on the steady-state level of ataxin-1. Similar results were also obtained with other DUSP proteins strongly associating with ataxin-1 (data not shown). These results indicate that the promotion of ataxin-1 degradation is probably the unique effects of DUSP8. Notably, the presence of ataxin-1 caused the increased partitioning of DUSP24 into insoluble fraction, suggesting that aggregation-prone polyQ proteins likely recruit DUSP proteins and as a result cause perturbations in the MAP kinase signaling pathways.

Finally, we investigated if the promotion of ataxin-1 degradation is dependent on the phosphatase activity of DUSP8. For this experiment, we generated a mutant version of DUSP8 in which the active site cysteine (C246) was replaced with serine. As shown in Figure 3, both the wild-type protein

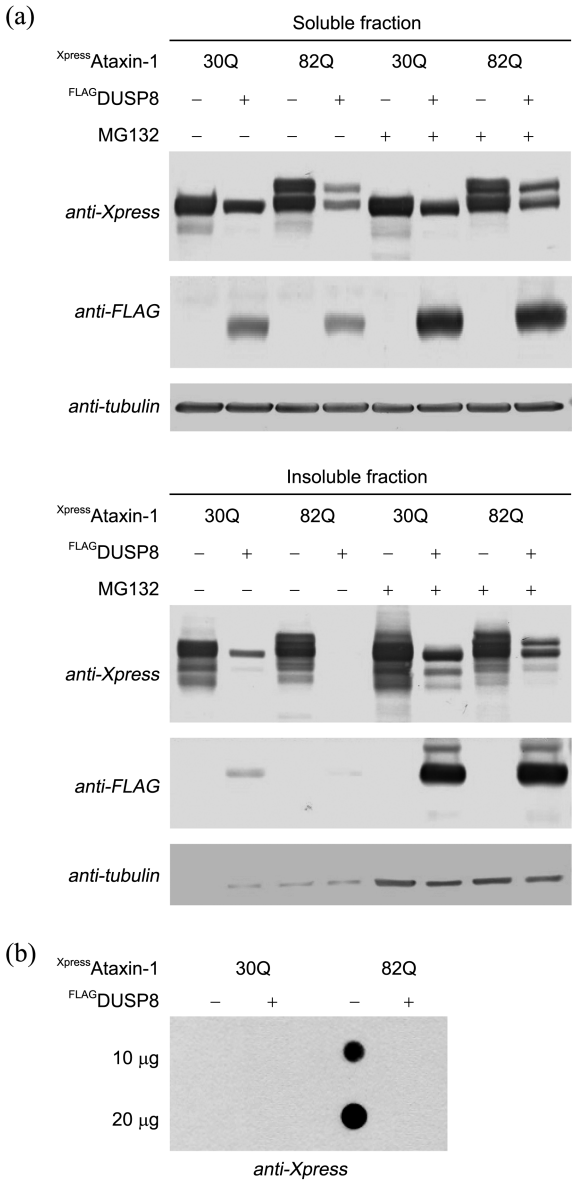

Figure 1. DUSP8 promotes the degradation of ataxin-1 and blocks the formation of large aggregates containing polyQ expanded ataxin-1. (a) To assess the effects of DUSP8 on the cellular level of ataxin-1, HEK293T cells were transfected with Xpress-ataxin-1 [30Q] or [82Q] together with FLAG-DUSP8. After 48 h of transfection, cells were collected and then subjected to fractionation as described in 'Experimental'. Cells were treated with or without 5 μM MG132 during 24 h of post-transfection period. Immunoblot analysis was performed using indicated antibodies. (b) For filter retardation assay, HEK293T cells transfected with DUSP8 and ataxin-1 were collected, lysed and then spotted onto a nitrocellulose filter using dot-blot microfiltration kit. Immunoblot analysis using anti-Xpress antibody was then carried out.

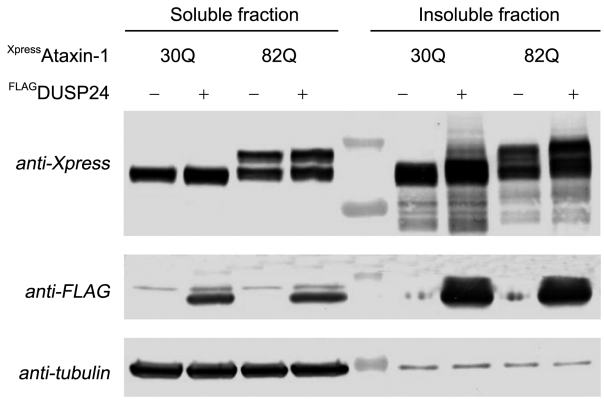

Figure 2. DUSP24, which can bind to ataxin-1, does not facilitates its degradation. To test the effects of other DUSP proteins interacting with ataxin-1 on the degradation of this polyQ protein, DUSP24 and ataxin-1 were co-transfected into HEK293T cells. Fractionation and subsequent immunoblot analysis was carried out described in 'Experimental' section.

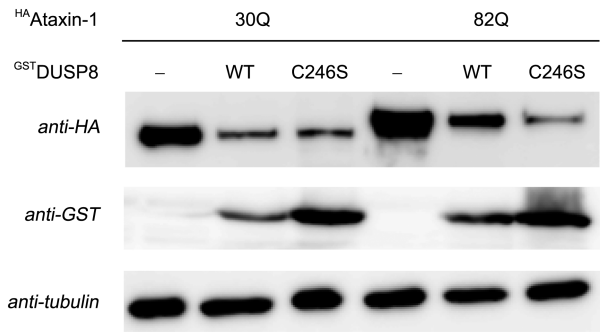

Figure 3. The catalytic mutant of DUSP8 is still capable of promoting degradation of ataxin-1. To test the effects of inactivation of active site (C246) in DUSP8 on the degradation of ataxin-1, the plasmids carrying HA-ataxin-1 and GST-DUSP8 (WT or C246S mutant) were co-transfected into HEK293T cells. Immunoblot analysis of ataxin-1 and DUSP8 proteins were carried out described in 'Experimental' section.

(WT) and the catalytic mutant (C246S) DUSP8 decreased the level of ataxin-1 to similar extents suggesting that the phosphatase activity of DUSP8 may not be required for the degradation of this polyglutamine-repeat protein; instead the protein-protein interaction between DUSP8 and ataxin-1 should be more crucial for the down-regulation.

In the previous report, we hypothesized that nuclear export signal (NLS) located within DUSP8 may prevent the nuclear translocation of ataxin-1 and thus increase its degradation in the cytoplasm.[12] Based on this assumption, we inferred that DUSP8 may indirectly influence the down-regulation of ataxin-1. However, in this study we provide evidence for more active roles of this dual-specificity phosphatase in the breakdown of misfolded polyQ proteins – *i.e.*, the findings that DUSP8 facilitates the degradation of ataxin-1 and blocks the formation of large aggregates containing polyQ-expanded ataxin-1 (Fig. 1). Recent studies have reported that laforin, a member of DUSP family, also promotes the degradation of cellular proteins involved in glycogen metabolism.[14,15] Laforin (encoded by *EMP2A*) and an E3 ubiquitin ligase malin (encoded by *EMP2B*), both of which are defective in the neurodegenerative Lafora disease, form a functional complex.[15] The laforin-malin complex interacts with substrate proteins and targets them for proteasomal degradation. This functional complex, in conjunction with Hsp70 proteins, facilitates the proteasomal degradation of

misfolded polyQ proteins and protects cells from cytotoxicity.[16] Such observations have similarities with our findings and suggest that DUSP8 probably have an analogous action mechanism. Although exact roles of DUSP8 in the degradation of polyQ proteins are not yet understood, DUSP8 (like laforin) may function as an adaptor protein channeling misfolded proteins into cellular proteolytic machinery. Our findings that the catalytic mutant of DUSP8 promoted the degradation of ataxin-1 to a similar extent as the wild-type protein (Fig. 3) also support such a notion that adaptor function of this protein, presumably mediated by protein-protein interaction, is more important than its phosphatase activity. Importantly, similar results were shown that the catalytic mutant of laforin (C266S) increased the degradation of its substrate (PTG) as effectively as the wild-type protein.[14]

It is noteworthy that laforin was originally suggeted to promote degradation of substrate proteins (including polyQ proteins) primarily through ubiquitin-proteasome system (UPS); however, more recent studies revealed that laforin also regulates autophagy through the mammalian target of rapamycin kinase (mTOR)-dependent pathway.[17,18] Our observation that MG132 treatment only marginally blocked the degradation of ataxin-1 also raises a possibility of similar action mechanism(s) for DUSP8. Whether over-expressed DUSP8 can increase autophagic degradation of polyQ proteins or if DUSP8 is functionally involved in the signaling pathways controlling autophagy (*e.g.*, mTOR pathway) will be the primary subjects to be investigated in the future studies.

Results from previous reports and our studies together implicate that certain DUSP proteins could promote the degradation of misfolded proteins, such as aberrant polyQ proteins, *via* UPS and/or autophagy, both of which are required for timely and prompt elimination of misfolded proteins and prevention of aggregation.[11] Despite such a similarity, the detailed action modes of individual DUSP proteins may differ significantly. Unlike laforin, which is recruited to aggresomes and then facilitates the degradation of misfolded proteins,[16] DUSP8 does not co-localize with inclusions containing polyQ proteins.[6] A possible explanation for such observations is that DUSP8 functions prior to the aggregation of misfolded proteins (observed as nuclear inclusions) whereas laforin involves in the later stage when inclusions are readily detected. In conclusion, our recent efforts to understand the roles of DUSP in the regulation of abnormal polyQ-repeat proteins have revealed somewhat unexpected effects of DUSP8 in the down-regulation of the misfolded proteins. To gain more insights about the roles of DUSP proteins in this process, it needs to be examined whether other DUSP proteins (*e.g.*, laforin) also promote the degradation of ataxin-1 or whether DUSP8 facilitates the degradation of other misfolded polyQ proteins, *e.g.*, ataxin-3 or huntingtin.

Experimental

Cell Culture and Transfection. Human embryonic kidney (HEK) 293T cells were maintained at 37 °C in DMEM supplemented with 10% fetal bovine serum and penicillin/streptomycin in the presence of 5% CO_2. For transient transfection, 1.4×10^6 cells were plated in 60-mm cell culture plates, grown overnight, and transfected with various plasmid DNA using Lipofectamine™ reagent (Invitrogen)

Immunoblot Analysis. After 48 h of expression of Xpress-ataxin-1 and FLAG-DUSP proteins (for experiments studying the effects of the catalytic mutant of DUSP8, HA-ataxin-1 and GST-DUSP8 were employed), HEK293T cells were collected and then lysed in NP-40 lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40) supplemented with Complete-MINI™ protease inhibitor mixture (Roche) for 20 min at 4 °C, followed by centrifugation at 13,000 × *g* for 20 min. Immunoblot analysis using anti-Xpress antibody (1:2,000; Invitrogen), anti-FLAG antibody (1:1,000; Sigma) or anti-GST antibody (1:1,000; Sigma) were performed to determine the relative amounts of individual proteins. The protein bands were visualized using ECL detection system (PIERCE).

Fractionation of Cell Lysate. To analyze the relative amounts of ataxin-1 and DUSP8 in the soluble and insoluble fractions, HEK293T cells expressing Xpress-tagged ataxin-1 with or without FLAG-tagged DUSP8 were lysed in NP-40 lysis buffer, clarified by centrifugation at 1,000 × *g* for 10 min and then subjected to the second round of centrifugation at 20,000 × *g* for 30 min. The supernatants were collected and referred as the soluble fraction. The pellets, referred as the insoluble fraction, were washed three times with NP-40 lysis buffer and solubilized with an equal volume of 1× SDS sample buffer. The relative amounts of individual proteins were determined by western-blot analysis.

Filter Retardation Assay. Filter retardation assay was performed using the protocol described in a previous study.[13] Cell lysate containing Xpress-tagged ataxin-1 was applied onto nitrocellulose membranes (0.2 μm; S & S) using a dot-blot microfiltration kit (Bio-Rad). The membranes were blocked in 5% non-fat dried milk in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) for 1 h and subsequently incubated with anti-Xpress antibody. After washing membranes with TBST, the presence of ataxin-1 in large aggregates was detected by using ECL detection system.

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References

1. Patterson, K. I.; Brummer, T.; O'Brien, P. M.; Daly, R. J. *Biochem. J.* **2009**, *418*, 475.
2. Jeffrey, K. L.; Camps, M.; Rommel, C.; Mackay, C. R. *Nat. Rev. Drug Discov.* **2007**, *6*, 391.
3. Keyse, S. M. *Cancer Metastasis Rev.* **2008**, *27*, 253.
4. Rininger, A.; Dejesus, C.; Totten, A.; Wayland, A.; Halterman, M.

W. *Cell Death Diff.* **2012**, *19*, 1634.
5. Okami, N.; Narasimhan, P.; Yoshioka, H.; Sakata, H.; Kim, G. S.; Jung, J. E.; Maier, C. M.; Chan, P. H. *J. Cereb. Blood. Flow Metabol.* **2013**, *33*, 106.
6. Merienne, K.; Helmlinger, D.; Perkin, G. R.; Devys, D.; Trottier, Y. *J. Biol. Chem.* **2003**, *278*, 16957.
7. Dickinson, R. J.; Keyse, S. M. *J. Cell Sci.* **2006**, *119*, 4607.
8. Oehrl, W.; Cotsiki, M.; Panayotou, G. *Cell Signal.* **2013**, *25*, 429.
9. Palacios. C.; Collins, M. K.; Perkins, G. R. *Curr. Biol.* **2001**, *11*, 1439.
10. Theodosious, A.; Ashworth, A. *Oncogene* **2002**, *21*, 2387.
11. Shao, C.; Diamond, M. I. *Hum. Mol. Genet.* **2007**, *16*, R115.
12. Lee, D. H.; Cho, S. *Bull. Kor. Chem. Soc.* **2013**, *34*, 1909.
13. Cotsiki, M.; Oehrl, W.; Samiotaki, M.; Theodosiou, A.; Panayotou, G. *Cell Signal.* **2012**, *24*, 664
14. Worby, C. A.; Gentry, M. S.; Dixon, J. E. *J. Biol. Chem.* **2008**, *283*, 4069.
15. Gentry, M. S.; Roma-Mateo, C.; Sanz, P. *FEBS J.* **2012**, *280*, 525.
16. Garyali. P.; Siwach, P.; Singh, P. K.; Puri, R.; Mittal, S.; Sengupta, S.; Parihar, R.; Ganesh, S. *Hum. Mol. Genet.* **2009**, *18*, 688.
17. Aguado, C.; Sarkar, S.; Korolchuck, V. I.; Criado, O.; Vernia, S.; Boya, P.; Sanz, P.; Rodriguez de Cordoba, S.; Knecht, E.; Rubinsztein, D. C. *Hum. Mol. Genet.* **2010**, *19*, 2867.
18. Puri, R.; Suzuki, T.; Yamakawa, K.; Ganesh, S. *Hum. Mol. Genet.* **2012**, *21*, 175.