RESEARCH NOTE

Does inactivation of USP14 enhance degradation of proteasomal substrates that are associated with neurodegenerative diseases? [version 1; referees: 1 approved, 2 approved with reservations]

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Abstract

A common pathological hallmark of age-related neurodegenerative diseases is the intracellular accumulation of protein aggregates such as α-synuclein in Parkinson's disease, TDP-43 in ALS, and tau in Alzheimer's disease. Enhancing intracellular clearance of aggregation-prone proteins is a plausible strategy for slowing progression of neurodegenerative diseases and there is great interest in identifying molecular targets that control protein turnover. One of the main routes for protein degradation is through the proteasome, a multisubunit protease that degrades proteins that have been tagged with a polyubiquitin chain by ubiquitin activating and conjugating enzymes. Published data from cellular models indicate that Ubiquitin-specific protease 14 (USP14), a deubiquitinating enzyme (DUB), slows the degradation of tau and TDP-43 by the proteasome and that an inhibitor of USP14 increases the degradation of these substrates. We conducted similar experiments designed to evaluate tau, TDP-43, or α-synuclein levels in cells after overexpressing USP14 or knocking down endogenous expression by siRNA.

This article is included in the Preclinical Reproducibility and Robustness channel.

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version 1 published 04 Feb 2016	? report	✓ report	? report

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Introduction

Research on the ubiquitin-proteasome system has far reaching implications for the development of drugs to treat illnesses associated with the accumulation of misfolded proteins, including Alzheimer's and Parkinson's disease (Ciechanover & Kwon, 2015). Ubiquitin-specific protease 14 (USP14), like its yeast ortholog Ubp6, is a proteasome-associated deubiquitinating enzyme (DUB) that is activated upon binding to the proteasome and catalyzes the cleavage of ubiquitin subunits from substrates before degradation by the proteasome (Borodovsky *et al.*, 2001; Hanna *et al.*, 2006; Hu *et al.*, 2005). By releasing ubiquitin molecules from the substrate, USP14/Ubp6 helps to prevent the rapid degradation of ubiquitin molecules together with the substrate protein (Hanna *et al.*, 2007). A critical role of USP14 in stabilizing cellular ubiquitin levels was demonstrated *in vivo* in USP14 deficient ax^J mice which display decreased ubiquitin levels in all tissues with the greatest loss observed at synaptic terminals (Anderson *et al.*, 2005; Wilson *et al.*, 2002).
In addition to maintaining cellular ubiquitin pools, USP14/Ubp6 has been shown to modulate substrate degradation. Goldberg and colleagues showed that upon binding to a substrate's polyubiquitin chain, activated USP14/Ubp6 facilitates gate-opening of the proteasome (Peth *et al.*, 2009). This mutual interaction of USP14/Ubp6 with the proteasome is thought to enhance selectivity of the proteasome for ubiquitinated proteins and couple deubiquitination to degradation. In contrast, Finley and colleagues found that USP14/Ubp6, and in some instances a catalytically inactive mutant (C114A in mammals), could cause an inhibition of the degradation of substrates (Hanna *et al.*, 2006; Lee *et al.*, 2010). For model substrates and ataxin3, this effect was shown to require USP14/Ubp6 protein but not its catalytic activity. For two proteins involved in neurodegenerative diseases, tau and TDP-43, inhibition of proteasomal degradation by USP14 was dependent on its deubiquitinating activity, since the catalytically inactive mutant had no effect (Lee *et al.*, 2010). This led to the hypothesis that deubiquitination of substrates by USP14 at a faster rate than the proteasome initiates degradation could cause rejection of otherwise competent substrates from the proteasome. Supporting this hypothesis, inhibition of USP14 by a small molecule inhibitor (IU1) enhanced proteasomal substrate degradation in cells overexpressing tau or TDP-43 (Lee *et al.*, 2010). Thus, inhibition of USP14 was proposed as a therapeutic strategy to enhance proteasomal function in neurodegenerative diseases in which these proteins accumulate.

Methods

Constructs. Human USP14 (hUSP14wt), V5-tagged hUSP14wt (V5-hUSP14wt), catalytically inactive mutant USP14-C114A (hUSP14CA), V5-tagged hUSP14CA (V5-hUSP14CA), human tau, and Flag-tagged human TDP- 43 (Flag-TDP-43) were cloned into the pTT5d expression vector by Amgen's Protein Sciences department and confirmed by sequencing. Human α-synuclein-Flag CMV6 expression vector was purchased from Origene (#RC221446) and confirmed by sequencing.

Cell lines. All cell lines were obtained from ATCC. HEK293 cells were grown in DMEM/10% fetal bovine serum/1% penicillin, streptomycin, glutamine. U2OS cells stably expressing Flag-tagged human α-synuclein (U2OS/synuclein) were generated by Amgen Neuroscience in San Francisco and grown in McCoy's 5A/10% fetal bovine serum/1% penicillin, streptomycin/2% glutamine and 0.5mg/mL G418. SH-SY5Y cells were grown DMEM/10% fetal bovine serum/1% penicillin, streptomycin, glutamine and 0.5mg/mL G418. All cells were grown in incubators at 5%CO_2/37°C. All cell culture reagents were purchased from Gibco.

Transfections. HEK293 cells were plated at a density of 10^{-6} cells/well in 6-well plates and transfected with plasmids using Lipofectamine™ 2000 (Thermofisher) for 4 hours, and analyzed 48 hours after transfection. U2OS/synuclein cells were plated at 5×10^{-4} cells/well in 24-well plates and SH-SY5Y cells were plated at 2×10^{-5} cells/well in 6-well plates. Cells were transfected with Opti-MEM™ (Thermofisher) containing 100nM siRNA, and analyzed 60, 72 or 96 hours after transfection. USP14 siRNAs were obtained from Ambion.

Western blot. Cells were lysed with Lysis Reagent (Roche) containing 1% SDS/1X Complete™ protease inhibitors cocktail tablets (Roche). Samples were boiled and Benzonase Nuclease (Sigma) was added following the manufacturer's instructions. 10ug of lysate was loaded on a 12% Bis-Tris gel (Life-Sciences) and proteins were separated by electrophoresis (100mA, 200V) and transferred onto 0.2µm nitrocellulose membrane (Life Sciences) for a minimum of 4hrs (100mA, 25V). Membranes were blocked with Odyssey Blocking Buffer (Li-Cor), incubated with primary antibodies diluted in Li-Cor buffer with 0.2% Tween-20 at 4°C shaking overnight, and washed 3× with phosphate-buffered saline/0.1% Tween-20 (PBST). Membranes were then incubated with secondary antibodies for 1 hour at room temperature in the dark, washed 3× with PBST, and analyzed with the Odyssey imaging system at a relative intensity setting of 2–2.5 for the 800 channel and 1–2 for the 700 channel. Beta-actin or GAPDH served as a loading control.

Antibodies. Mouse monoclonal anti-tau5 (1µg/ml; Invitrogen AHB0042), mouse monoclonal beta-actin (1:1000; Cell Signaling 3700S), mouse monoclonal anti-flag (1:500; Sigma-Aldrich F1804), mouse monoclonal anti-V5 (1µg/ml, Sigma-Aldrich V8012), mouse monoclonal anti-GAPDH (1µg/ml; Invitrogen 39-8600), chicken polyclonal anti-USP14 (5µg/ml; Lifesensors AB505), IRDye 680 or 800 anti-mouse or anti-chicken infrared secondary antibodies (1:10000; Li-Cor).

Data analysis. Ratios of the intensity readings for the protein of interest and the loading control were calculated in Microsoft Excel 2010 and plotted using GraphPad Prism 6.05.

Results

A key experiment from Lee *et al.*, 2010, (Figure 1g) showed that recombinantly expressed tau or TDP-43 levels in HEK293 cells were higher when coexpressed with wild type as compared to catalytically inactive (C114A) USP14. We cotransfected V5-pTT5d-USP14 or V5-pTT5d-USP14 (C114A) plasmids (ranging from 0.5 to 2µg) and 2µg pTT5d-Tau or pTT5d-Flag-TDP-43 plasmids in HEK293 cells. Note that we used a pTT5d vector to express proteins, while Finley and colleagues used a pcDNA3.1 vector (Invitrogen). Despite robust expression of USP14 or the catalytically inactive mutant as detected by anti-V5 antibody

(Figure 1A, C), no decrease was observed in the levels of tau (Figure 1A, B) or TDP-43 (Figure 1C, D) in cells transfected with the catalytically inactive mutant compared to wild type USP14. These experiments were repeated twice with similar results.

To exclude the possibility that the V5-tag rendered the USP14 constructs non-functional, we validated an anti-USP14 antibody (Supplementary material) and tested untagged USP14 constructs in TDP-43 overexpressing cells. HEK293 cells were transfected with USP14 or USP14(C114A) plasmids at concentrations ranging from 31ng to 4μg and tau and Flag-TDP-43 at concentrations of 2 or 4μg (3 independent experiments were run for tau and Flag-TDP-43 each); representative blots are shown in Figure 2. Despite robust expression of USP14 or its catalytically inactive mutant as detected by the USP14 antibody, no decrease was observed in tau or Flag-TDP-43 protein levels in cells transfected with the catalytically inactive mutant compared to wild type USP14 (Figure 2A, B).

Because there was a possibility that even the untagged-USP14 constructs were not functional, we tested whether siRNA knock down of endogenous USP14 would increase turnover of substrate. Lee *et al.* (2010) showed that *Usp14*$^{-/-}$ mouse embryonic fibroblasts had lower levels of tau or TDP-43 than those overexpressing wild-type USP14. Therefore, we reasoned that USP14 knockdown should result in lower levels of substrate. To avoid variability resulting from transient transfections, we tested USP14 knockdown in a stable Flag-tagged α-synuclein U2OS cell line. As shown in Figure 3, four different siRNAs (A58, A59, A60 and A90; 100nM) caused a 50–75% decrease in endogenous USP14 protein levels at 60 or 96 hours post-transfection (Figure 3A, B). No changes in Flag-α-synuclein were detected (Figure 3A, C).

Finally, to eliminate the concern that the artificial levels of the transiently or stably overexpressed substrates caused the lack of effect, we repeated the siRNA knockdown experiment in SH-SY5Y cells that endogenously express tau using siRNAs from Ambion (A58, A59, A60 and A90; 100nM) in two independent experiments. As shown in a representative western blot in Figure 4, no changes in endogenous tau levels were observed despite a 50–75% knockdown of endogenous USP14 protein levels.

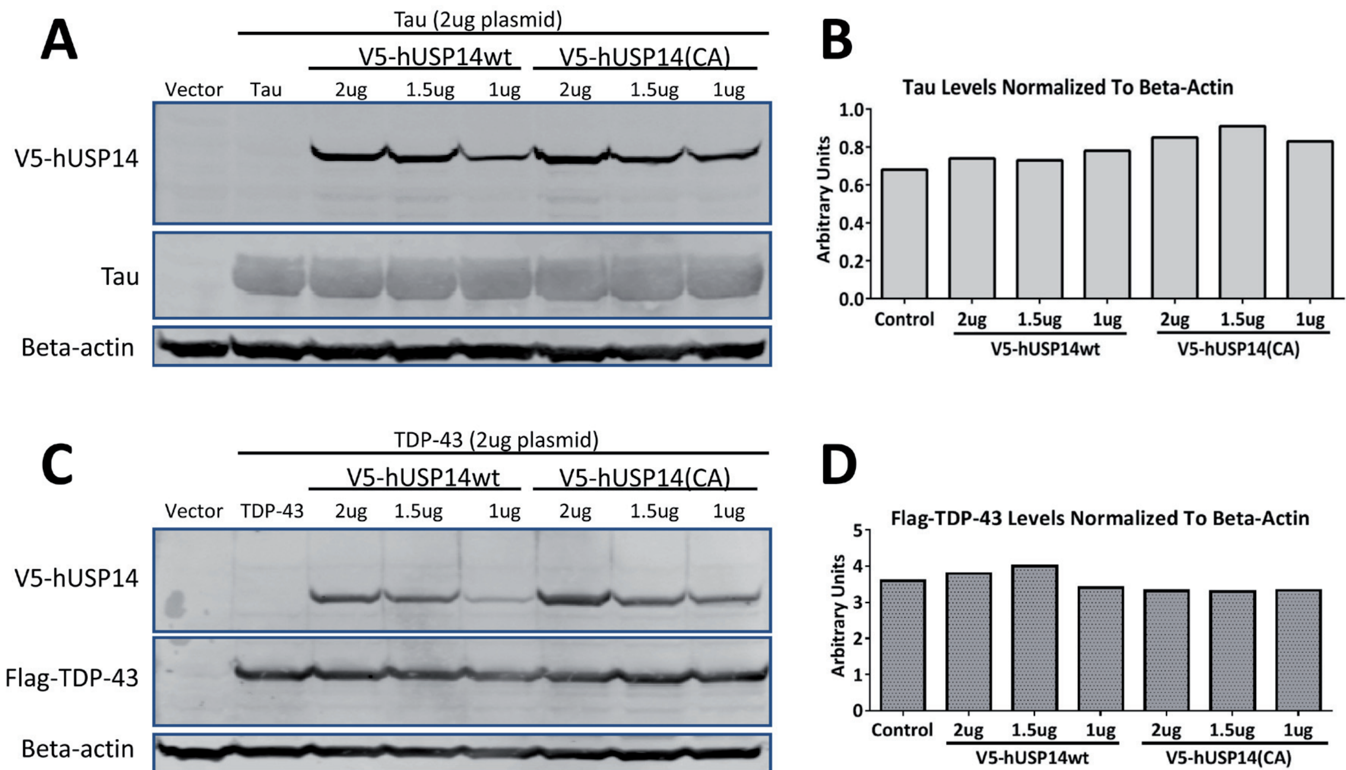

Figure 1. Tau and TDP-43 levels were not increased when coexpressed with V5-tagged hUSP14wt versus V5-tagged hUSP14(CA). 1, 1.5 or 2ug of V5-hUSP14wt (wt = wild type) or V5-hUSP14(CA) (CA = C114A, catalytically inactive) were cotransfected with 2ug Tau or Flag-TDP-43 plasmid in HEK293 cells. Cells were lysed after 48 hours and analyzed by western blot using a standard protocol. Actin served as loading control. Despite robust expression of USP14 or its catalytically inactive mutant as detected by the V5-tag (**A**, **C**), no differences were observed in Tau (**A**, **B**) or Flag-TDP-43 (**C**, **D**) protein levels. Note that we did not observe differences in the expression levels of USP14 versus USP14(CA). Control = empty vector control.

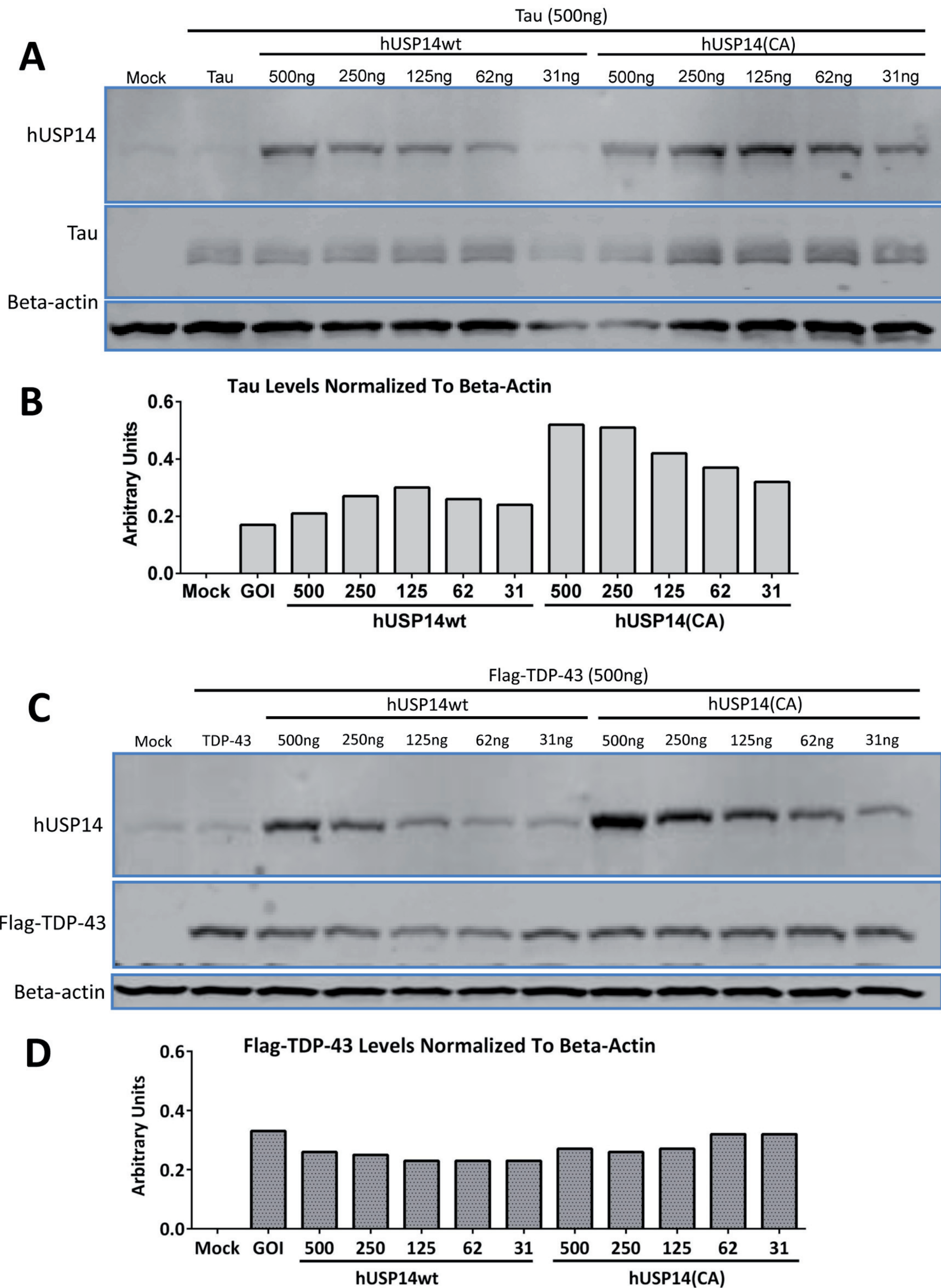

Figure 2. No decreases observed in tau or TDP-43 levels after cotransfection with untagged hUSP14(CA) versus untagged hUSP14wt. 31 to 500ng of hUSP14wt or hUSP14(CA) plasmids were cotransfected with 2ug tau or TDP-43 plasmid in HEK293 cells. Cells were lysed after 48 hours and analyzed by western blot using a standard protocol. Actin served as loading control. Despite robust expression of USP14 or the catalytically inactive mutant as detected by anti-USP14 antibody (**A**, **C**), no decreases were observed in tau (**A**, **B**) or TDP-43 (**C**, **D**) protein levels in the cells transfected with hUSP14CA. Note that we did not observe differences in the expression levels of USP14 versus USP14(CA). Mock = empty vector control, GOI = gene of interest and refers to either tau or TDP-43 in the absence of USP14 cotransfection.

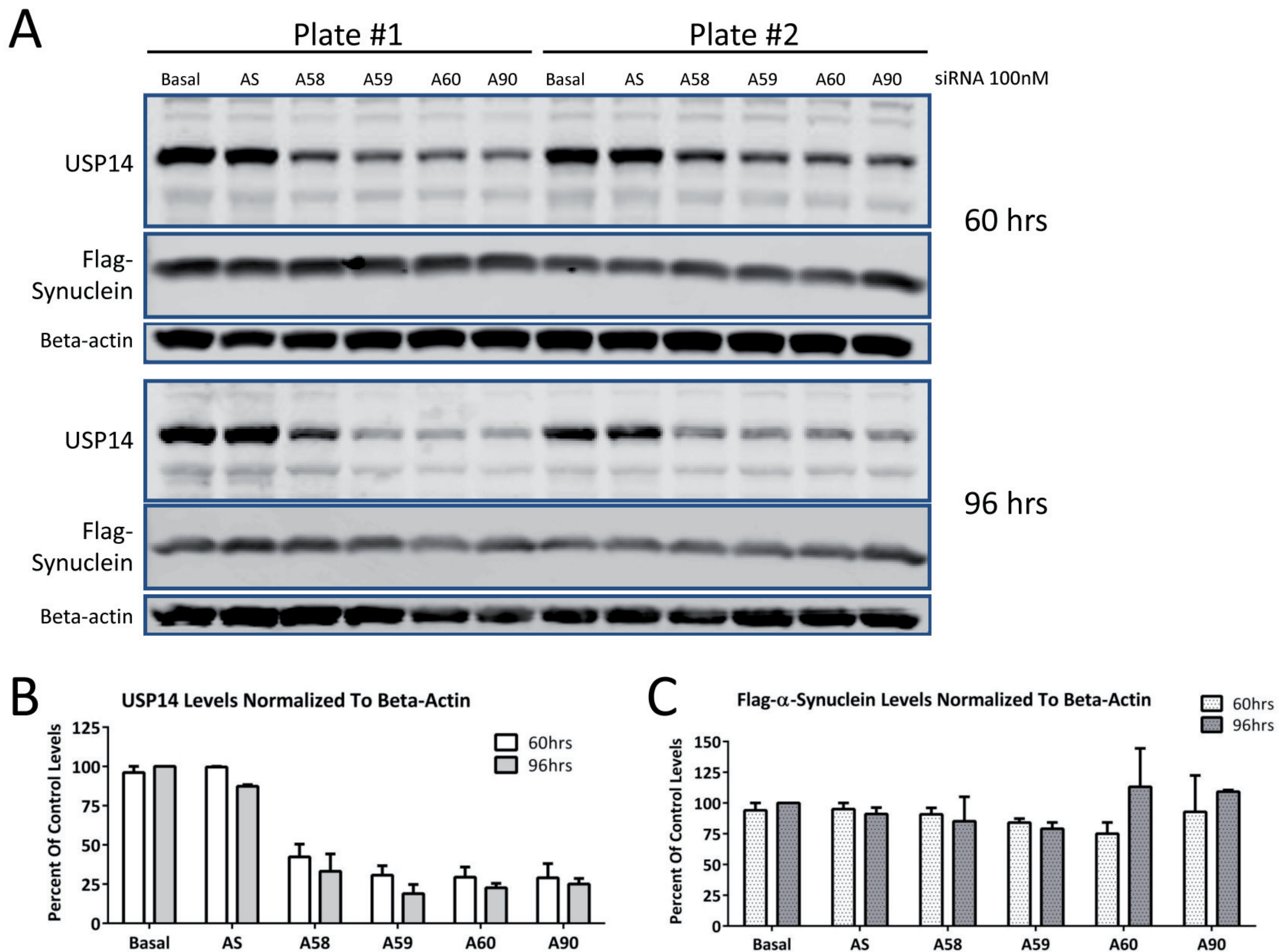

Figure 3. siRNA knockdown of endogenous USP14 does not decrease α-synuclein levels in U2OS cells stably expressing α-synuclein. U2OS cells stably expressing Flag-α-synuclein were treated with 100nM USP14 siRNA from Ambion (A58, A59, A60 or A90) for 60 or 96 hours (**A**). Scrambled siRNA (AS) served as control for the specificity of the siRNA knockdown. Despite 50–75% knockdown of basal USP14 protein levels (**B**), no changes in Flag-α-synuclein expression were detected (**C**).

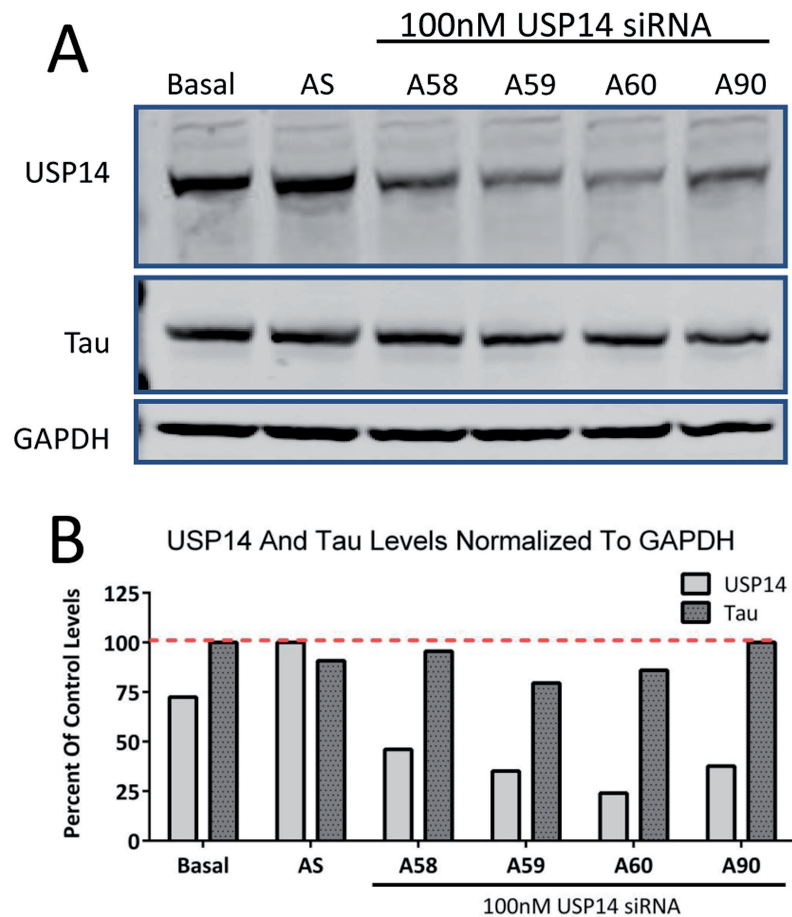

Figure 4. siRNA knockdown of endogenous USP14 does not decrease levels of endogenous tau in SH-SY5Y cells. SH-SY5Y cells endogenously expressing tau were transfected with 100nM USP14 siRNAs from Ambion (A58, A59, A60 or A90) or scrambled siRNA (AS) for 72 hours. Cells were lysed and analyzed by western blot using a standard protocol. A 50–75% decrease in USP14 protein levels was achieved compared to scrambled control, but no change in basal tau protein levels (**A**, **B**).

Conclusions

Though we took several different approaches to assay the effects of USP14 on substrate levels, we were unable to confirm a robust role for USP14 in tau or TDP-43 degradation in our experimental systems. The possibility remains that differences in our methods (such as using a different expression vector) caused the discrepancies between our data and those in Lee *et al.* (2010). After these data in immortalized cell lines were generated, Wilson and colleagues published *in vivo* data from USP14-deficient ax^J mice. They found no changes in endogenous tau or ataxin-3 protein levels, but did observe a difference in phosphorylated tau (Jin *et al.*, 2012). They also generated mice expressing catalytically inactive USP14 and could not detect altered proteasomal function in these mice, although tau levels were not analyzed (Vaden *et al.*, 2015). However, whether pharmacological or genetic inhibition of USP14 could improve degradation of aggregate-prone proteins in a disease state is still unknown. We hope our findings serve as a starting point for further discussion, collaboration, and research in this field.

Data availability

Open Science Framework: Dataset: Does inactivation of USP14 enhance degradation of proteasomal substrates that are associated with neurodegenerative diseases?, doi 10.17605/OSF.IO/8HWUB (Ortuno *et al.*, 2016).

Author contributions

DO conducted all experiments. DO, HC and SM conceived of the experimental design. HC and SM wrote the article.

Competing interests

All authors were full-time employees at Amgen Inc. at the time the experiments were conducted.

Grant information

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Acknowledgements

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Supplementary material

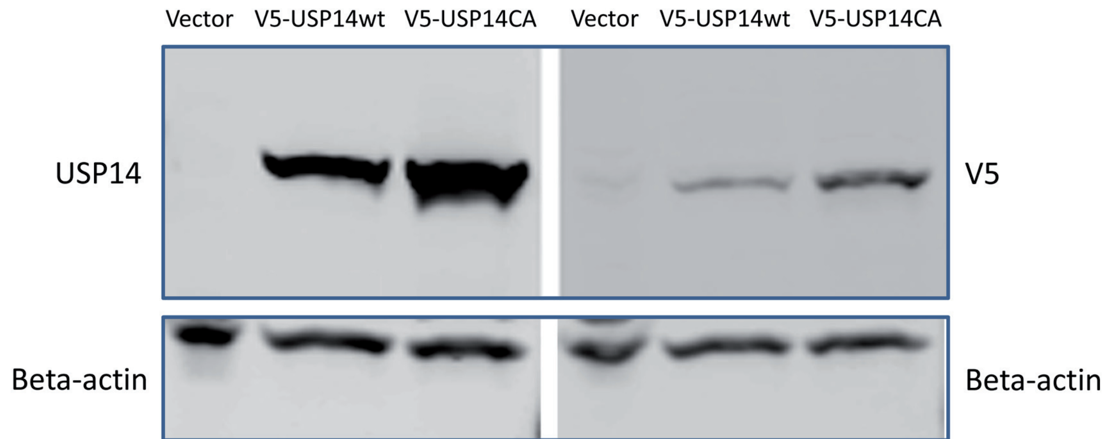

Figure S1. Validation of chicken polyclonal USP14 antibodies. 1µg V5-tagged USP14, V5-tagged USP14(CA) or empty vector control constructs were transfected in HEK293 cells and probed with V5 or chicken polyclonal anti-USP14 antibodies. Beta-actin served as loading control.

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Current Referee Status:

Version 1

Referee Report 14 March 2016

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Ubiquitin-dependent protein degradation involves the assembly of ubiquitin chains on specific proteins followed by their recognition and subsequent degradation by the proteasome. Since ubiquitination is a reversible reaction, there has been great interest in understanding the role of protein deubiquitination as a mechanism to regulate protein degradation. This is particularly true in post-mitotic neurons where lowering the burden of ubiquitinated aggregates of tau, Htt, alpha-synuclein and TDP-43 observed in Alzheimer's Disease, Huntington's Disease, Parkinson's Disease and Amyotropic lateral sclerosis, respectively, is an attractive therapeutic intervention for the treatment of these diseases.

Ubiquitinated proteins stably associate with the proteasome through their interaction with proteasomal ubiquitin binding proteins. Following binding, and prior to degradation by the proteasome, ubiquitinated proteins are stripped of their ubiquitin tag. The disassembly of ubiquitin chains by proteasomal deubiquitinating enzymes serves multiple functions, including maintaining ubiquitin pools and determining whether a substrate will be released or degraded by the proteasome. The ubiquitin-specific protease 14 (USP14) is a proteasome-associated deubiquitinating enzyme that is required to maintain ubiquitin levels by preventing conjugated ubiquitin from entering the proteasome. Previous studies have also indicated that either a pharmacological block or genetic inactivation of USP14's ubiquitin-hydrolase activity can reduce the steady-state levels of overexpressed aggregate-prone proteins tau, TDP-43 and ataxin-3 in immortalized cell lines. These findings suggested that blocking USP14's deubiquitinating activity would lead to enhanced degradation of ubiquitinated substrates by preventing the substrates from being released by the proteasome prior to their commitment to degradation.

Studies in mice provide support for an essential role for USP14 in controlling ubiquitin pools. Analysis of USP14-deficient mice revealed a significant loss of ubiquitin in multiple tissues, including the brain, and even greater loss of ubiquitin at synaptic terminals. Restoration of ubiquitin pools in these mice restored some of the synaptic transmission deficits caused by the loss of USP14, indicating a requirement for USP14 in ubiquitin homeostasis. Contrary to what was observed in immortalized cell lines, there was no detectable change in the steady state levels of the aggregate-prone proteins tau and ataxin-3 in the USP14-deficient mice. However, increased levels of phosphorylated tau were observed in the USP14-deficient mice and correlated with elevated levels of activated JNK, ERK and AKT. While USP14 still remains an interesting target for therapeutic intervention in protein-aggregate diseases, its role in controlling the degradation of specific proteins is not clear.

This study by Ortuno *et. al.* aims to further investigate a role for USP14 in controlling proteasomal

degradation of aggregate-prone proteins. To do this, the authors first investigated if either overexpression of wild type USP14 or ubiquitin-hydrolase inactive USP14 would alter the levels of transfected tau or alpha-synuclein in an HEK293 cell line. If USP14 acts as an inhibitor of protein degradation, then overexpression of wild type USP14 should lead to increased levels of these aggregate-prone proteins while overexpression of ubiquitin-hydrolase inactive USP14 should reduce their levels. However, increasing either wild type or ubiquitin hydrolase inactive USP14 did not result in a detectable change in the steady-state levels of tau or alpha-synuclein.The authors then investigated if lowering the levels of USP14 in the neuronal SHSY5Y cell line, which expresses endogenous tau, would result in decreased tau levels. Although the authors were able to significantly decrease the expression of USP14, they did not observe any significant change in the level of endogenous tau. The authors were therefore unable to confirm a role for USP14 in controlling the degradation of aggregate-prone proteins.

The title is appropriate and the abstract provides a suitable summary. The experiments conducted in this study all generated high quality data and included appropriate controls. The authors provided a reasonable conclusion and potential reasons for differences between their results and those previously reported on USP14.

Concerns:

1. The entire premise of this paper is based on the manipulation of proteasome-bound USP14. Unfortunately, the authors did not determine the level of proteasome-bound USP14. This is particularly important for the transfection of USP14(CA) and the *Usp14* siRNA knockdown experiments. If proteasome-bound USP14 has a long half-life, then these manipulations may not have significantly displaced endogenous USP14 from the proteasome.

2. The steady state level of any protein depends on the rates of synthesis and degradation. However, the measurements in this report did not take into account either of these variables. While highly unlikely, changes in protein turnover due to manipulation of USP14 may have caused increased turnover of tau, alpha-synuclein or TDP-43 with a corresponding increase in synthesis, resulting in no change in protein abundance.

3. There are no error bars in figures 1, 2 and 4.

4. It is not clear if the quantitations represent averages from replicate immunoblots or if a single blot was performed for each experiment.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Competing Interests: No competing interests were disclosed.

Referee Report 09 March 2016

doi:10.5256/f1000research.8397.r12829

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This study tests the previously published assertion that reducing the activity of USP14, a proteasome-associated deubiqtuitylase, results in decreased levels of neurotoxic proteins such as TDP43 and Tau. The bottom line of the study is that manipulation of USP14 level and activity in a cell model system has no discernable effect of the levels of these proteins, contrary to expectations based on a previously published 2010 study[1]. While this is obviously a model system with unknown relevance to *bona fide* neurons *in vivo*, the experiments appear to be well done and the data support the conclusions. The authors are careful to point out that there are minor differences between some of their protocols and those used in the 2010 study and call for increased communication and collaboration between interested laboratories to determine if USP14 is truly a good drug target for neurodegenerative diseases. This is entirely appropriate.

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I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Referee Response (*Member of the F1000 Faculty*) 11 Mar 2016

Thomas Kodadek, Department of Chemistry, Scripps Florida Research Institute, USA

Since posting my review of this F1000Research article, a colleague made me aware of two manuscripts that are highly relevant to this topic, but were not cited by Ortuno *et al.*

They are:

Homma T, Ishibashi D, Nakagaki T, *et al.*: Ubiquitin-specific protease 14 modulates degradation of cellular prion protein. *Sci Rep*. 2015; **5**:11028.

McKinnon C, Goold R, Andre R*, et al*.: Prion-mediated neurodegeneration is associated with early impairment of the ubiquitin-proteasome system. *Acta Neuropathol*. 2016; **131**(3): 411-425.

Both of these papers report that levels of prion proteins in neurons are strongly influenced by USP14 activity.

Thus, while the experiments conducted by Ortuno *et al*. reported in this communication do not seem to indicate that manipulation of USP14 has a major effect on TDP-43 and α-synuclein levels under their conditions, the major findings of the 2010 Nature paper by Lee *et al.* are strongly supported by these two studies. Thus, these papers should have been cited by Ortuno *et al*. in their F1000Research article. I apologize for not being aware of these two studies when I reviewed this work.

Competing Interests: No competing interestsNo competing interests

Referee Report 07 March 2016

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Many adult-onset neurodegenerative diseases are associated with aggregates of misfolded proteins or peptides. A number of groups have proposed that those aggregates that are intracellular, such as tau, synuclein and TDP-43, may result from defects in the protein-degradation pathways like the proteasome which slows normal protein turnover. Such proposals lead naturally to the idea that enhancing endogenous protein degradation pathways is a potential therapeutic strategy to reduce aggregate levels, thereby slowing or blocking disease progression. This manuscript focuses on USP14, a deubiquinating enzyme associated with the proteasome that catalyzes the release of ubiquitin for proteins targeted for degradation and allow the ubiquitin to be recycled for targeting other proteins to the proteasome. Recycling of ubiquitin is particularly important in domains far removed from sites where newly synthesized ubiquitin is available. The need to transport ubiquitin to synaptic terminals is an obvious example. Studies in a mouse model deficient in USP14 found reduced tissue ubiquitin levels in all tissues with a particularly significant loss in synaptic terminals and there is evidence of altered synaptic transmission in these mice. Curiously, there were no obvious accumulations of specific proteins or increased aggregates in brains noted in descriptions of this USP14-deficient mouse, despite its putative role in proteasome function. Subsequent studies failed to show a difference in endogenous levels of tau in the USP14-deficient mouse and a second mouse line expressing a catalytically dead USP14 did not find altered proteasomal activity.

Understanding the role of USP14 in clearance of pathogenic proteins is complicated by the fact that different proteins may involve different actions of USP14. For example, degradation of some proteins is normal in the presence of catalytically dead USP14, while others require catalytic activity. Tau and TDP-43 were both reported to be in the latter category as recombinant proteins accumulated to a higher level when expressed with wild type, but not with catalytically inactive USP14. This finding was the basis for suggestions that inhibition of USP14 might enhance clearance of these proteins.

Experiments described in this report sought to further characterize the ability of USP14 to modulate the clearance of tau, TDP-43 and α-synuclein. Unfortunately, increased levels of either wild type or catalytically inactive USP14 had no effect on levels of tau or TDP-43 and siRNA knockdown of endogenous USP14 failed to affect cellular levels of α-synuclein or alter endogenous expression of tau protein in a differentiated neuroblastoma cell line. A variety of different approaches to alter levels of USP14 failed to confirm the previous reports of altered clearance. The experiments are carefully documented and well controlled, suggesting that USP14 does not play a role in modulating the clearance of these proteins, consistent with the mouse studies. The conclusion is that inhibition of USP14 is not a promising therapeutic target for enhancing clearance of pathogenic proteins in adult-onset neurodegeneration. Although these cell-based assays make a strong case for this conclusion, data from the mouse models were never consistent with this proposal, since they showed no obvious evidence of proteasomal dysfunction or reduced tau levels. Indeed, given the fact that loss of USP14 catalytic activity in the mouse led to defects in synaptic transmission, it is hard to see how inhibition of USP14 was a plausible therapeutic strategy.

Minor Points.

1. The quantitative data in figures 1 and 2 are expressed as being normalized to beta actin levels. No indication is given as to the number of replicates or whether any statistical analysis was done. The raw data is shown as immunoblots with epitope tags, so the conclusions appear justifiable.

Nevertheless, the number of experimental replicates must be given and the case would be more compelling with statistics.

2. Technically, the bar graphs show ratios, which are dimensionless, not arbitrary units.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Competing Interests: No competing interests were disclosed.