TRAF6 promotes atypical ubiquitination of mutant DJ-1 and alpha-synuclein and is localized to Lewy bodies in sporadic Parkinson’s disease brains

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INTRODUCTION

Parkinson’s disease (PD) is a neurodegenerative disorder characterized by loss of dopaminergic neurons in the Substantia Nigra and the formation of ubiquitin- and alpha-synuclein (aSYN)-positive cytoplasmic inclusions called Lewy bodies (LBs). Although most PD cases are sporadic, families with genetic mutations have been found. Mutations in PARK7/DJ-1 have been associated with autosomal recessive early-onset PD, while missense mutations or duplications of aSYN (PARK1, PARK4) have been linked to dominant forms of the disease. In this study, we identify the E3 ubiquitin ligase tumor necrosis factor-receptor associated factor 6 (TRAF6) as a common player in genetic and sporadic cases. TRAF6 binds misfolded mutant DJ-1 and aSYN. Both proteins are substrates of TRAF6 ligase activity in vivo. Interestingly, rather than conventional K63 assembly, TRAF6 promotes atypical ubiquitin linkage formation to both PD targets that share K6-, K27- and K29- mediated ubiquitination. Importantly, TRAF6 stimulates the accumulation of insoluble and polyubiquitinated mutant DJ-1 into cytoplasmic aggregates. In human post-mortem brains of PD patients, TRAF6 protein colocalizes with aSYN in LBs. These results reveal a novel role for TRAF6 and for atypical ubiquitination in PD pathogenesis.

Parkinson’s disease (PD) is one of the most common neurodegenerative disorders. It is characterized by loss of dopaminergic (DA) neurons in the Substantia Nigra (SN) and the presence of cytoplasmic inclusions called Lewy bodies (LBs) (1). Most PD cases are sporadic, but a minority of forms is associated to familial transmission. Among them, PARK7/DJ-1 has been linked to autosomal recessive PD, whereas PARK1/PARK4/alpha-synuclein (aSYN) is found in dominant forms. aSYN is a major component of LBs providing a molecular link between sporadic and genetic cases. The accumulation of misfolded proteins into cellular aggregates is a prominent feature common to most neurodegenerative diseases. These insoluble proteinaceous deposits contain ubiquitin and components of the ubiquitin–proteasome system, including those encoded by genes mutated in familiar cases, like the ubiquitin ligase parkin/PARK2 and ubiquitin C-terminal hydrolase-L1 (UCH-L1/PARK5) (2–5). These data suggest that cellular handling of misfolded and aggregation-prone proteins play a central role in PD pathogenesis. Substrate ubiquitination is a signal for diverse cellular functions. Polyubiquitin chains can be formed through covalent conjugation using any of the seven lysines present in the ubiquitin moiety. Ubiquitination through K48 generally targets proteins for degradation, whereas K63 linkage plays a role in signaling and protein trafficking (6). Recent data indicate...

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that degradative and nondegradative functions of protein ubiquitination can be also associated with atypical chain formation. All lysine residues, with the exception of K63, accumulate in response to proteasome inhibition (7). K11 has been implicated in endoplasmic reticulum-associated degradation (7), while K27 and K29 appear to be connected to lysosomal localization and degradation (8,9). K6, K27, K29 and K33 have also been proven to have nondegradative functions on selected substrates (10–12).

We previously showed that tumor necrosis factor-receptor associated factor 6 (TRAF6)-binding protein TRAF- and TNF-receptor-associated protein (TTRAP) (13) is a novel interactor of DJ-1 able to bind the PD-associated mutant L166P more strongly than wild type (wt) (14). TRAF6 is an E3 ubiquitin ligase that promotes K63-specific chain assembly in the signal transduction pathway that ultimately leads to nuclear factor-kB (NFkB) activation (15). Although in the immune system it has an essential role in tumor necrosis factor and interleukin-1/Toll-like receptors signaling, in the brain, TRAF6 activity has been associated with the transduction cascade of the neurotrophins receptors p75 and TrkA (16–18). So far, the only evidence that supports a role for TRAF6 in neurodegeneration has been limited to its colocalization with tau in brains of Alzheimer’s disease (AD) patients (19).

In the current study, we provide evidence that TRAF6 interacts with and ubiquinates mutant DJ-1 and aSYN. Unexpectedly, TRAF6 promotes an atypical mode of polyubiquitin chain formation onto its PD-associated target proteins that share K6, K27 and K29 ubiquitination. We also show that TRAF6-mediated ubiquitination stimulates the accumulation of mutant DJ-1 into insoluble aggregates. In post-mortem brains of sporadic PD patients, TRAF6 is present in LBs.

Altogether, our data imply a novel role for TRAF6 in mediating atypical ubiquitination of proteins relevant for sporadic and familial PD.

RESULTS

TRAF6 binds to and ubiquinates misfolded mutant DJ-1

We have previously shown that TTRAP is a potent interactor of PD-associated mutant DJ-1, with misfolded L166P being the stronger partner (14). Since TTRAP was originally identified as a TRAF-interacting protein with highest selectivity for TRAF6 (13), we analyzed whether TRAF6 itself could associate with DJ-1.

We performed coimmunoprecipitation experiments using HEK cells transfected with HA–TRAF6 and FLAG–DJ-1 wt or PD-linked L166P mutant. Although protein levels were significantly lower than those of wt protein, misfolded DJ-1 mutant could specifically associate with TRAF6 in untreated conditions (Fig. 1A). Since proteasome inhibition stabilizes L166P levels, we then analyzed the binding capabilities of wt and mutant proteins in conditions of proteasome block. Coimmunoprecipitation in cells treated with MG132 revealed an enhanced binding of TRAF6 to L166P and confirmed not detectable binding with wt DJ-1, proving that TRAF6 is able to discriminate misfolded mutant DJ-1. Interaction data were confirmed by reverse immunoprecipitation with HA–TRAF6 and FLAG–DJ-1 (Fig. 1B) as well as by

the use of different protein tags (Fig. 1C). To identify which portion of TRAF6 binds to misfolded DJ-1, we expressed wt and mutant form of TRAF6 lacking the N-terminal RING domain in HEK cells and verified binding to L166P. Similar to other TRAF6-binding proteins (16), the C-terminal TRAF domain was sufficient to mediate its association with mutant DJ-1 (Fig. 1D). Together, our data indicate that TRAF6 binds misfolded mutant DJ-1, but not wt protein.

Since TRAF6 is an E3 ubiquitin ligase, we investigated whether mutant DJ-1 might be substrate of TRAF6 activity. To test this hypothesis, we performed in vivo ubiquitination assays. HEK cells were transfected with MYC–DJ-1 wt or L166P with HA–ubiquitin in the presence or absence of FLAG–TRAF6. Controls were included with HA–ubiquitin alone or with FLAG–TRAF6 to avoid artifacts due to a general increase in polyubiquitination. We found that, in the absence of exogenous TRAF6, both wt and mutant DJ-1 could be ubiquitinated, even though at very low levels (Fig. 2A). Overexpression of TRAF6 had no effect on the ubiquitination of wt DJ-1, but significantly enhanced ubiquitination of L166P, as expected from their interaction pattern. Mutant DJ-1 was mainly observed in the polyubiquitinated form. To provide further support on the role of TRAF6 in mediating misfolded DJ-1 ubiquitination, we performed in vivo ubiquitination assays with a form of TRAF6 lacking the N-terminal ubiquitin ligase RING domain (DN) (Supplementary Material, Fig. S1A). The effects on wt DJ-1 were used as internal control. We found that overexpression of TRAF6–DN abolished L166P ubiquitination (Fig. 2B). Therefore, E3 ligase activity of TRAF6 is required for mutant DJ-1 polyubiquitination.

To examine the specificity of TRAF6-mediated effects on L166P, we compared TRA6 activity with that of TRAF2, another E3 ligase that belongs to the TRAF family. MYC–L166P was transfected with HA–ubiquitin and FLAG–TRAF2 wt or DN mutant. FLAG–TRAF6 wt and DN were included in the same experiment as internal reference. The amount of polyubiquitinated L166P by TRAF2 was much less than by TRAF6 (Fig. 2C). These results indicate that the effects of TRAF6 are specific, but not exclusive.

TRAF6-mediated ubiquitination of L166P involves atypical chain linkage

E3 ligases can promote the formation of polyubiquitin chains on target substrates through any of the lysines present on ubiquitin molecules. TRAF6 activity has been widely associated with chain formation mainly via K63-specific linkage. Therefore, we investigated whether K63 or canonical K48 linkages were used for mutant DJ-1 ubiquitination by TRAF6. We used ubiquitin mutants in which either of these residues was substituted with an arginine (K48R and K63R). An ubiquitin mutant with all lysines substituted with arginine was also included as negative control (K0). HEK cells were thus transfected with MYC–L166P with HA–wt or mutant ubiquitin in the presence of FLAG–TRAF6. As expected, a robust polyubiquitination of L166P with wt ubiquitin was promoted by TRAF6 overexpression, while it was abolished in the presence of K0 mutant. Strikingly, we found that TRAF6-mediated ubiquitination of L166P was maintained with K48R and K63R
mutants (Fig. 3A). We then generated ubiquitin mutants with only one lysine available for polymerization. Consistent with the data obtained with K48R and K63R, neither K63 nor K48 were used by TRAF6 for mutant DJ-1 ubiquitination. Instead, TRAF6 promoted a robust polyubiquitination of L166P in the presence of K27 and K29 mutants. Weaker but reproducible ubiquitination was also induced with K6 and K33 ubiquitins. Background signals were observed with K0 (Fig. 3B).

Altogether, our data indicate that TRAF6 promotes atypical polyubiquitination of misfolded mutant DJ-1 by using K6, K27, K29 and K33 as isotype linkages.

TRAF6 ubiquitination promotes the accumulation of mutant DJ-1 into insoluble aggregates

One fundamental question was to determine whether TRAF6-mediated atypical ubiquitination of L166P might trigger its degradation. Human neuroblastoma SH–SY5Y cells stably expressing FLAG–L166P were transfected with FLAG–TRAF6 wt or DN, and the steady-state levels of misfolded DJ-1 were followed by western blot. TRAF6 had no effect on L166P protein levels, indicating that its activity has a non-degradative role (Supplementary Material, Fig. S2A). Similarly, pulse chase experiments with cyclohexamide showed no evident effects of TRAF6 ubiquitination on L166P stability (Supplementary Material, Fig. S2B). Since nondegradative ubiquitination has been previously associated with altered solubility of PD-associated aSYN and synphilin-1 (20,21), we next investigated whether TRAF6-mediated ubiquitination of L166P might have an impact on its biochemical status. MYC–L166P and HA–ubiquitin were co-expressed in HEK cells in the presence or absence of FLAG–TRAF6 wt or DN. Lysates were separated into Triton X-100 soluble and insoluble fractions. We found that nonubiquitinated forms of L166P were distributed in both fractions and TRAF6 had no effect on total levels of soluble mutant DJ-1. In contrast, polyubiquitinated misfolded DJ-1
was present only in Triton-insoluble fraction (Fig. 4A). Consistent with our in vivo ubiquitination data, we observed a potent increase in the amount of ubiquitinated L166P only when TRAF6 wt, but not DN, was overexpressed.

To assess whether the increased insolubility of L166P induced by TRAF6 is associated with a propensity to form protein aggregates, we monitored the effects of TRAF6 overexpression on L166P inclusion formation. We created wt–TRAF6 and DN–TRAF6 fused to GFP (Supplementary Material, Fig. S1B) and used these constructs with FLAG–L166P and HA–ubiquitin. The formation of ubiquitin- and L166P-positive aggregates was followed by double immunofluorescence coupled with GFP autofluorescence. To detect only insoluble inclusions, a permeabilization protocol was performed (14). L166P showed a diffused pattern when expressed alone or with ubiquitin (Fig. 4B). Addition of ligase-competent TRAF6 generated larger insoluble L166P-containing aggregates in >90% of the cells. These were positively stained for TRAF6 and ubiquitin. No aggregates were observed when TRAF6 DN was used, proving that inclusion
formation is a consequence of TRAF6 activity on mutant DJ-1. As expected, wt DJ-1 staining was not altered by ubiquitin or TRAF6 expression (Fig. 4B). To prove that TRAF6 action on L166P involved atypical ubiquitination, we carried out analogous experiments with ubiquitin mutants that were shown to be involved in L166P ubiquitination (K27 and K29). As controls, K0 was used as well as K11 and K48 ubiquitins. These are mutants that mediate poly-chain formation, but are not TRAF6 substrates. K27 and K29, but not K0, K11 and K48, were able to recapitulate the phenotype observed with wt ubiquitin, proving the specificity of TRAF6 activity (Fig. 4C). Experiments were also carried out with differentially tagged mutant DJ-1 and all the remaining ubiquitin mutants (Supplementary Material, Fig. S3). Together, these
Figure 4. TRAF6 promotes the accumulation of ubiquitinated mutant DJ-1 in insoluble aggregates. (A) HEK cells were transfected with HA–ubiquitin, MYC–L166P and FLAG–TRAF6 (wt or DN). After treatment with 10 μM MG132 for 3 h, Triton X-100 soluble and insoluble fractions were separated. Lysates were analyzed with anti-MYC, anti-HA, anti-FLAG and anti-actin antibodies. (B) Cells were transfected with HA–ubiquitin wt, FLAG–L166P and GFP–TRAF6 (wt or DN) and treated as in (A). Before fixation, cells were permeabilized with Triton X-100. Insoluble aggregates were analyzed by double immunofluorescence coupled with GFP autofluorescence. (C) HA–ubiquitin wt or K0, K11, K27, K29 and K48 mutants were used with FLAG–L166P and GFP–TRAF6 wt to follow insoluble aggregate formation. Double immunofluorescence coupled with GFP autofluorescence was performed as in (B) on permeabilized cells.
data provide evidence that TRAF6-mediated atypical ubiquination of DJ-1 L166P increases its propensity to accumulate into insoluble aggregates.

**TRAF6 binds to and ubiquinatates aSYN with atypical linkage formation**

It has been shown that the ubiquitin ligase seven in absentia homolog (SIAH)-1 binds to and ubiquinatates aSYN and aSYN-binding protein synphilin-1 (4). Structural studies proved that members of the SIAH family possess a domain highly similar to the TRAF-C region of TRAF proteins (22). To study a potential role of TRAF6 in PD sporadic cases, we analyzed the interplay between TRAF6 and aSYN. Coimmunoprecipitation experiments were carried out using MYC-aSYN and FLAG-TRAF6. Both wt and PD-associated A53T mutant aSYN constructs were tested. aSYN was found to specifically interact with TRAF6 (Fig. 5A). No preferential binding toward the wt or mutant form of aSYN could be observed, suggesting a potential role of TRAF6 in both sporadic and aSYN-linked genetic cases.

The interaction between TRAF6 and aSYN raised the possibility that aSYN might be an additional PD-associated target of TRAF6 ligase activity. We thus performed in vivo ubiquination assay with HA-ubiquitin and MYC-aSYN wt or A53T with or without FLAG-TRAF6 wt and DN. In this experiment, the level of aSYN polyubiquitinated species was significantly enhanced by TRAF6 overexpression (Fig. 5B). No accumulation of ubiquitinated aSYN was observed when TRAF6 DN was used. Both wt and A53T mutant were targets of TRAF6.

We then evaluated whether TRAF6 activity on aSYN might involve atypical ubiquitin chain formation (Fig. 5C). We thus performed ubiquination assay with all ubiquitin mutants. TRAF6 supported aSYN ubiquinatation mainly via K6, K27 and K29 chains. Importantly, these are shared motifs with misfolded mutant DJ-1.

**TRAF6 is present in LBs and accumulates in PD brains**

To gain further insights into the relevance of TRAF6 for PD pathogenesis, we analyzed the presence of endogenous TRAF6 in SN DA neurons in human post-mortem brains. As shown in Fig. 6A, TRAF6 is expressed in the cytoplasm of DA neurons, as identified by tyrosine hydroxylase (TH) staining. Some TRAF6 immunoreactivity was also found in non-DA neurons, proving a quite broad distribution in midbrain cell populations.

Several E3 ligases, including Parkin, carboxyl terminus of Hsc70-interaction protein (CHIP) and SIAH, have been shown to be part of LBs in surviving DA neurons in PD brains. Therefore, we examined human post-mortem brains of pathologically confirmed cases of sporadic PD. Experiments were performed on brain samples from two different patients. LBs were identified using an aSYN antibody. Interestingly, virtually all nigral LBs were immunoreactive for an anti-TRAF6 antibody in both individuals (Fig. 6B and Supplementary Material, Fig. S4). TRAF6 was expressed throughout the cytoplasm, but the protein was clearly accumulated at the border of LBs, a site where aSYN staining was more prominent. The specificity of TRAF6 accumulation in LBs was verified using three antibodies directed against N- or C-terminal epitopes of the protein (Supplementary Material, Fig. S4). No immunoreactivity was detected with unrelated IgG (data not shown).

In response to an excess burden of misfolded proteins, neurons tend to increase the expression of E3 ligases and downregulate the level of deubiquitinating enzymes as previously shown in PD post-mortem brains for Parkin and UCH-L1 (23–26). Therefore, we tested TRAF6 mRNA levels in sporadic PD (n = 6) and healthy control (n = 3) brains (27). RNA was extracted selectively from SN, the site of PD pathogenesis. Quantitative real-time polymerase chain reaction (qPCR) amplification showed that the amount of TRAF6 mRNA was statistically increased in sporadic PD samples when compared with normal controls (P < 0.02), with an average 1.7-fold upregulation (Fig. 6C).

To strengthen the significance of the increased amount of TRAF6 expression in PD, we also checked for the status of regulators of TRAF6 activity. By qPCR analysis, we found no significant differences in the expression of p62, CYLD and A20 between PD and control samples (Supplementary Material, Fig. S5). Overall, our results show that the ubiquitin ligase TRAF6 is present in DA neurons in the midbrain of both normal and PD brains. In sporadic patients, TRAF6 colocalizes with aSYN within LBs and the amount of its mRNA seems increased in SN.

**DISCUSSION**

The description of the molecular components of LBs and the discovery of new players in the recruitment of these molecules to aggregates are important for the understanding of the molecular mechanisms of PD and for the identification of new drug targets. More than 76 proteins have been described so far as localized in LBs (28–30) and the significance of their relocalization may depend on protein identity.

Ubiquitin ligases are responsible for the formation of polyubiquitin chains in substrate proteins and are frequently associated with neurodegenerative diseases for their presence in intracellular aggregates and their role in aggregate formation. The E3 ligases Parkin, CHIP and SIAH have been found to accumulate in LBs of PD patients (2,4,5). While their enzymatic activities may be important for protein recruitment into the aggregates, their sequestration may decrease the quantity of soluble, active enzymes inducing a ‘loss of function’ phenotype for the lack of appropriate physiological ubiquitination of targets. Interestingly, they ubiquitinate misfolded L166P DJ-1 and aSYN with degradative or nondegradative effects on either of PD substrates (4,5,21,31).

Structural studies proved that members of the SIAH family possess a domain highly similar to the TRAF-C region of TRAF proteins (22). In this study, we demonstrate that the ubiquitin E3 ligase TRAF6 is a component of LBs. Virtually all LBs in the post-mortem brains of two individuals were positive for TRAF6. Three different antibodies against both the N- and C-terminals of the protein have been used.

TRAF6 ligase activity has been extensively characterized in the context of NFkB activation (15). In the brain, TRAF6 has
been previously linked to K63-specific polyubiquitination of AD-associated protein tau (19) and in nerve growth factor-receptor signaling (18). Most importantly, TRAF6 localizes in tau-containing, intracellular aggregates in AD post-mortem brains, suggesting that TRAF6 sequestration may be a common mechanism in neurodegeneration (19).

In post-mortem brains of non-PD individuals, we show that TRAF6 protein is expressed in DA cells of SN. In the mouse brain, TRAF6 mRNA is present at comparable levels in DA cells from SN and Ventral Tegmental Area, a site that is spared in PD (32). This is consistent with the hypothesis that TRAF6 expression per se is not related to cellular vulnerability.

TRAF6 interacts with mutant L166P DJ-1 and aSYN to promote their ubiquitination via atypical ubiquitin chain formation involving residues K6, K27, K29 and K33. Surprisingly, none of the PD targets that we tested were modified via TRAF6 canonical activity with K63 specificity. Nonconventional ubiquitination of L166P is sufficient for its accumulation into insoluble inclusions.

In a quantitative proteomic analysis of atypical ubiquitin chains in yeast, substrates for K6, K27, K29 and K33 have been shown to accumulate upon proteasome block, although to a lower extent than for K48, suggesting their potential role in protein degradation (7). Interestingly, these four ubiquitin moieties showed some functional redundancy. Unfortunately, the low cellular concentrations of their substrates have limited so far their identification in yeast or in ex vivo mammalian samples (7).

TRAF6 substrate L166P DJ-1 is very unstable and its expression level, both in transfection studies and in patient lymphoblasts, is lower than wt (33). This suggests that L166P mutation may induce a loss of DJ-1 function. However, L166P disrupts DJ-1 protein conformation resulting in aSYN aggregation and ubiquitination.

Figure 5. TRAF6 binds aSYN and enhances its ubiquitination with atypical chains. (A) HEK cells were transfected with FLAG–TRAF6 and MYC–aSYN wt or A53T (mut). Lysates were immunoprecipitated with anti-FLAG agarose beads and bound proteins were revealed with anti-MYC and anti-FLAG antibodies. Inputs were tested for the expression of TRAF6 and aSYN proteins. (B) Cells were transfected with HA–ubiquitin, MYC–aSYN (wt or A53T) and FLAG–TRAF6 (wt or DN). For in vivo ubiquitination assay, lysates were immunoprecipitated with anti-MYC antibody. Immunoprecipitates and lysates were analyzed with anti-MYC, anti-FLAG and anti-HA antibodies. (C) HA–ubiquitin mutants in which only the indicated lysine residue is available for chain formation were used. Wt and K0 ubiquitin were included as controls. In vivo ubiquitination of aSYN wt was performed as in (B).
in a misfolded protein that is prone to assemble into multimeric structures (34–36). Indeed, by gel filtration and other assays, L166P has been previously shown to be present mostly as a high-molecular-weight complex that may contain either DJ-1 oligomers and/or aggregates with other proteins (31,33,36). According to this model, L166P may be prone to aggregation in both healthy individuals heterozygous for the mutation and in PD patients. Several hypotheses may be formulated to account for these phenotypes. L166P may be recruited to cytoplasmic aggregates by TRAF6 to avoid toxicity in heterozygosity so that neurodegeneration occurs only in the absence of a functional DJ-1. In this context, wt DJ-1 may protect against cellular stress elicited by L166P. In physiological conditions, aggresomes may then be cleared in vivo by autophagy. The aggresome–autophagy pathway is increasingly recognized as a specialized type of induced autophagy that mediates selective clearance of misfolded and aggregated proteins under the conditions of proteotoxic stress (37,38). While TRAF6 has been recently involved in autophagy in macrophages (39), the E3 ligase parkin seems to be a key regulator of this pathway triggering L166P aggregation in an HDAC6-dependent manner (31). One can speculate that atypical ubiquitination might function in the recruitment to autophagic and/or storage vesicles as proved for K27 ubiquitination of Jun and K29 of Deltex (8,9). It must be noted that, to the best of our knowledge, no investigation of DJ-1 L166P has been done in post-mortem brains of PD patients carrying this mutation or in knock-in animal models.

Components of LBs are candidate aSYN interactors. Despite major efforts, the mechanisms of aSYN-induced neurodegeneration in vivo remain unclear. Although some genes have been isolated for their ability to suppress aSYN-mediated toxicity in invertebrate models (40–42), there is still the need to identify aSYN interactors that may regulate aSYN conformation, subcellular localization and biochemical properties.

Figure 6. TRAF6 colocalizes with aSYN in LBs and accumulates in PD post-mortem brains. (A) Cryo-sections of post-mortem brain tissues were taken from healthy individuals and endogenous TRAF6 was visualized by immunohistochemistry. DA neurons were identified with anti-TH antibody. (B) TRAF6 was analyzed by immunohistochemistry in SN of PD patients. LBs were visualized with anti-aSYN antibody. (C) Total RNA was extracted from SN of six PD and three control brains. TRAF6 mRNA was measured by qPCR relative to β-actin. Significance between PD and control group was calculated with t-test.
Both SIAH (4) and CHIP (5) bind to aSYN and regulate its levels and aggregation properties (21,43,44). Here we identify TRAF6 as a new aSYN modifier through unconventional ubiquitination.

LBs contain phosphorylated, ubiquitinated and nitrated aSYN (45–47). The type of ubiquitin moieties covalently attached to aSYN in PD and control brains remains unclear. Interestingly, only 10% of aSYN is ubiquitinated with evidences for conjugation of one, two or three ubiquitins (44). Monoubiquitination by SIAH has been proposed to act as a seed for further protein aggregation (48).

In our experimental settings, we failed to trigger aSYN aggregates by TRAF6 overexpression. Its induction in cell cultures depends on many factors including a permissive cellular environment, expression of synphilin-1 (49) and a proteolytic cleavage that releases an inhibitory domain at the C-terminal (50). It will be interesting to assess whether TRAF6 may trigger aggregation of aSYN in these conditions. It also remains unclear the role of TRAF6-mediated atypical ubiquitination in the degradation of aSYN via the lysosomal/autophagy pathways (51).

Interestingly, TRAF6 expression seems increased in SN of sporadic PD brains. These data need to be validated in a larger sample size since the reproducibility of gene expression analysis of human post-mortem brains may suffer from sampling a heterogeneous tissue. If confirmed, this expression may be part of a cellular response to an excess burden of unfolded proteins, as shown for other E3 ubiquitin ligases.

However, TRAF6 may also ubiquitinate soluble aSYN in the brain and thus be important for aSYN physiological function and not necessarily for its recruitment to LBs. In this model, the increased expression of TRAF6 may be a response to its sequestration to LBs to maintain the appropriate post-translational modifications of its targets including aSYN. Further experiments are needed to verify this hypothesis.

In conclusion, our data indicate a role for TRAF6 in PD for its localization in LBs and handling of aggregation-prone misfolded proteins as atypical ubiquitin E3 ligase. These results pose new exciting questions about the role of atypical ubiquitination in cellular physiology and dysfunction.

**MATERIALS AND METHODS**

Further experimental details are provided as Supplementary Material.

**Immunoprecipitation and western blot**

For coimmunoprecipitation experiments, cells were lysed in TRAF6 (200 mM NaCl, 50 mM Tris, pH 7.5, 0.5% NP40, 10% glycerol) or aSYN (150 mM NaCl, 50 mM Tris, pH 7.5, 0.5% CHAPS) buffers supplemented with anti-protease cocktail (Roche) and 5 mM N-ethyl-maleimide (NEM). Cell lysates were incubated with anti-FLAG agarose beads (Sigma) or with the appropriate antibody. After washing, immunoprecipitated proteins were eluted with 2x sodium dodecyl sulphate (SDS) sample buffer, boiled and analyzed by western blot.

The following antibodies were used: anti-FLAG 1:2000 (Sigma), anti-MYC 1:4000 (Cell Signaling), anti-HA 1:1000 (from ibridoma supernatant, kindly provided by Dr Licio Collavini), anti-β-actin 1:5000 (Sigma). For detection, antimouse-horseradish peroxidase (HRP) and anti-rabbit-HRP (Dako) or protein A-HRP (Upstate) in combination with enhanced chemiluminescence (ECL) (GE Healthcare) were used.

**In vivo ubiquitination assay**

For in vivo ubiquitination assays, HEK cells were transfected with HA–ubiquitin and the indicated DJ-1, aSYN, TRAF6 or TRAF2 constructs. After transfection, cells were either left untreated or incubated with 10 μM MG132 for 3 h. Cells were then lysed with radioimmunoprecipitation assay (RIPA) buffer (150 mM NaCl, 50 mM Tris, pH 7.5, 1% Triton X-100, 1% deoxycholic acid and 0.1% SDS) and samples were briefly sonicated. After centrifugation, clear lysates were immunoprecipitated with anti-MYC, anti-FLAG or anti-GFP (Invitrogen) antibodies. Immunocomplexes were analyzed by western blot using anti-HA antibody to detect ubiquitin conjugates. For development, protein A-HRP was used.

**Cell fractionation**

Transfected HEK cells were lysed in a buffer containing 150 mM NaCl, 50 mM Tris, pH 7.5, and 0.2% Triton X-100, supplemented with protease inhibitor cocktail and 5 mM NEM (Sigma). Lysates were centrifuged at 20,000 g for 30 min at 4°C and separated into Triton X-100 soluble (supernatant) and insoluble (pellet) fractions. Insoluble pellets were resuspended in boiling sample buffer, sonicated and used for western blot analysis.

**Immunocytochemistry and immunohistofluorescence**

To detect insoluble aggregates of mutant DJ-1, immunocytochemistry was performed as previously described (14). After permeabilization, cells were fixed in 4% paraformaldehyde and indirect immunofluorescence was carried out following standard methods. Anti-FLAG (1:1000) and anti-ubiquitin (Dako) (1:50) antibodies were used. For detection, Alexa Fluor-405-labeled (blu) or -594-labeled (red) (Invitrogen) anti-mouse or anti-rabbit antibodies were used while GFP fluorescence was evidenced at 488 laser. Immunohistofluorescence on human post-mortem brain sections was performed as reported (52). Anti-TRAF6 antibodies were from Abnova (PAB0279) and Santa Cruz (sc-8409 and sc-7221). Mouse monoclonal (#2647) and rabbit polyclonal (#2628) anti-aSYN antibodies were from Cell Signaling. All images were collected using a confocal microscope (LEICA TCS SP2).

**Post-mortem human brain samples**

Brain samples are from the brain bank at the Institute of Neuropathology, Bellvitge Hospital (University of Barcelona, Spain). Samples were dissected at autopsy with the informed consent of patients or their relatives and the institutional approval of the Ethics Committee of the University of Barcelona. Brains were obtained from Caucasian, pathologically confirmed PD cases and age-matched controls (27). Briefly,
all cases of PD had suffered from classical PD, none of them had cognitive impairment and their neuropathological character-
ization was made according to the established criteria. Control healthy subjects showed the absence of neurological symptoms and of metabolic and vascular diseases, and the neuropathological study disclosed no abnormalities, including lack of AD and related pathology. The time between death and tissue preparation was in the range of 3–5 h. The ventral mid-
brain region was sectioned horizontally. The dark–pigmented zones of the SN were readily apparent from all surrounding structures and were then isolated from the ventral midbrain. SN autopsies tissues were immediately frozen for RNA extraction. For histological analysis, samples were cryoprotected with 30% sucrose in 4% formaldehyde, frozen in dry ice and stored at −80°C until use.

**Quantitative real-time polymerase chain reaction**

Total RNA from SN of six pathologically confirmed and three control subjects (27) was isolated using the TRIZOL reagent (Invitrogen). cDNA was obtained from 1 μg of purified RNA using the iSCRIPT™ cDNA Synthesis Kit (Bio-Rad). qPCR was performed using SYBR-Green PCR Master Mix (Applied Biosystem). Expression of TRAF6, CYLD, A20 and p62/SQSTM1 was analyzed using specific oligonucleo-
tides.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

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**Conflict of Interest statement.** None declared.

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