Supplementary Materials and Methods

Constructs

IRATp970G0432D plasmid encoding the full-length sequence of human TRAF6 was purchased from ImaGene-RZPD (Germany). Coding sequence was corrected by site directed mutagenesis (Quick Change Mutagenesis Kit, Stratagene) to match NCBI reference sequence (NM_145803.1). Open reading frame was produced by PCR with specific oligonucleotides and cloned into EcoRI and Xhol sites of pcDNA3-2XFLAG or pcDNA-HA vectors (1). To obtain dominant negative TRAF6 (TRAF6 DN) lacking the N-terminal RING domain (TRAF6 300-524), similarly to what previously reported (2), the sequence between XmnI and Xhol was excised and subcloned into pCDNA3-2XFLAG. GFP-tagged TRAF6 wt and DN constructs were obtained by restriction of coding sequence from corresponding pCDNA3-2XFLAG vectors with EcoRI and Apal and subcloning into pEGFP-C2 (Clonetech).

pRK5-HA-ubiquitin wt, K0, K33, K48, K63, and K48R were received from Addgene Inc (Cambridge, MA). Ubiquitin mutants K6, K11, K27 and K29 were generated by site directed mutagenesis (Stratagene) from pRK5-HA-ubiquitin K0 as template. K63R mutant was obtained by mutagenesis of pRK5-HA-ubiquitin wt construct.

Human TRAF2 wt and DN plasmids were kindly provided by Dr. Sandro Goruppi (Tufts University, Boston, MA). Myc-His-aSYN wt and A53T constructs were kindly provided by professor Peter Lansbury (Columbia University, NY). FLAG- and MYC-tagged DJ-1 wt and L166P vectors were described elsewhere (1).

Oligonucleotides for cloning

TRAF6 constructs:

1) Generation of NBCI-matched human TRAF6 sequence by site-directed mutagenesis:

hTRAF6 fwd GTGAAAAACAGCTGTGGATCCAGCCAGTCTGAAAGTGA
hTRAF6 rev TCACTTTCAGACTGGCTGGATCCACAGCTGTTTTCAC

2) Cloning human TRAF6 open reading frame into pcDNA3-2XFLAG and pcDNA3-HA vectors:

hTRAF6 fwd ATATAGAATTCAGCTGTGGATCCAGCCAGTCTGAAAGTGA
hTRAF6 rev GCGCCTCGAGCTATACCCCTGCATCAGTAC
Ubiquitin constructs:
1) Generation of K63R mutant from pRK5-HA-ubiquitin wt template:
   K63R fwd GACTACAACATCCACAGAGAGTCCACCCTGCAC
   K63R rev GTGCAGGGCTTCTCTCTGATGTTAGTC
2) Generation of single lysine mutants from pRK5-HA-ubiquitin K0 template:
   K6 fwd GACCATGCAGATCTTCGTCAAGACGTTAACCGGTAGAACCA
   K6 rev TGGTTCTACCGGTTAACGTCTTGACGAAGATCTGCATGGTC
   K11 fwd CGTCAGAACGTTAACCGGTAAGACCATAACTCTAGAAGTTG
   K11 rev CAACGTGTTATCGGTTTACCGGTTAACGTTCTGACG
   K27 fwd CCGATACCATCGAAAACGTAAAGCTAGAATTCAGACAGAGA
   K27 rev TCTCTGTCTGAATTCAGTCTTACCGGTTAACGTTCTGACG
   K29 fwd CCATCGAAAACGTAGACAGAGAAGGTCCATG
   K29 rev ATGCGCTCTCTTCAGTCTCTAGTAACTCTACGGTATGG

Cell culture
Human embryonic kidney (HEK) 293 (SIGMA) and human neuroblastoma SH-SY5Y cells (ATCC) were maintained in culture in DMEM (Invitrogen) with 10% FBS (SIGMA) and F12:EMEM (1:1) (Invitrogen) with 15% FBS, respectively. SH-SY5Y cells stably transfected with control vector of FLA G-DJ-1 wt and L166P were described elsewhere (1). Transfections were performed with standard calcium phosphate method or with LipofectAmine 2000 (Invitrogen), as required.

Buffers for immunoprecipitation, in vivo ubiquitination and cell fractionation assays
TRAF6 lysis: 200mM NaCl, 50mM Tris pH 7.5, 0.5% NP40, 10% glycerol supplemented with anti-protease cocktail (Roche) and 5mM NEM (SIGMA).
aSYN lysis: 150mM NaCl, 50mM Tris pH 7.5, 0.5% CHAPS supplemented with anti-protease cocktail (Roche) and 5mM NEM (SIGMA).
RIPA buffer: 150mM NaCl, 50mM Tris pH 7.5, 1% Triton X-100, 1% deoxycholic acid and 0.1% SDS supplemented with anti-protease cocktail (Roche) and 5mM NEM (SIGMA).
Triton buffer: 150mM NaCl, 50mM Tris pH7.5 and 0.2% TRITON X-100, supplemented with protease inhibitor cocktail (Roche) and 5mM NEM (SIGMA).

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Oligonucleotides for qPCR

The following oligonucleotides were designed on human reference sequences and were used for quantitative real time PCR:

TRAF6 fwd AAGAGATCCATGACCAGAACT
TRAF6 rev GGCGTGCCAAGTGATTCC
A20 fwd GCGTTCAGGACACAGACTTG
A20 rev TTCATCATTCCAGTTCCGAGTATC
CYLD fwd CTGGAGTTTGTACGCTTCAGAGGAC
CYLD rev CACGCCACAATCTTCATCACACTG
SQSTM1 fwd AGGATGACATCTTCCGAATCTAC
SQSTM1 rev CTCGCAGACGCTACACAAG
beta-actin fwd CGCCGCCAGCTCACCATG
beta-actin rev CACGATGGAGGGGAAGACGG
Supplementary References


**Supplementary legends to figures**

**Figure S1. Analysis of TRAF6 wt and DN mutant for ubiquitin ligase activity.** A) HEK cells were transfected with constructs encoding for HA-ubiquitin wt alone (c) or in combination with FLAG-TRAF6 wt or dominant negative (DN). *In vivo* ubiquitination assay was performed immunoprecipitating lysates with anti-FLAG agarose beads. Auto-ubiquitinated TRAF6 was revealed with anti-HA immunoblot. Immunoprecipitates were also tested with anti-FLAG antibody. Lysates were analyzed for expressed proteins with anti-HA and anti-FLAG antibodies. Molecular weight markers are indicated on the left (KDa). B) Activity of GFP- TRAF6 wt and DN constructs was verified by immunoprecipitation with anti-GFP antibody. Immunoprecipitates and lysates were analyzed with anti-HA and anti-GFP antibodies. Asterisk indicates an unspecific band. HC, heavy chains.

**Figure S2. TRAF6 does not alter mutant DJ-1 stability.** A) Steady state levels of L166P protein were analyzed by western blot. Human neuroblastoma SH-SY5Y cells stably expressing FLAG-L166P were transfected with empty control (c) or with plasmids encoding for TRAF6 wt or DN. Mutant DJ-1 levels were tested 48 hours after transfection. Lysates were also checked for expression of TRAF6. Quantity of loaded proteins was verified with β-actin. Results are representative of three independent experiments. B) The half-life of mutant DJ-1 was followed in HEK cells upon cyclohexamide pulse chase experiments. Cells were transfected with L166P, then divided and further transfected with control (c) or TRAF6 wt plasmids. The amount of L166P protein was verified by western blot at 0, 5, 10 and 24 hours after cyclohexamide treatment. Expression of transfected TRAF6 was also monitored. β-actin was used as loading control. The experiment was repeated four times.

**Figure S3. Effects of TRAF6-mediated L166P atypical ubiquitination on aggregate formation.** HEK cells were transfected with HA-ubiquitin wt or indicated mutants, MYC-L166P and GFP-TRAF6 (wt) and treated with 10 μM MG132 for 3 hours. Before fixation, cells were permeabilized with Triton X-100. Insoluble aggregates were analyzed by double immunofluorescence coupled to GFP autofluorescence at confocal microscope.
Figure S4. Analysis of TRAF6 localization in LBs of PD post-mortem brains. TRAF6 localization was analyzed by immuno-histochemistry in the SN of PD patients. LBs were identified with anti-aSYN antibody. Nuclei were visualized with DAPI. A) Lower magnification (40X). Arrows indicate representative LBs. B) Higher magnification of LBs (63X) stained with different anti-TRAF6 antibodies, as indicated on the left.

Figure S5. mRNA levels of TRAF6 regulators p62/SQSTM1, A20 and CYLD are unchanged in PD post-mortem brains. Total RNA was extracted from SN of six PD and three control brains. p62/SQSTM1, A20 and CYLD mRNA levels were measured by qPCR relative to β-actin. Significance between PD and control group was calculated with t-test.