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protocols in WDR81 paper

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ABSTRACT

Experimental Procedures

Cell culture, reagents, and transfection

Cells were cultured at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (HyClone, Novato, CA), 100 U/ml penicillin and 100 mg/ml streptomycin. Transfections were performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturers' instructions. MG132 and doxycycline were obtained commercially. Fibroblasts from control individuals and HD patients with the Htt47Q and Htt68Q expansions were obtained from Dr. Boxun Lu's lab in Fudan University.
In brief, primary cortex neurons were isolated from appropriately timed pregnant female (16.5 days). Using forceps, we isolated cortex from each hemisphere of mouse brains under microscope. Once the cortex regions were isolated, they were cut into approximately 10 smaller pieces for digestion (1 ml prewarmed Trypsin, at 37°C for 20 min). Then serum was added to inactivate trypsin. After aspirating the supernatant, we added 1 ml prewarmed DNase solution for 10 min at 37°C. Then, the cells were dissociated by pipetting up and down, and all supernatant were transferred to new tubes for subsequent centrifugation (800xg for 5 min). We resuspended cell pellet gently and plated them on coated dishes (Poly-D-Lysine, BD, #354210). The neuronal culture medium was changed every 2 days. After about 14 days of neuronal culture, we performed transient transfection of Htt97Q-EGFP (1 mg) or/and siWDR81 (100 pmol, three times at an interval of 24 h) using Lipofectamine MessengerMAX (Thermo Fisher Scientific) as manufacturing introduction.

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Htt polyQ clearance assay

To assess the effect of siRNA knockdown of WDR81 on clearance of Htt polyQ inclusions, HeLa cells stably expressing Tet-on Htt97Q-EGFP were transfected twice at an interval of 24 h with siRNA oligos against WDR81. 12 h afterthe second siRNA transfection, Htt97Q-EGFP expression was induced with doxycycline (1 mg/ml). The medium was changed 12 h later to remove the doxycycline and the cells were split into several dishes for the remaining siRNA treatments. Time-course quantification of polyQ foci was performed 12 h after the induction of Htt97Q-EGFP expression. To rescue WDR81 siRNA-induced accumulation of polyQ foci, cells were first treated with control or WDR81 siRNA, then 3 mg of vector expressing siRNA-resistant WDR81 were transfected into the same cells simultaneous with the induction of Htt97Q-EGFP expression by doxycycline. siRNA treatments were performed twice more as above and polyQ foci were scored 48 h post induction of Htt97Q-EGFP expression.

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Brain samples from 12 control individuals and 12 patients with indicated neurodegenerative diseases (AD, n=4; HD, n=3; PD, n=5) were obtained from theChinese Brain Bank of Zhejiang University (Table 1). The samples are paraffin-embedded sections of hippocampus and cortex of frontal lobe. The controls and patients were strictly paired according to parameters including age, gender, reason of death and phase of disease. All procedures were performed according to protocols approved by Human Ethics Committees at both Fudan University and Zhejiang University.

Paraffin-embedded sections were dewaxed, rehydrated, and rinsed in PBS. After being boiled for 15 min in 0.01 mol/liter sodium citrate buffer (pH 6.0), sections were blocked in 5% guinea pig (Gp) or rabbit pre-immune serum in PBS for 1 h at room temperature and then incubated overnight with WDR81 antibody (Gp, 1:200) or p62 antibody (rabbit, 1:1000). Sections were then incubated in horseradish peroxidase-conjugated secondary antibody (1:200) at room temperature for 3 hours. After 4 washes in PBS, 3,3-diaminobenzidine (DAB) was added for 1 min at room temperature. Sections were counterstained with hematoxylin for 2 min. The IHC pictures were obtained by Nikon microscope (Ts2R). Results were calculated by software Image J and NIS-Element, BR. 3.00 (Nikon). For quantification measurements, images were randomly acquired from the hippocampus and cortex of frontal lobe and analyzed by researchers in a double-blind manner.

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pcDNA4-Htt97Q-GFP	Htt97Q-GFP	EcoRI/XhoI	pcDNA4-TO-myc-His
P *Y37A1B.5Myc-sorf-2*	*sorf-2* ORF with Myc	XmaI	pPD49.26
P *Y37A1B.5*WDR81	WDR81	KpnI/EcoRV	pPD49.26
P*dat-1 sorf-2*	*dat-1* promoter and *sorf-2* ORF	PstI/XmaI for *Pdat-1* XmaI /NcoI for *sorf-2*	pPD95.77
P*dat-1* WDR81	*dat-1* promoter	SphI/BamHI	P *Y37A1B.5*WDR81
pmCherry-c1-ATG5	Atg5	XhoI/BamHI	pmCherry-c1
pmCherry-c1-ATG12	Atg12	XhoI/BamHI	pmCherry-c1

The following vector were kindly provided by other scientists: pcDNA3.1-Htt97Q-EGFP (Dr. Xiaojiang Li, Institute of Genetics and Developmental Biology, CAS, China).

Small interfering RNAs (siRNAs)

The oligos used for siRNA knockdown of WDR81 were as described previously [14]. Other oligos were as follows:
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ATG12: 5'-AUGAGCUUCAAUUGCAUCCtt-3' [38] ;
Control siRNA: 5'-UUCUCCGAACGUGUCACGUTT-3'.
Cells were transfected with 100 pmol siRNA oligos twice at an interval of 24 h. Cells were subjected to further analysis 24 h after the last transfection. For the transfection of primary neurons, we performed the assay using Lipofectamine MessengerMax reagent (Invitrogen).

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Western blotting and immunoprecipitation

To analyze levels of proteins of interest, cells were lysed in ice-cold Triton X-100 buffer (20 mM Tris-HCl, pH7.5, 100 mM NaCl, 1% TritonX-100, 1 mM phenylmethanesulfonyl fluoride (PMSF)) or RIPA buffer (20 mM Tris-HCl pH7.5, 100 mM NaCl, 0.1% SDS, 0.5% Sodium deoxycholate, 1 mM PMSF) containing one Complete Protease Inhibitor Cocktail Tablet (Roche, Basel, Switzerland). Cell lysates were spun down at 12000 rpm for 10 min. 50 g of supernatants were resolved onsodium dodecyl sulfate polyacrylamide gels (SDS-PAGE) and blotted with the indicated antibodies. b-actin was used as the loading control.

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MTT assay for cell viability

The MTT kit was purchased from Promega (#G4002). Cells were cultured in 96-well plates with DMEM containing 10% FBS. After various treatments, 15 mL of dye solution was added into each well. Then the plates were incubated at 37°C for 1.5 hours in a humidified CO_2 incubator. Stop solution (100 mL) was added into each well, and the absorbance was recorded at 570 nm using plate reader. 630 nm was used as a reference wavelength.

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