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Experiments in HEK-293 cells

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We use this protocol and it's working

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Abstract

The end-stage pathology of Parkinson's disease (PD) involves the loss of dopamine-producing neurons in the substantial nigra compacta, but synaptic deregulation of these neurons begins much earlier in the disease process. Understanding the mechanisms of axonal deficits may provide opportunities for early therapeutic intervention, yet they remain largely unknown.

Given that increased kinase activity is an accepted pathogenic mechanism for the action of mutant LRRK2, our data in mice and findings in human showing higher LRRK2 levels in vulnerable dopamine neurons suggest that the aberrant phosphorylation of downstream targets in these neuronal populations may contribute to presynaptic dysfunctions. To investigate the molecular mechanisms by which the LRRK2 kinase activity may mediate presynaptic defects in dopamine neurons, we performed a phosphoproteomics screen using striatal synaptosomes from Lrrk2^{G2019S} mice with or without LRRK2 kinase inhibitor treatment. We identified Rab3 proteins as differentially phosphorylated presynaptic LRRK2 substrates, consistent with previous reports indicating that Rab proteins serve as robust LRRK2 substrates across various cell types. Rab3 proteins are known to play a role in presynaptic vesicle trafficking events, and we found Rab3a and Rab3c to be amongst the most highly expressed proteins in our vulnerable dopamine neuron-specific proteome dataset. We demonstrated that LRRK2 phosphorylates these Rab3 proteins in synaptosomes from the SNc and striatum. Our unbiased MS data using mouse brain extracts indicate that increased Rab3 phosphorylation diminishes its interaction with Rim1 and Rim2 proteins. This is only observed with phosphorylated Rab3 but not with overexpressed phosphomimetic Rab3 constructs, widely used to probe consequences of altered phosphorylation across several studies.

Cell culture

1 HEK-293 cells were cultured in low glucose DMEM (Gibco, #11885-084) supplemented with 10% fetal bovine serum (Gibco, #A5256801), 1% MEM non-essential amino acids (Gibco, #11140050), and 1% penicillin/streptomycin (Gibco, #15140122).

2 At 90% of confluency, cells were trypsinized with 0.25% trypsin/EDTA (Gibco, #25200072) and plated at a 1:4 dilution into a 100 mm tissue culture dish.

Plasmids 12

1d 12h

3 Rab3, RIMS1 and pCMV plasmids were transformed into competent bacteria (One Shot TOP10 chemically competent E. coli, Invitrogen, #C404003), and single colonies were selected and cultured in 3 ml of LB media with the corresponding antibiotics for 8 h at 37 °C

20h

4 1 ml of bacterial culture was grown in 200 ml of LB media plus antibiotics overnight at 37 °C.

12h

5 Plasmids were purified using ZymoPURE II Plasmid MidiPrep kits (Zymo Research, #D4201) following manufacturer's instructions.

4h

Transfection

1d 0h 21m

6 For cotransfection of Rab3 plasmids and RIMS1, HEK-293 cells at 80% confluency were plated into 6-wells at a 1:20 split ratio.The following day, cells were co-transfected with HA-Rab3 constructs and myc-flag-RIMS1, or with myc-flag-RIMS1 alone.

6.1 For each 6-well, 200 ng of HA-Rab3 plasmids and 2 ug of myc-flag-RIMS1 plasmid were mixed in a 1.5 ml centrifuge tube in 50 ul high glucose DMEM (Gibco, #11965092) , and 6 ul of LipoD293 (Signagen Laboratories, #SL100668) was mixed in another 1.5 ml centrifuge tube in 50 ml high glucose DMEM.

3m

6.2 Three minutes later, contents were mixed, briefly vortexed and incubated for 15 min at room temperature.

15m

6.3 Media was exchanged to fresh complete medium, and the plasmid/LipoD293 mix added dropwise to wells.

3m

6.4 24 h after transfection, cells were transferred into 100 mm dishes and grown for another 24 h.

7 For determination of isoform-specificity of the Rab3 antibodies, HEK-293 cells at 80% confluency were plated into 6-wells. The following day, they were co-transfected with HA-Rab3 constructs (200 ng) and pCMV (2 ug), or with pCMV alone (2 ug) as described above

7.1 After 24 h, transfected cells were transferred into 100 mm dishes and grown for another 24 h. 1d

Immunoprecipitation 3h 16m

8 For HA-Rab3 and myc-flag-RIMS1 co-transfections, HEK293 cells were collected 48 h after transfection.

8.1 For each 100 mm dish, cells were washed with ice-cold 1 x PBS 3m

8.2 Cells then lysed with 1 ml of IP lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA pH 8.0, 0.3% Triton-X100, 10% glycerol, and 1X cOmplete, Mini, EDTA-free protease inhibitor cocktail) in a 1.5 ml low protein binding tube for 30 min on a rotary wheel at 4 °C. 30m

8.3 Lysates were centrifuged at 10,000 rpm for 10 minutes at 4 °C, and 50 ul of the supernatant set aside as immunoprecipitation input. 10m

8.4 Magnetic anti-HA beads (25 ul) (Pierce) were washed for 30 min with IP wash buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA pH 8.0, 10% glycerol) on a rotary wheel at 4 °C. 30m

8.5 Beads were pulled down with magnet, followed by equilibration in IP lysis buffer for 3 min on ice. 3m

8.6 Beads were pulled down again and incubated with 1 ml of lysed supernatant for 1h at 4 °C on a rotary wheel. 1h

8.7 Beads were washed three times for 5 min with 1 ml of IP wash buffer, and proteins eluted with 40 ul
of 2x Laemmli sample buffer and boiled at 95 °C for 5 min to release bound proteins. 20m

9 For determination of Rab3 antibody specificity, cells were washed and collected 48 h after transfection in 1 ml of IP lysis buffer.

9.1 Lysates were incubated for 30 min on a rotary wheel at 4 °C, and centrifuged for 10 min at 10,000 rpm at 4 °C. 40m

10 For each transfection, five ul of supernatant was resolved on SDS-PAGE gels and analyzed by
western blotting as described above.