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FLAG-tag mediated co-immunoprecipitation of phosphorylated Rab GTPases with cellular interacting proteins V.1

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We use this protocol and it's working

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Abstract

Leucine Rich Repeat Kinase 2 phosphorylates a subset of Rab GTPases at a conserved Ser/Thr residue in their switch 2 region. This modification disrupts interactions between Rabs and effectors/regulators while enabling binding to novel phospho-specific effectors. We use purified phosphorylated and non-phosphorylated FLAG tagged Rab3a as 'bait' to immunoprecipitate interacting proteins. This is followed by mass spectrometry to identify proteins that bind preferentially to either phospho- or non-phospho Rab3a (found in another protocol).

Guidelines

Note on lysate preparation: Interacting proteins are immunoprecipitated from both A549 cell lysate and whole mouse brain lysate treated with the LRRK2 inhibitor MLi2. Lysates are prepared with cold lysis buffer freshly supplemented with cOmplete Mini (EDTA-free) protease inhibitor (Roche), PhosSTOP phosphatase inhibitor (Roche) and clarified by centrifuging at 17,000 x g for 15 min at 4°C. Protein concentrations are determined by Bradford Assay and the lysates stored at -80°C.

Materials

Buffers

PBS-T: PBS supplemented with 0.01% Tween-20, 5mM MgCl2
Lysis buffer: 50mM Tris-HCl pH 7.4, 150mM NaCl, 10% (w/v) glycerol, 10mM sodium-glycerophosphate, 10mM sodium pyrophosphate, 0.5% (v/v) NP40 - supplemented with cOmplete Mini (EDTA-free) protease inhibitor (Merck), PhosSTOP phosphatase inhibitor (Merck) and 5mM MgCl2
High salt lysis buffer: lysis buffer supplemented with 500mM NaCl

Reagents

Phosphorylated FLAG-Rab
FLAG-Rab
PBS (GIBCO brand)
Pierce™ Anti-DYKDDDDK Magnetic Agarose (supplied as 25% slurry in PBS, 0.01% Tween-20 detergent, 0.02% sodium azide, pH 7.2 with a confirmed binding capacity of ~3.2mg FLAG Rab (~22kDa)/ml settled beads
cOmplete Mini (EDTA-free) protease inhibitor (Roche)
PhosSTOP phosphatase inhibitor (Roche)

Equipment

Magnetic separation stand

Troubleshooting

Immunoprecipitation of Interacting Proteins

1 6 replicates are performed for each phospho-FLAG Rab and FLAG Rab, following the steps below for each replicate.

2 Aliquot 5µl settled volume of Pierce™ Anti-DYKDDDDK Magnetic Agarose in low binding Eppendorf tube and wash with 500µl PBS-T on a rotator for 5 minutes.

3 Place tube in magnetic separation stand and remove supernatant. Repeat washing step 3 times.

4 Add 20µg of FLAG protein to the washed beads and make up the volume to 500µl with PBS-T. Incubate at 4°C on a rotator for 1 hour.

5 Place tube in magnetic separation stand and remove supernatant without disturbing the bead pellet.

6 Wash the beads with 500µl PBS-T twice and remove supernatant completely.

7 Add cell lysate (~400µg A549 lysate or ~750µg brain tissue lysate) to the beads on ice and make up the volume to 500µl with cold lysis buffer. Incubate on a rotator for 1 hour at 4°C.

Note

Note on lysate preparation: Interacting proteins are immunoprecipitated from both A549 cell lysate and whole mouse brain lysate treated with the LRRK2 inhibitor MLi2. Lysates are prepared with cold lysis buffer freshly supplemented with cOmplete Mini (EDTA-free) protease inhibitor (Roche), PhosSTOP phosphatase inhibitor (Roche) and clarified by centrifuging at 17,000 x g for 15 min at 4°C. Protein concentrations are determined by Bradford Assay and the lysates stored at -80°C until required for the IP.

8 On a magnetic stand, remove supernatant and wash twice with 500µl cold high salt lysis buffer followed by twice with 500µl cold lysis buffer.

Note

Ensure supernatant is removed completely after the final wash.

9 Store samples at -80°C for mass spectrometry analysis or immunoblotting.