Reconstitution of Parkin ubiquitin ligase activity using mouse and human mitochondria.

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ABSTRACT

Analysis of Parkinson's linked genes PINK1 and Parkin has uncovered a mechanism by which upon loss of mitochondrial membrane potential, Parkin E3 ubiquitin ligase activity is activated by PINK1 kinase activity, to trigger mitochondrial membrane protein ubiquitylation, leading to removal of damaged mitochondria (mitophagy). We and other groups have previously reported *in vitro* assays of Parkin E3 ligase activity using recombinant Parkin and PINK1 expressed in *E.* coli. This provided evidence of Parkin activation by PINK1 phosphorylation of Ser65 in both ubiquitin and UBL domain of Parkin.
Herein, we report a reconstitution assay in which addition of recombinant Parkin to mitochondria isolated from cells after treatment by combination of Antimycin A and Oligomycin (to induce PINK1 activation on the outer mitochondrial membrane), enables robust ubiquitylation of multiple substrates at the mitochondria. This assay represents a robust tool to study Parkin E3 ligase activity and the functional interplay between ubiquitylation and phosphorylation mediated by PINK1 and Parkin and their role in reshaping the endogenous mitochondrial proteome.

KEYWORDS

MEFs, mitochondria, PINK1, Parkin, Ubiquitin

MATERIALS

For Mouse Embryonic fibroblast culture

1. E13.5 mouse embryos (8–10 embryos, either sex; we used PINK1 wild-type and knockout mice) **CRITICAL!** All experiments must be conducted in accordance with the relevant institutional and governmental guidelines and regulations.

2. **Digestion medium**: 0.025% Trypsin-EDTA (Gibco™ #25300054); 0.125mg/mL DNase I (Merck #11284932001) in HBSS (Gibco™, #14025050).

3. **Culturing medium:** DMEM (Gibco™ #11960-085); 20% Foetal Bovine Serum (FBS) heat-inactivated (Gibco™ #10500064); 1% Penicillin-Streptomycin (Gibco™ # 15140122), 1% L-Glutamine (Gibco™ #25030024); 1X Non-essential Amino acid (Gibco™ #11140-035), 1X Sodium pyruvate (Gibco™ #11360-039).

4. DPBS, no calcium, no magnesium (Gibco™ #14190094).

5. Trypan Blue solution (Sigma-Aldrich #T8154).

Cell lines

1. Hela ATCC (Catalog# CCL-2)
   Culturing medium: DMEM (Gibco™ #11960-085); 10% Foetal Bovine Serum (FBS) (Sigma-Aldrich #F7524); 1% Penicillin-Streptomycin (Gibco™ # 15140122), 1% L-Glutamine (Gibco™ #25030024).

For mitochondrial depolarisation and isolation

1. **Mitochondrial depolarisation:** 10 µM Antimycin A (Sigma-Aldrich #A8674); 1 µM Oligomycin (Sigma-Aldrich #75351) in DMSO (Sigma-Aldrich #D2650).

2. **Hypotonic Buffer**: 20 mM HEPES (pH7.8), 5 mM KCl, 1.5 mM $MgCl_2$, 2 mM DTT, 1 mM PMSF, phosphate inhibitors PhosSTOP and protease inhibitor cocktail.

3. **2.5X MSH (Mannitol-Sucrose-HEPES) Buffer**: 525 mM Mannitol, 175 mM Sucrose, 20 mM HEPES (pH 7.8), 5mM EDTA, 2mM DTT, 1mM PMSF.

4. **1X MSH Buffer**: 210 mM Mannitol, 70 mM Sucrose, 20 mM HEPES (pH 7.8), 2mM EDTA, 1mM PMSF, phosphate inhibitors PhosSTOP and protease inhibitor cocktail.

5. **Mito Ubi Buffer (MUB)**: 50 mM Tris-HCl pH 7.5; 70mM Sucrose; 210 mM Sorbitol, 5mM sodium pyrophosphate, 50 mM Sodium Fluoride, 10mM sodium-2-glycerophosphate.

6. DPBS, no calcium, no magnesium (Gibco™ #14190094).

7. **Table of reagents**

REAGENT	COMPANY	CAT. NUMBER
D (+)-SACCHAROSE (SUCROSE)	VWR	27480.360
D-SORBITOL	Merck (Sigma-Aldrich)	S1876
D-MANNITOL	Merck (Sigma-Aldrich)	M4125
EDTA DISODIUM SALT DIHYDRATE	Fisher BioReagents	BP120-500
TRIS (TROMETAMOL)	VWR	103157P
POTASSIUM CHLORIDE	VWR	26764.298
MAGNESIUM CHLORIDE HEXAHYDRATE	Merck (Sigma-Aldrich)	13152
DTT	Formedium	DTT010
HEPES	Formedium	HEPES10
2-GLYCEROPHOSPHATE DISODIUM SALT HYDRATE	Merck (Sigma-Aldrich)	G9422
PMSF	Merck (Sigma-Aldrich)	93482
SODIUM FLUORIDE	Merck (Sigma-Aldrich)	S7920
SODIUM PYROPHOSPHATE DECAHYDRATE	Merck (Sigma-Aldrich)	221368
PHOSPHATASE INHIBITORS (phosSTOP)	Merck (Sigma-Aldrich)	4906845001
COMPLETE PROTEASE INHIBITORS	Merck (Roche)	11873580001

For Ubiquitylation assay

1. Tris-Base – 103157P VWR Prepare a 1 M Tris-HCl pH 7.5 stock in deionised water with the pH adjusted using 37.5 % HCl.

2. MgCl2.6H2O – M2670-500G Sigma.

3. Adenosine Triphosphate (ATP) – ab14730 Abcam.

1. Tris(2-carboxyethyl) phosphine (TCEP) – BIT0122 Apollo Scientific.

2. His-Ube1 (MRC-PPU Reagents & Services, DU32888).

3. UbE2L3 (MRC-PPU Reagents & Services, DU3772).

4. Ubiquitin (MRC-PPU Reagents & Services, DU20027).

5. (His-SUMO-cleaved) Parkin (MRC-PPU Reagents & Services, DU42598)

For biochemistry

8. Coomassie Protein Assay (Thermo Scientific™ #1856209).

9. 4X NuPAGE™ LDS Sample Buffer (Invitrogen™ #NP0008).

10. 2-Mercaptoethanol (Sigma-Aldrich M6250).

11. PageRuler™ Prestained Protein Ladder (Thermo Scientific™ #26616).

12. Immobilon-P PVDF Membrane (Merck #IPVH00010).

13. Amersham™ Protran® Nitrocellulose membranes (Merck #GE10600041).

14. NuPAGE™ 4 to 12%, Bis-Tris Mini Protein Gel, 10-well or 20-well (Invitrogen™ #NP0321BOX, #WG1402BOX).

15. NuPAGE™ MOPS SDS Running Buffer-20X(Invitrogen™ #NP000102) or MES SDS Running Buffer-20X (Invitrogen™ #NP0002).

16. 1 X Towbin transfer buffer:25mM Tris, 192 mM Glycine, 20% methanol.

17. 1X Tris Buffered-Saline (TBS): 500mM Tris, 150mM sodium chloride, pH 7.6, at 25 °C.

18. 1X Tris-Buffered Saline, 0.1% Tween® 20 Detergent (TBST).

19. 5 % non-fat milk in TBST.

20. 5% bovine serum albumin (BSA) in TBST (Sigma-Aldrich #10735094001).

21. **Primary antibodies**: Phospho-Ubiquitin (Ser65) (E2J6T) Rabbit mAb (Cell signalling technology #62802), Ubiquitin Antibody P4D1 (Biolegend #646302), Anti-Parkin phospho-Ser65 Rabbit mAb by Epitomics in collaboration with the Michael J Fox Foundation for Research, Parkin Antibody PRK8 (Santa Cruz Biotechnology #sc-32282), CISD1 Antibody (Cell signalling technology #83775), CTP1α Antibody (Abcam #ab128568), CYB5B Antibody (Novus biological #NBP1-88039), HK1 Antibody (Cell signalling technology #2024), MFN2 Antibody (Abcam #ab124773), VDAC Antibody (Cell signalling technology #4661).

22. **Secondary Antibodies**: Goat anti-Rabbit IgG (H+L) Secondary Antibody, HRP conjugate (Invitrogen™ #31460), Rabbit anti-Mouse IgG (H+L) Secondary Antibody, HRP conjugate (Invitrogen™ #31450).

23. ECL™ Western Blotting Reagents (Merck, Cytiva, #RPN2106).

24. Super signal West Dura (Thermo Scientific, #34075).

25. Amersham Hyperfilm ECL (Merck, Cytiva, #28906837)

STOCK SOLUTION PREPARATION

- **DNaseI:** Dissolve 100mg/mL (wt/vol) DNase in sterile double-distilled water; filter, aliquot and store at − 20 °C. The solution is stable for 2–3 months.

- **Antimycin A**: Prepare 50mM of Antimycin A in DMSO; aliquot and store at − 20 °C.

- **Oligomycin**: Prepare 10 mM of Oligomycin in DMSO; aliquot and store at − 20 °C.

EQUIPMENT

1. Dumont #5 Forceps Biologie Inox (Fine Science Tool #11252-20).
2. Dumont #5XL Forceps Standard Inox (Fine Science Tool #11253-10).
3. Dumont #7 Fine Forceps Biologie Inox (Fine Science Tool #11274-20).
4. Student Vannas Spring Scissors Straight (Fine Science Tool #91500-09).
5. Fine Scissors ToughCut Straight 9cm (Fine Science Tool #14058-09).
6. Cell Counter-DeNovix CellDrop™.
7. 37 °C water bath.
8. Laminar flow cell culture hood.
9. Cell culture incubator 5% CO2, 95% humidity HERAcell®CO2 incubator (150 L).
10. Microcentrifuges, Micro Star 17R (VWR #521-1647).
11. Dounce Dura Grind® Tissue Grinder (EMS #64791-07).

12. XCell *SureLock*™ 4Midi-Cell running tank (Invitrogen™ #WR0100).
13. XCell *SureLock*™ Mini-Cell running tank (Invitrogen™ #EI0001).
14. Mini Trans-Blot® Cell transfer tank (BIORAD # 1703930).
15. Trans-Blot® Cell transfer tank (BIORAD # 1703939).
16. ChemiDoc MP Imaging System (BIORAD).
17. ECOMAX™ X-ray Processor.
18. Eppendorf ThermoMixer – 5382000031 Eppendorf.

CONSUMABLES

1. 10cm and 15cm tissue culture Petri Dishes (ThermoFisher # 172931 and 168381 respectively).
2. Cell strainer 70µM (FisherScientific #10788201).
3. Stericups 0.22um, 250 mL and 500 mL (Merck #SCGPU02RE, #SCGPU05RE).
4. 50mL Stripette® Serological Pipets (Corning #4490).
5. 25mL Stripette® Serological Pipets (Corning #4489).
6. 10mL Stripette® Serological Pipets (Corning #4488).
7. 5mL Stripette® Serological Pipets (Corning #4487).
8. 15mLCELLSTAR® tubes (Greiner bio-one #188271).
9. 50mL CELLSTAR® tubes (Greiner bio-one # 227261).
10. Standard 1mL and 200µL Pipette tips (Greiner bio-one # 686271, #685261).
11. Syringe filter (0.22µm. Sartorius, Item # ST16541-Q).
12. Syringes (50mL) (Terumo™# 8SS50L1).
13. 1.5mL Eppendorf tubes (Eppendorf™ # 0030120086).
14. Disposable Cell Lifter (FisherBrand #08100240).

PROCEDURE TO ISOLATE AND CULTURE MOUSE EMBRYONIC FIBROBLASTS

Dissection of E13.5 mouse embryos ◊TIMING 20 min

1. Use sterilized instruments by autoclave or washing them with 70% (vol/vol) ethanol. Dry thoroughly if ethanol is used. Soak dissection tools in 70% ethanol between embryos to prevent contamination.
2. In the hood. Prepare 10-cm dishes with cold PBS. Separate embryos from uterus and placenta. Place each embryo into a single dish with cold PBS.

3. Number dishes and Eppendorf tubes for tissue collection for genotyping.

4. Euthanize the embryos by decapitation and separate the head from the body.

5. Wash the bodies twice with PBS to minimise contamination and collect a small piece of tail for genotyping.

6. Place the body on a dish with PBS and remove the red spot (bowel) with forceps.

7. Place the body on a clean dish and mince the tissue with a spring scissors (or with a sterile scalpel blades).

Cell dissociation and plating ◊TIMING ~30min-1 h

8. Prepare digestion medium by adding 125 µL of DNase I (stock solution 10mg/mL) to 10 mL of Trypsin 0,025% (1:1 Trypsin 0,05%-HBSS).

9. Add 5mL of digestion medium to the tissue and transfer in a 15 mL falcon tube.

10. Incubate at 37 °C in a water bath for 15 min.

11. Pipette to mechanically dissociate the tissue, gentle and sequential pipetting (using 10mL, 5mL and 1 mL pipettes) until cells are completely suspended. **Note:** The number of trituration is approximative, it may vary depending on the size of the unbroken tissues.

12. Inactivate trypsin digestion by 5 mL of culturing medium.

13. Centrifuge at 1200 rpm for 5 min.

14. Remove media and resuspend in 5 mL culturing media.

15. Filter the cells through a 70 µm filter. **Note:** The cell suspension should be filtered in order to exclude any undigested tissue pieces or aggregates from the newly prepared cell suspension.

16. Take a 15 µL aliquot, add 1:1 ratio Trypan Blu and determine the density of cells and cell viability to the cell counter.

17. 3.0×10^6 cells/well plates are plated out on 10cm dishes, containing 10 mL of pre-warmed culturing media.

18. Change media every 5 days, grow to 90% confluence and split (minimum 25% confluence to keep cells within the range to promote growth). The growth rate progressively declines when transformation occurs and the cells become immortal, at approximately after 18 passages (it can be variable). **Note:** In this assay we use primary MEFs between 8-10 passages.

MITOCHONDRIAL ISOLATION FOR MEFs

Mitochondrial depolarisation ◊TIMING 4h, day of experiment

To depolarize or uncouple mitochondrial membrane potential in MEFs, cultures were treated for 4 hours with a combination of 10 µM Antimycin A and 1 µM Oligomycin dissolved in DMSO

at 37°C. **Note:** Before the experiment, MEFs were plated in 15 cm dishes and stimulated at 80-90% confluence.

Mitochondrial isolation ◊TIMING 1-1.5h

1. Gently aspirate the medium from wells.
2. Wash twice by adding 5 mL of warmed DPBS (room temperature) containing protease inhibitors and phosphatase inhibitors.
3. Place the 15 cm dish on ice and add 1mL of Hypotonic Buffer. Carefully scrape the cells and collect the cells in a 15 mL microcentrifuge tube. Add 2mL of Hypotonic Buffer in each tube (for a total of 3 mL).
4. Stand on ice for 15 min in the cold room.
5. Homogenise cells using a stainless steel Dounce homogeniser with 45 strokes. Note: Check cell disruption with light microscope, 80-90 % cells should be disrupted.
6. Add to the disrupted cells 2.5X MSH buffer and mix. Note: Mixing 2.5X MSH volume to the initial volume of hypotonic buffer will give 1X MSH.
7. Centrifuge the homogenate at 700*g* in a refrigerated centrifuge for 10 min, to remove cell debris and nuclei.
8. Transfer supernatant into a new 15 mL tube and centrifuge at 700g x 10 min at 4°C to remove residual nuclei and cell debris.
9. Centrifuge at 9000g x 10 min at 4°C to pellet mitochondria.
10. Resuspend mitochondria in 1 mL of 1X MSH buffer and centrifuge at 9000g x 10 min at 4°C to pellet mitochondria. Repeat twice to remove any cytosolic proteins.
11. Centrifuge at 9000g x 10 min at 4°C to pellet mitochondria and resuspend in 150µL of MUB Buffer.
12. Protein quantification: take a small aliquot of mitochondria (10 µL), add 1% Triton, vortex and estimate protein concentration by using the Coomassie Protein Assay.
13. Usually from a 15 cm dish of MEFs at 90% confluence it is possible to isolate 200µg of crude mitochondria.

MITOCHONDRIAL ISOLATION FOR HeLa

Mitochondrial depolarisation ◊TIMING 2h, day of experiment

To depolarize or uncouple mitochondrial membrane potential in MEFs, cultures were treated for 2 hours with a combination of 10 µM Antimycin A and 1 µM Oligomycin dissolved in DMSO at 37°C. **Note:** Before the experiment Hela were plated in 15 cm dishes and stimulated to 80-90% confluence.

Mitochondrial isolation ◊TIMING 1-1.5h

1. Gently aspirate the medium from wells.

2. Wash twice by adding 5 mL of warmed DPBS (room temperature) containing protease inhibitors and phosphatase inhibitors.
3. Place the 15 cm on ice and add 1mL of Hypotonic Buffer. Carefully scrape the cells and collect the cells in a 15 mL microcentrifuge tube. Add 2mL of Hypotonic Buffer in each tube (for a total of 3 mL).

4. Stand on ice for 15 min in the cold room.

5. Homogenise cells using a stainless steel Dounce homogeniser with 25 strokes. **Note:** Check cell disruption with light microscope, 80-90 % cell should be disrupted.

6. Add to the disrupted cells 2.5X MSH buffer and mix. **Note:** Mixing 2.5X MSH volume to the initial volume of hypotonic buffer will give 1X MSH.

7. Centrifuge the homogenate at 700*g* in a refrigerated centrifuge for 10 min, to remove cell debris and nuclei.

8. Transfer supernatant into a new 15 mL tube and centrifuge at 700g x 10 min at 4°C to remove residual nuclei and cell debris.

9. Centrifuge at 9000g x 10 min at 4°C to pellet mitochondria.

10. Resuspend mitochondria in 1 mL 1X MSH buffer and centrifuge at 9000g x 10 min at 4°C to pellet mitochondria. Repeat twice to remove any cytosolic proteins.

11. Centrifuge at 9000g x 10 min at 4°C to pellet mitochondria and resuspend in 150µL-250 µL of MUB Buffer.

12. Protein quantification: take a small aliquot of mitochondria (10 µL), add 1% Triton, vortex and estimate protein concentration by using the Coomassie Protein Assay.

13. Usually from one 15 cm dish of Hela at 80-90% confluence it is possible to isolate 600-800µg of crude mitochondria.

UBIQUITYLATION ASSAY ◊TIMING 2.5h-3h

14. Resuspend isolated mitochondria in MUB buffer at concentration ~1mg/mL, in order to use a volume of mitochondria < 10% of reaction volume.

15. 5 µg of mitochondria were used for a total volume reaction of 50 µL.

16. Defrost proteins on ice and prepare a master mix considering that for one single reaction it is required 1µM Parkin, 0.1 µM His-UbE1, 1 µM UBE2L3 and 30 µM Ubiquitin (as negative control prepare a master mixer without Parkin).

17. Prepare reaction buffer with 50mM Tris pH 7.5, 5 mM MgCl2 and 0.5mM TCEP.

18. Combine reaction buffer, master mix of proteins and mitochondria.

19. Aliquot in order to distribute 50µL in 1.5 mL Eppendorf tube and start the ubiquitylation reaction by adding 2mM ATP in each Eppendorf tube.

20. Place the eppendorf tubes in a thermomixer and incubate the reaction at 30°C, shaking for 2 hours (MEFs) or 90 min (Hela) at 1000rpm.

21. Stop the reaction by the addition of 4 x LDS loading buffer containing 10% of 2-mercaptoethanol.

Immunoblotting of ◊TIMING 5h-2d

1. Samples are boiled for 3 min at 97°C.

2. Analyse samples by running 20µL of reaction on Nu-page Bis-Tris 4-12% gels for a better resolution of ubiquitin chains, at 120 V for ~2h. Use MES SDS Running Buffer or MOPS SDS Running Buffer according to the size of protein to be analysed. For phospho-parkin and Parkin blots, it is recommended to dilute the final reaction 1 in 25 with 1X LDS containing 2.5 % 2-mercaptoethanol.

3. Transfer gel on PVDF membrane for phospho-ubiquitin and ubiquitin signal and nitrocellulose membrane for phospho-Parkin and Parkin signal. Transfer in Towbin buffer at 80 V for 1.5 h on ice or in cold room (for HK1 it is recommended to transfer at 90 V for 1.5h) Note: Prepare only 1 membrane per transfer tank –avoid multiple membranes for transfer in same tank as this reduces ubiquitin transfer.

4. Incubate membrane with blocking buffer 5% milk in 0.1% TBS-Tween for 1 h at Room Temperature.

5. Remove blocking buffer, if primary antibodies are in 5% BSA, rinse twice with 0.1% TBS-Tween to remove any traces of milk, add primary antibodies and incubate overnight at 4°C,
   Note: Prepare phospho-Ubiquitin Antibody (1:2000), Ubiquitin Antibody (1:1000), CISD1 Antibody (1:1000), CPT1α Antibody (1:1000), CYB5B Antibody (1:1000), HK1 Antibody (1:1000), MFN2 Antibody (1:1000), VDAC Antibody (1:1000) and Parkin Antibody (1:1000) in 5% BSA (TBS-Tween). Prepare phospho-Parkin Antibody (1:2000) in 5% milk (TBS-Tween). To avoid non-specific signal, it is recommended to preincubate phospho-Parkin antibody with a membrane for 2 days before using it.

6. Remove primary antibody and wash 3 times with 0.1%TBS-Tween for 10 min.

7. Add secondary antibodies, HRP-conjugate for 1 hour at RT diluted 1:5000 in 1% BSA (0,1% TBS-Tween). Use 1:10000 dilution in 1% BSA for Parkin antibody and 1:10000 dilution in 5% milk for and phospho-Parkin antibody.

8. Remove secondary antibody and wash 3 times with 0.1%TBS-Tween for 10 min.

9. Develop signal using ECL western Blotting reagents and analysing with Chemidoc.
   Note: Depending on signal, film can be best for sensitivity. To improve detection of HK1 and VDAC ubiquitylation, it is recommended to develop signal using Super signal West Dura reagents.