Mitophagy analysis (monitoring mitochondrial protein degradation in Parkin-mediated mitophagy)

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METHODS

Western blotting of mitochondrial matrix proteins

1. Seed cells stably expressing exogenous Parkin in 6 cm dishes and culture in DMEM supplemented with 10% (vol/vol) FBS, and 2 mM L-glutamine, and maintain the cells in a CO_2 incubator (5% CO_2, 37°C).
2. Incubate the cells in regular medium in the presence or absence of 20 μM CCCP and/or 0.1 μM bafilomycin A_1 for 12-24 hours dependent on the purpose) in the CO_2 incubator.
3. Wash cells with ice-cold PBS, and harvest cells with ice-cold lysis buffer supplemented with protease inhibitors (protease inhibitor cocktail and 1 mM PMSF).

NOTE Cells can be harvested in PBS, pelleted by centrifugation at 3, 000 rpm, and stored at -80°C.

NOTE The rest of the protocol follows a standard Western blotting method.

4. Incubate the cell lysates in centrifuge tubes for 10 min on ice.
5. Centrifuge the lysates at 15, 000 rpm for 15 min at 4°C.
6. Take 5 μl out of the supernatants and add to tubes containing 20 μl milliQ (final x5 dilution), which will be used for protein quantification.
7. Take the rest of supernatant, add 1/5 volume of 6x sample buffer and immediately proceed to boiling for 5 min. The lysates can be stored at -30°C after boiling.
8. Load 15 μg protein/well on a polyacrylamide gel of appropriate concentration (e.g. 10% for complex III core 1) and run at 15 mA/mini gel (constant current) for ~2 h until the bromophenol blue reaches the bottom.

NOTE Boil the frozen lysates again for 1 min before loading.

9. Semi-dry transfer at 100 mA (constant current) for 1 h.
10. Block the membrane with 5% skim milk/TBSt for 1 h at room temperature.
11. Incubate with a primary antibody (e.g. anti-complex III core 1 antibody, 1:2,000 dilution) in 5% skim milk/TBSt overnight at 4°C.

NOTE It is critically important to use a mitochondrial matrix protein to monitor mitophagy, because outer membrane proteins and sometimes inter membrane

space proteins can be degraded by the proteasome [1, 2]. Complex III localizes in the inner mitochondrial membrane, and core 1 subunit (the target of the antibody described above) localizes at the matrix side of inner membrane. Other matrix proteins such as HSP60 can also be used [1].

12. Wash the membrane with TBSt for 10 min. Repeat 3 times.
13. Incubate with an HRP-conjugated secondary antibody (e.g. anti-mouse IgG HRP-conjugated goat antibody (1: 10,000) for complex III core 1) for 1 h at room temperature.
14. Wash the membrane with TBSt for 10 min. Repeat 4 times.
15. Add ECL detection mixture onto the membrane and detect with a LAS-3000 mini image analyzer.
16. Representative data are shown in Figure 1.

Immunofluorescence microscopy of mitochondrial matrix proteins

1. Place cover slips into a 24-well plate and coat with gelatin.
2. Seed cells stably expressing Parkin in the 24-well plate and culture in DMEM supplemented with 10% (vol/vol) FBS and 2 mM L-glutamine, and maintain the cells in a CO_2 incubator (5% CO_2, 37°C).
3. Incubate the cells in regular medium in the presence or absence of 20 μM CCCP and/or bafilomycin A_1 for 12-24 hours in the CO_2 incubator.
4. Wash cells with PBS, and fix them in 4% (w/vol) paraformaldehyde/PBS for 10 min at room temperature.
5. Wash cells with PBS three times.

NOTE Fixed cells can be stored in PBS at 4°C for several days.

6. Permeabilize cells with 0.1% Triton X-100/PBS for 5 min at room temperature.
7. Wash cells with PBS three times.
8. Antigen retrieval (Figure 2. OPTIONAL, necessary for complex III core 1 staining). Put an empty 24 well dish in a heat resistant container, fill ion-exchange water in the container, and preheat and boil water by microwave. Carefully put cover slips in each well and boil for 5 min by microwave, and let the water cool down to room temperature. Wash cells with PBS.
9. Incubate cells with 3% (w/vol) BSA/PBS for 30 min at room temperature.
10. Incubate cells with primary antibody in 3% BSA/PBS (x400 for Complex III core 1) for 1 h at room temperature.

NOTE It is critically important to use a mitochondrial matrix protein to monitor

mitophagy.

11. Wash cells four times with PBS.

12. Incubate cells with secondary antibody solution (e.g. Alexa Fluor 488 goat anti-mouse IgG (x1000) for complex III core 1) in 3% BSA/PBS for 1 h at room temperature in the dark.

13. Wash cells five times with PBS.

(OPTIONAL. Stain nuclei by incubating cells in Hoechst/PBS (0.1 µg/ml) for 3 min, wash cells twice with PBS and proceed to mounting. Hoechst staining as well as DIC imaging will allow visualization of intact cells which have lost mitochondria.)

14. Rinse the cover slips briefly in distilled water and mount them on slide glass in a small droplet (~3 µl) of *SlowFade* Gold antifade solution. Seal with manicure and let dry. Store the slides at 4°C.

15. Observe cells under a canonical microscope or a confocal microscope according to the purpose of the study. Canonical microscopes are more suitable for observing total mitochondria and confocal microscopes can give clearer images for colocalization study.

16. Representative data are shown in Figure 3.

Monitoring mitochondrial protein degradation by flow cytometry

1. Seed cells stably expressing exogenous Parkin and mitochondrial matrix-targeting GFP (e.g. su9-GFP, GFP fused to the mitochondrial matrix-targeting signal of subunit 9 of F_o-ATPase) in 6 well dishes and culture in DMEM supplemented with 10% (vol/vol) heat-inactivated FBS, 50 U/ml penicillin-streptomycin and 2 mM L-glutamine, and maintain the cells in a CO_2 incubator (5% CO_2, 37°C).

NOTE GFP fused to outer mitochondrial membrane proteins such as GFP-Omp25 can be degraded by the proteasome under the mitophagy-inducing conditions. Therefore, GFP has to be fused to mitochondrial matrix-targeting signal [2].

2. Incubate the cells in regular medium in the presence or absence of 20 µM CCCP and/or bafilomycin A_1 for 12-24 hours in the CO_2 incubator.

3. Wash cells with PBS, incubate with 0.05% trypsin-EDTA at 37°C for 5 min to detach cells.

4. Suspend cells in ice-cold PBS, centrifuge cells at 3,000 rpm for 3 min and discard the supernatant.

5. Resuspend cells in ice-cold PBS and place the cell suspension into flow cytometry tubes. Always keep the tubes on ice.
(OPTIONAL. Let the cells pass through nylon mesh (30-60 µm pore) to get rid of aggregated cells)
6. Immediately proceed to detection of GFP fluorescence by a flow cytometer.

MATERIALS

dimethyl sulfoxide (DMSO) (Wako, 045-24511)
carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (SIGMA, C2759)
bafilomycin A_1 (SIGMA, B1793)
Complete, EDTA-free protease inhibitor cocktail (Roche, 11873580001)
phenylmethanesulfonyl fluoride (PMSF) (SIGMA, P7626)
anti-complex III core 1 mouse monoclonal antibody (Life Technologies, 459140, clone #16D10AD9AH5)
peroxidase-conjugated AffiniPure goat anti-mouse IgG (H+L) antibody (Jackson ImmunoResearch Laboratories, 315-035-003)
Alexa Fluor® 488 goat anti-mouse IgG (H+L), highly cross-absorbed (Life technologies, A-11029)
Immobilon Western (Millipore, WBKLS0500)
SlowFade® Gold (Life technologies, S36936)
Hoechst 33342 1 mg/ml H_2O (Wako, 346-07951)

EQUIPMENT

CO_2 incubator (Panasonic, MCO-175-PJ)
Fluorescence microscope (Olympus, IX81) equipped with a 60X PlanApo N oil immersion lens (Olympus, 1.42NA) and a charge-coupled device camera (Hamatsu Photonics, ORCA ER)
Confocal laser microscope (Olympus, FV1000D IX81), equipped with a 60X PlanApo oil immersion lens (Olympus, 1.42NA)
Image analyzer (Fujifilm, LAS-3000mini)
Flow cytometer (BD Biosciences, FACSCalibur HG)

REAGENT PREPARATION

CCCP stock solution (20 mM in DMSO); dissolve 4.09 mg CCCP in 1 ml DMSO
bafilomycin A_1 (0.1 mM in DMSO); add 160.6 ul DMSO to 10 ug bafilomycin A_1

lysis buffer; 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-00, 0.5 M EDTA
100 mM PMSF; dissolve 0.174 g PMSF in 10 ml ethanol
6x sample buffer; 280 mM Tris-HCl (pH 6.8), 30% glycerol, 10% SDS, 9.3% dithiothreitol, a trace of bromophenol blue to add color

TROUBLESHOOTING TIPS

1. No induction of mitophagy.

Observe the shape of mitochondria by fluorescence microscopy. If mitochondria are not fragmented, CCCP treatment is not successful. Mitotracker Red staining after CCCP treatment is also a good indication for whether or not mitochondria properly lost their membrane potential. When mitochondria have properly lost the membrane potential but still mitophagy is not observed, the expression level of Parkin may not be enough. Obtain another cell line stably expressing a higher level of exogenous Parkin.

Observation of mitochondrial outer membrane proteins may give a good indication since they are ubiquitinated dependently on Parkin under mitophagy-inducing conditions and degraded by the proteasome [1-3]. If degradation of neither outer mitochondrial membrane proteins nor matrix proteins is observed, the induction has most likely failed. The effect of CCCP and the expression level of Parkin should be checked.

2. Unable to detect LC3 staining around mitochondria

The most common way to detect autophagosomes by fluorescence microscopy is to stain LC3 which is used as an autophagosome marker. However, TritonX-100 treatment influences LC3 immunostaining, while staining of mitochondrial matrix protein requires permeabilization by TritonX-100. If it is necessary to observe mitochondria and LC3 at the same time, Mitotracker Red staining (before CCCP treatment) is recommended and cells should be permeabilized by digitonin (for LC3 staining, see the protocol by Nishimura and Mizushima).

<u>IMPORTANT NOTE</u> LC3 puncta can sometimes be observed in the absence of isolation membranes/autophagosomes and the LC3 signals can colocalize with damaged mitochondria, for instance in FIP200 KO cells which lack isolation membranes [4]. Thus, colocalization between LC3 and damaged mitochondria does <u>NOT</u> necessarily indicate the presence of mitophagosomes. The only

conclusive way to observe mitophagosomes at the moment is by electron microscopy (without immunostaining to preserve membrane structures).

3. Mitochondria seem to disappear in the absence of autophagy
Outer mitochondrial membrane proteins such as TOM20 can be rapidly degraded by the proteasome under mitophagy inducing conditions [1, 2]. On the other hand, the degradation of matrix proteins and mitochondrial DNA is almost exclusively by mitophagy under the same condition. It is essential to observe either matrix proteins or mitochondrial DNA (see the article by Matsuda) to monitor mitophagy.

4. Mitophagy seems to be retarded by disruption of a certain gene
Mitophagy induction can significantly vary among cell lines. One of the factors which can affect the mitophagy induction is the expression level of exogenous Parkin. It is, therefore, recommended to re-express the gene of interest by stable expression of the gene itself (for knockout) or an siRNA-resistant construct (for knockdown).

REFERENCES

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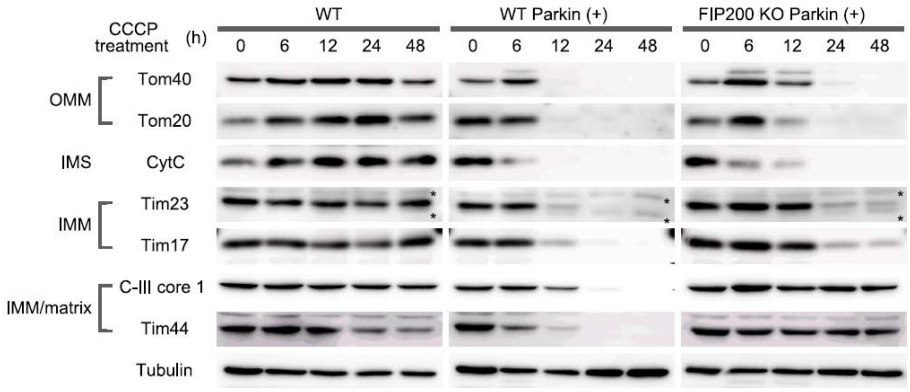

Figure 1. Mitochondrial protein degradation detected by Western blotting.
Wild-type (WT) MEFs (with or without stable Parkin expression) and autophagy-deficient FIP200 KO MEFs (with stable Parkin expression) were treated with 20 μM CCCP for indicated time periods. The cells were analyzed by SDS-PAGE and subsequent immunoblotting with antibodies against Tom40 and Tom20 (outer mitochondrial membrane (OMM) proteins), cytochrome *c* (CytC) (an intermembrane space (IMS) protein), Tim23 and Tim17 (inner mitochondrial membrane (IMM) proteins), and complex III (C-III) core 1 and Tim44 (IMM/matrix proteins) as well as α-tubulin (a loading control). Asterisks indicate nonspecific immunoreactive bands. The data were modified from Yoshii et al. (2011)[2].

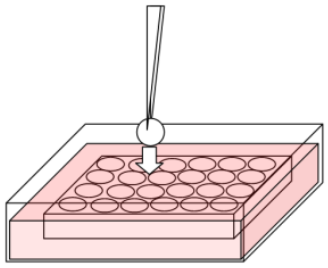

Figure 2. Antigen retrieval for C-III core 1
Put an empty 24 well dish in a heat resistant container, fill ion-exchange water in the container, and preheat and boil water by microwave. Carefully put cover slips in each well and boil for 5 min by microwave, and let the water cool down to room temperature

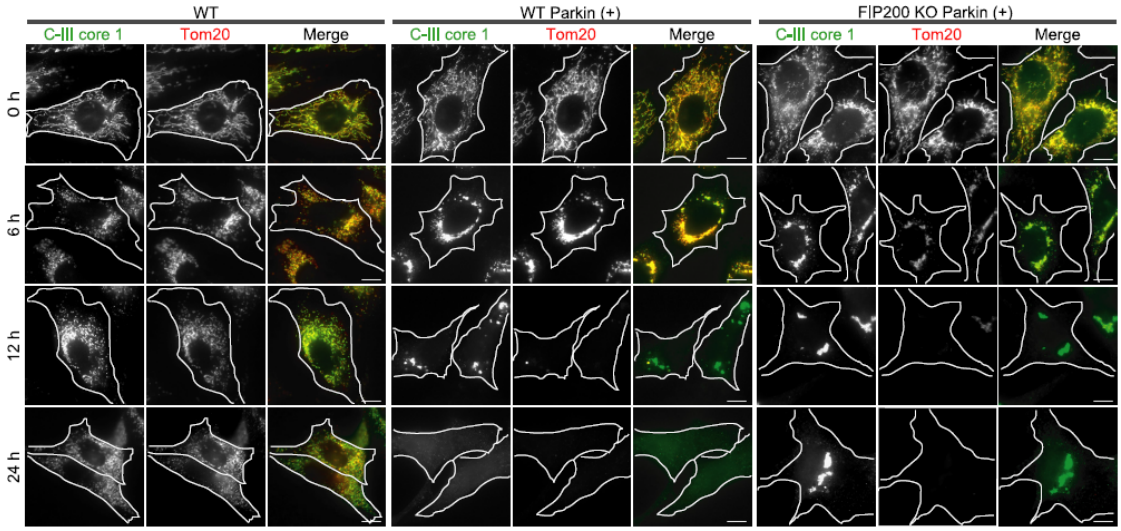

Figure 3.

Wild-type (WT) MEFs (with or without stable Parkin expression) and autophagy-deficient FIP200 KO MEFs (with stable Parkin expression) were treated with 20 μM CCCP for indicated time periods. Cells were immunostained for C-III core 1 (an IMM/matrix protein) and Tom20 (an OMM protein). The data were modified from Yoshii et al. (2011)[2].