Depletion of mitochondria in mammalian cells through enforced mitophagy

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Abstract

Mitochondria are not only the “power-house” of the cell, but are also involved in a multitude of processes that include calcium storage, cell-cycle and cell-death. Traditional means to investigate mitochondrial importance in a given cellular process have centred upon depletion of mitochondrial DNA (mtDNA) through chemical or genetic means. While these methods severely disrupt mitochondrial electron transport chain, mtDNA-depleted cells still maintain mitochondria and many mitochondrial functions. Here, we describe a straightforward protocol to generate mammalian cell populations with low to non-detectable levels of mitochondria. Ectopic expression of the ubiquitin E3 ligase Parkin, combined with short-term mitochondrial uncoupler treatment, engages widespread mitophagy, and effectively eliminates mitochondria. In this protocol, we explain how to generate mitochondria-depleted cells and a variety of methods to confirm mitochondrial clearance. Furthermore, we describe culture conditions to maintain mitochondrial-depleted cells with minimal loss of viability for longitudinal studies. This method should prove useful to investigate the importance of mitochondria in a variety of biological processes.

Introduction

Mitochondria have traditionally been viewed as the powerhouse of the cell due to their central role in metabolism and energy production. More recently, mitochondria have been implicated in a wide-variety of other cellular roles including calcium storage, migration, cell death, senescence and inflammation[1]. Equally, impairment of mitochondrial homeostasis has been heavily linked to a wide range of diseases[2,3]; for this reason understanding the mechanisms that regulate mitochondrial health is of fundamental importance. To this end, eukaryotic cells are equipped with a range of mitochondrial quality control (QC) mechanisms [4,5].

Mitochondria-specific autophagy, hereafter termed mitophagy, is a key mitochondrial QC mechanism that helps maintain mitochondrial fitness by efficiently removing dysfunctional organelles. While several different mechanisms regulating mitophagy have been described in mammalian cells, the best understood is PINK1/PARKIN-mediated mitophagy[6]. In this process, mitophagy occurs primarily following a complex interplay between the Ser/Thr kinase PINK1 and the E3 ubiquitin ligase PARKIN[4]. In healthy mitochondria PINK1 is subject to constant turnover; cleavage of its mitochondrial targeting sequence by the mitochondria intermembrane space protease PARL reveals a destabilizing N-terminal residue that targets PINK for proteasomal degradation[7,8]. Disruption of mitochondrial function causes an accumulation of PINK1 on the mitochondrial outer

membrane where it phosphorylates ubiquitin and Parkin leading to activation of the PARKIN E3 ubiquitin ligase at the mitochondria[9-11]. Parkin recruitment to mitochondria requires mitofusin 2 (MFN2) in some settings [12,13] but is dispensible in others [14]. In a feed-forward mechanism, Parkin ubiquitinates mitochondrial substrates that, in turn, leads to more PINK1 substrate phosphorylation and Parkin activity. Ubiquitinated mitochondria are targeted for autophagy following recognition by specific ubiquitin binding adaptor proteins[9]. Importantly, experimentally induced PINK1/Parkin-mediated mitophagy can reduce mitochondrial levels to below the level of detection, thereby opening its use as an experimental tool to study mitochondrial function[15,16].

Development of an assay that allows the generation of mitochondria-depleted cells

A well-established approach to study mitochondrial function in various processes centres upon depleting mitochondrial DNA (mtDNA) to generate so-called ρ^0 cells [17]. Typically this is achieved by long-term culture in the presence of ethidium bromide (EtBr) that inhibits replication of mitochondrial but not nuclear DNA. Nevertheless, this method has various drawbacks including the extended time required to generate ρ^0 cells (typically weeks) and potential unwanted mutagenic effects on genomic DNA caused by EtBr treatment. Alternative methods to deplete mtDNA and generate ρ^0 cells include mitochondrial targeting of the restriction endonuclease EcoRI [18] or expression of a dominant negative form of Polγ, a gene that codes for the catalytic subunit of the mitochondrial DNA polymerase [19]. However, these solutions do not circumvent a major drawback, which is that ρ^0 cells still contain functional mitochondria. For example, mitochondrial apoptosis proceeds efficiently in ρ^0 cells [20]. This prompted us to develop a method to remove mitochondria, allowing the study of cellular functions in the absence of all aspects of mitochondrial biology.

Herein, we describe a facile protocol that exploits PINK1/Parkin-mediated mitophagy as a tool to investigate mitochondrial function. In short, the method entails overexpression of Parkin and short-term mitochondrial depolarization to stimulate widespread mitophagy. This generates mammalian cells with low to non-detectable mitochondrial content allowing comparison of matched cells proficient or deficient in mitochondria (Figure 1). Demonstrating the protocol's effectiveness mitochondria-depleted cells show no detectable mitochondrial respiration, proteins or mtDNA, and strikingly no mitochondrial organelles were observed by 3D electron microscopy [15,16]. Following mitochondrial depletion, cells fail to repopulate their mitochondrial population over extended periods of time (16 days post removal of CCCP), demonstrating effective depletion. Furthermore, we have developed culture conditions to keep mitochondrial-depleted cells in culture with minimal loss of viability for long periods of time ranging from 5 days to 1 month (depending on the cell type) allowing extensive longitudinal studies.

Applications of the widespread-mitophagy protocol

1. *Investigating the role of mitochondria in cell death*

Mitochondria are often essential in the execution of programmed cell death (or apoptosis). The intrinsic pathway of apoptosis is also called "mitochondrial apoptosis" since the permeabilisation of mitochondria is required for caspase activation and cell death [21]. A recently described non-apoptotic type of cell death is necroptosis (programmed necrosis) that requires the executioner protein mixed lineage kinase domain-like (MLKL) [22]. Mitochondria, through the generation of ROS, were considered essential for the execution of necroptosis [23-25]. To directly test this, we used Parkin-mediated mitophagy to efficiently remove mitochondria and assess its effect on necroptosis and apoptosis. As expected, mitochondrial-depleted cells were resistant to mitochondrial apoptosis [15]. However, while Parkin-mediated mitophagy completely prevented ROS production, it did not impact on necroptosis execution. This provided the first evidence that mitochondria are dispensable for the execution of necroptosis [15].

2. *Investigating the role of mitochondria in cellular senescence*

Senescence is a cellular response to a variety of stresses, including oncogene activation, DNA damage and telomere dysfunction, that permanently arrests the cell. Senescence has been involved in several processes including development, cancer and tissue remodelling. Furthermore, increasing evidence suggests that senescence are major contributors to age-related tissue dysfunction and pathologies, mostly by the development of a pro-oxidant and pro-inflammatory phenotype [26]. Mitochondrial dysfunction is a feature of senescence [27] and has been shown to induce and stabilise the senescence arrest mostly via generation of ROS-driven cellular damage [28]. Despite the fact that multiple biochemical reactions occur within mitochondria, it is still relatively unknown what other factors apart from ROS impact on the senescent phenotype. By inducing Parkin-mediated widespread mitophagy our group has demonstrated that these organelles are required for the development of both the pro-oxidant and pro-inflammatory senescence-associated phenotypes [16]. This approach demonstrated the requirement of mitochondria for the development of several features of the senescent phenotype which are considered drivers of the ageing process, suggesting these organelles as putative therapeutic targets for interventions to inhibit senescence and ageing.

3. *Mitophagy as readout of autophagic flux*

Autophagy is a recycling process delivering various cytosolic cargoes (including whole organelles such as mitochondria or the peroxisomes) to lysosomes for degradation. Autophagy has lately received considerable attention due to its involvement in a multitude of human diseases, ranging from neurodegenerative disorders to cancer [29,30]. It is therefore important to develop reliable methods for assessing the autophagic flux *in vitro*. This is especially relevant when testing new autophagy inhibitors that could be translated into clinical trials. The most commonly used method to characterize autophagy is the western blotting or immunofluorescence detection of lipidated LC3 (or LC3-II) which is indicative of autophagosome assembly. Nevertheless, inhibition of autophagosome breakdown or stimulation of autophagy can both increase LC3 II levels thereby complicating analysis. As such, a quantitative measure of autophagic flux is a much preferable measure. To address this issue, our lab has recently used the Parkin-mediated mitophagy protocol as a read-out of autophagic flux[31]. Briefly, mitophagy was used to detect differences in the autophagic flux between various cell lines with accurate detection and characterization of the effect of pharmacological or genetic inhibition/promotion of autophagy[31].

4. Additional Applications

Generation of transmitochondrial cybrids. Using this method, mitochondria can be eliminated from cells followed by cell fusion with cytoplasts containing different degrees of mtDNA mutations. This method allows the investigation of mitochondrial diseases, without the need to use of ρ^0 cells- which still contain dysfunctional mitochondrial organelles.

Investigating **mitochondrial biogenesis**: We have found that if lower concentrations of CCCP are used and incomplete Parkin-mediated mitochondrial clearance occurs, mitochondria can repopulate. This will allow cell studies to investigate kinetics and determinants of mitochondrial replication.

Drug/genetic screens aiming to identify novel pathways that are dependent/independent of mitochondrial function.

Advantages, limitations and adaptations

Traditional methods to perturb mitochondrial function have relied on deletion of mtDNA, typically through long-term ethidium bromide treatment [17]. Nevertheless, while this effectively depletes mtDNA, mitochondria themselves and many of its associated functions remain in the cell. Moreover, it takes a considerable amount of time to achieve mtDNA depletion (weeks to months) and ethidium bromide may exert unwanted effects upon nuclear DNA. The protocol described below, using the Parkin-mediated mitochondrial depletion, addresses these issues by completely removing mitochondria in a quick (24 - 48 hours) and specific

manner. Furthermore, this approach enables the generation of cell populations that show high penetrance of mitochondrial depletion (over 95% of cells with no detectable mitochondria) and therefore greatly facilitates the study of mitochondria in various cellular processes.

Potential limitations of this assay are that some cells do not readily survive mitochondrial depletion. In our own experience, all cell lines we have used show minimal cytotoxicity in reponse to short-term CCCP or antimycin A/oligomycin treatment. However, activation of Parkin-mediated mitophagy can cause extensive caspase-dependent apoptosis in some cell lines (HeLa for example). For unclear reasons, this cytoxicity appears cell-type dependent nevertheless it can be prevented by the co-application of caspase inhibitors. Secondly, the use of chemicals such as CCCP to induce mitophagy can have unwanted off-target effects[32]. Additionally, effective mitochondrial depletion requires ectopic expression of Parkin which may exert unwanted effects on the biological process being investigated. An approach to circumvent this may be through the use of an inducible Parkin expression vector, such that Parkin expression is switched off post-mitophagy. Finally, while the transduction of immortalised cells is often highly efficient, generation of stable cell lines using primary cells can be more troublesome - below we describe a protocol for both immortalised and primary cell lines.

Experimental design

The protocol can be divided into three main parts: generation of a stable YFP-Parkin-expressing cell line (Steps 1–10); induction of widespread mitophagy (Steps 11-13); and long-term culture of mitochondria-depleted cells. We finish with a brief summary for readouts of widespread mitophagy.

Generation of a stable YFP-Parkin-expressing cell line. In this protocol, we describe how to generate a stable YFP-Parkin-expressing cell line in primary human fibroblasts. Nevertheless, similar procedures have been successfully tested in other human primary (e.g., IMR-90 primary) and immortalised cells (e.g. KP-4 and HeLa cells) and murine cells (e.g. SVEC and 3T3 cell). We also describe cell culture conditions were primary fibroblasts can be maintained viable for up to a month following mitochondrial depletion allowing for kinetics assays and long-term studies.

In this protocol we use Phoenix Amphotropic cells to produce retroviral particles. Phoenix™ Amphotropic packaging cell lines are second generation retrovirus producer lines that were generated from human embryonic kidney (HEK) 293T cells[33,34]. In addition to the temperature sensitive T antigen co-selected with neomycin already present in HEK293T

cells, Phoenix™ Amphotropic cells contain two extra constructs: a construct capable of producing the gag-pol and a construct for the codification of the envelope protein for amphotropic viruses. Both the gag-pol and the envelope protein sequences were introduced with hygromycin and diphtheria toxin resistance as the co-selectable markers, respectively. The expression of each constructs is under regulation of different non-Moloney promoters to minimize recombination potential. The incorporation of these constructs in the Phoenix cells makes the transfection process relatively easy in these cells, where packaging cells (Phoenix™ Amphotropic cells), retroviral vectors and the transfection reagent are the only required transfection components (no requirement of additional packaging constructs). This cell line is highly transfectable with either calcium phosphate mediated transfection or lipid-based transfection protocols, with ≥ 50% cells being transiently transfected. In this protocol we use and describe a lipid-based transfection protocol using Lipofectamine™.

Next, we describe a protocol for transduction of YFP-Parkin retroviral particles into human primary fibroblasts. Human diploid fibroblasts, such as MRC5 fibroblasts, undergo a finite number of cell divisions or population doublings (PD) before permanently arresting in the cell cycle in a state termed replicative senescence [35]. Therefore, following a precise protocol when culturing and generating stable primary human fibroblasts cell lines is essential. For this reason, calculating the PD of human primary fibroblasts is required to evaluate the numbers of cell divisions that these cells have undergone and avoid transducing cells that are reaching their proliferative limit. Human fibroblasts are also sensitive to cell confluency: under confluency (≤ 40% cell density) or over confluency (≥100 % cell density) can induce cellular stress and lead to premature senescence. It is thus necessary to calculate cell density for optimised cell fitness, transduction and posterior selection with antibiotics (e.g. Zeocin™).

Induction of widespread Mitophagy. In this protocol, widespread mitophagy is achieved by inducing overall mitochondrial depolarisation. Carbonyl cyanide 3-chlorophenylhydrazone (CCCP) is a protonophore (H^+ ionophore) and uncoupler of oxidative phosphorylation in mitochondria that promotes loss $\Delta\psi m$ and morphological swelling [36]. Treatment of YFP-Parkin-expressing cells with CCCP ensues mitochondrial dysfunction and translocation of Parkin to mitochondria, where it binds to PINK1 that has accumulated in the outer-mitochondrial membrane. An alternative to CCCP, as it can show off-target effects on lysosomal function [32], is a mix of Antimycin A and Oligomycin A (in equimolar amounts) which are specific mitochondrial uncouplers. Typically, the widespread mitophagy protocol takes 48 hours. While immortalized cells require treatment with the mitochondrial membrane uncoupler every 12 hours, primary cells only require treatment every 24 hours for 48 hours.

Note, the mitochondrial uncoupler dose and treatment duration should be tested for each cell line in order to efficiently induce widespread mitochondrial depletion.

Long-term culture of mitochondria-depleted cells

Here we describe the culture conditions that allow long-term culturing of cells depleted of mitochondria. This is mainly achieved by supplementing the growth media with uridine.

Assessment of mitophagy. The effectiveness of induced mitophagy can be assessed in multiple ways but typically relies upon detection of loss of mitochondrial protein(s) signal or loss of mtDNA. Importantly, Parkin has been shown to target some mitochondrial proteins for proteasomal degradation independently of mitophagy [37,38]. Therefore, multiple approaches should be used, at least in initial experiments, to verify the method is functional.

Materials

REAGENTS

Cell culture

Critical: The cell lines used should be regularly checked to ensure that they are authentic and not infected with Mycoplasma. Although mycoplasma contamination does not affect induction of mitophagy it can affect several cellular functions and viability.

The generation of a YFP-Parkin expressing cell line involves the culturing of a packaging cell line for YFP-Parkin retroviral production, in this case Phoenix™ Amphotropic cells, and a cell line that will be subject to YFP-Parkin-retroviral transduction and become the YFP-Parkin expressing cell line (Table 1).

The indicated reagents or suppliers listed below can be substituted with appropriate alternatives if necessary.

- Dulbecco's modified Eagle's medium (DMEM, Cat. Number D5796, Sigma)
- Penicillin-Streptomycin (Cat. Number P4333, Sigma)
- Fetal bovine Serum (FBS, Cat. Number F9665, Sigma, Dorset)
- L-Glutamine (Cat. Number G7513, Sigma, Dorset)
- Trypsin-EDTA (TE) (Cat.Number T3924, Sigma)

- Opti-MEM® I Medium (Cat. Number 11058-021, Thermo Fisher Scientic)
- MEM NEAA (Minimum Essential Medium Non-Essential Amino Acids) (Cat. Number 11140, Life Technologies)
- Sodium pyruvate (Cat. Number S8636, Sigma-Aldrich)
- 2-Mercaptoethanol (Cat. Number 21985, Life Technologies)
- Phosphate Buffer Saline (Cat. Number D1408, Sigma)

YFP-Parkin vector transfection and retroviral transduction

- YFP-Parkin-IRES-zeocin (Addgene, ID number 61728)
  Note: mCherry-Parkin-IRES-zeocin is also available (Addgene, ID number 61727)
- Lipofectamine™ 2000 (Cat. Number 11668-019, Invitrogen,)
- Polybrene (Cat . Number sc-134220, Santa Cruz)
  Caution: Polybrene is toxic if swallowed or in contact with the skin/eyes. Avoid contact with skin and eyes. Avoid formation of aerosols and provide appropriate exhaust ventilation at places where aerosols are formed.

Selection of efficiently transduced cells

- Zeocin™ (Cat. Number R25001, Invitrogen)

  Caution: Zeocin™ is toxic if swallowed or in contact with the skin/eyes. Zeocin™ is also suspected of causing genetic defects (mutagen). Avoid direct contact, and use gloves while preparing and using Zeocin™. Avoid formation of aerosols and provide appropriate exhaust ventilation.

Induction of mitophagy

- Carbonyl cyanide 3-chlorophenylhydrazone (CCCP, Cat number C2759, Sigma)

  Caution: CCCP is toxic if swallowed, in contact with the skin/eyes or if inhaled. Avoid direct contact, and use gloves while preparing and using CCCP. Avoid formation of dust and aerosols. Provide appropriate exhaust ventilation.

- Antimycin A (Cat number A8674, Sigma)

  Caution: Antimycin A is fatal if swallowed and toxic if in contact with the skin/eyes or if inhaled. Avoid direct contact, and use gloves while preparing and using Antimycin A. Avoid formation of dust and aerosols. Provide appropriate exhaust ventilation at

places where dust/ aerosols is formed. Antimycin A is also toxic to aquatic life and therefore requires disposal of contents/ container to an approved waste disposal plant.

- Oligomycin A (Cat number 75351, Sigma)

  Caution: Oligomycin A is harmful if swallowed, in contact with the skin/eyes or if inhaled. Avoid direct contact, and use gloves while preparing and using Oligomycin A. Avoid formation of dust and aerosols. Provide appropriate exhaust ventilation at places where dust/aerosols is formed.

Assessment of widespread mitophagy

Detection of mitochondrial proteins

Detection of mitochondrial proteins using western blot and/or immunofluorescence staining can be performed using conventional protocols and reagents. In Table 2 we describe the antibodies used for mitochondrial proteins detection used in this protocol.

Detection of mitochondrial proteins can also be achieved by live-cell imaging of YFP-Parkin cells expressing a mitochondrial targeted protein (for instance mito-dsRRed; Clontech, Cat. Number 632421).

Quantification of mtDNA abundance

Detection of mtDNA is performed by qPCR. Below we describe the mtDNA and nuclear (control) primers for mtDNA analysis used in this protocol (Table 3).

EQUIPMENT

- Centrifuge tubes, 15 ml (Cat. Number E1415-0200, Starlab)
- Centrifuge tubes, 50 ml (Cat. Number E1450-0200, Starlab)
- Microcentrifuge tubes, 1.5 ml (Cat. Number E1415-1510, Starlab)
- Refrigerators, 4 and −20 °C
- Heracell^{TM} 150i CO incubator (ThermoScientific)
- Microcentrifuge 5415R (Eppendorf)

- Corning® cell culture flasks surface area 75 cm2, canted neck (Cat. Number CLS430641-100EA, Sigma)
- Corning® Costar® cell culture plates 6 well (Cat. Nu,mber CLS3506-100EA, Sigma)
- Corning® Costar® cell culture plates 12 well (Cat. Nu,mber CLS3512-100EA, Sigma)
- Corning Tissue Culture Treated Culture Dishes 100mm X 20mm (Cat. Nubmber CLS430167-500EA, Sigma)
- Cover Glass, Ø 19mm, Thickness 0.16mm (Cat. Number 631-0156, VWR)
- Millex-HV 0.45 µm PVDF filters (Millipore, cat. no. SLHVR25LS)
- 35 mm glass-bottom dishes (Cat Number P35G-1.5-20-C, MatTek)
- Nikon TE2000 microscope with Perfect Focus System (PFS). The system is equipped with a Sutter Lambda 10-3 controller, ASI MS2 XY stage with linear encoders, Photometrics CoolSnap HQ CCD camera and Metamorph 7.5 software.

REAGENT SETUP

Culture medium for human Phoenix Amphotropic cells

Supplement DMEM medium with 10% (vol/vol) FBS, 100 units/ml penicillin, 100 µg.ml^{-1} streptomycin, 2 mM glutamine, 1mM sodium pyruvate, 300 µg.ml^{-1} Hygromycin B and 1 µg.ml^{-1} Diphtheria toxin. This medium can be stored at 4 °C for up to 1 month.

Culture medium for human MRC5 fibroblasts

Supplement DMEM medium with 10% (vol/vol) FBS, 100 units/ml penicillin, 100 µg.ml^{-1} streptomycin and 2 mM glutamine. This medium can be stored at 4 °C for up to 1 month.

Culture medium for human MRC5 fibroblasts without mitochondria

Supplement DMEM medium with 10% (vol/vol) FBS, 100 units/ml penicillin, 100 µg.ml^{-1} streptomycin, 2 mM glutamine and 50 µg.ml^{-1} uridine. This medium can be stored at 4 °C for up to 1 month.

Mitochondrial membrane uncouplers

- Dissolve CCCP in DMSO to a stock concentration of 125 mM. Dilute to a final concentration of 12.5 µM (1:10000) in complete medium, just before adding it to the

cells (add the same volume of DMSO to complete medium if needed a vehicle control). CCCP (stock solution of 125 mM) can be stored at -20 °C, protected from light for 6 months.

Critical: Prepare small aliquots of CCCP stock solution (125 mM) and freeze them at -20°C. Avoid freeze/thawing cycles as this may reduce CCCP efficiency.

- Dilute Antimycin A and Oligomycin A in ethanol (100%) to a stock concentration of 1 mM and small volume aliquots (20-50 µl) are kept at -20°C. Dilute to a final concentration of 1 µM (1:1000) in complete medium, just before adding to the cells (add the same volume of ethanol to complete medium if needed a vehicle control). Antimycin A and Oligomycin A solutions can be stored at -20 °C for 6 months.

Procedure

Generation of YFP-Parkin-expressing cell line

Steps 1 to 4 summarize the transfection of YFP-Parkin vector into Phoenix™ Amphotrophic cell and generation of retroviral particles. Timing: 4-5 days

Critical step: Transfection protocols are performed following Biosafety level 2 (BL-2) procedures.

1. Day 1

   Seed 5×10^6 Phoenix™ Amphotropic cells in 10 ml of growth medium containing serum (no antibiotics) in a 10 cm tissue culture plate so that they will be 90–95% confluent on the next day (day of transfection).

2. Day 2

Before transfection, remove the culture medium from the Phoenix™ Amphotropic cells and replace with 5 ml of Opti-MEM® I Medium containing serum (no antibiotics). For each transfection sample (pLZRS-YFP-Parkin and pLZRS-empty vector (EV) expression plasmid DNA), prepare DNA-Lipofectamine™ 2000 complexes as follows:

2.1 In sterile 15 ml tubes, dilute 3 µg of each expression plasmid DNA

(pLZRS-EV and pLZRS-YFP-Parkin) in 1.5 ml of Opti-MEM® I Medium without serum and mix gently.

2.2 In a separate sterile 15 ml tube, dilute 9 µl of Lipofectamine™ 2000 in 1.5 ml of Opti-MEM® I Medium without serum. Mix gently and incubate for 5 minutes at room temperature (20°C). **Note:** Mix Lipofectamine™ 2000 gently before use.

2.3 After a 5-minute incubation, combine the diluted DNA with the diluted Lipofectamine™ 2000 and mix gently. Allow for DNA-Lipofectamine™ 2000 complexes formation by incubating for 20 minutes at room temperature. The solution may appear cloudy, but this does not impede the transfection.

2.4 Add the DNA-Lipofectamine™ 2000 complexes dropwise to each plate of cells. Mix the plate gently by rocking back and forward and incubate cells overnight at 37°C in a humidified 5% CO_2 incubator.

Critical: Plasmid DNA for transfection into eukaryotic cells should very clean and free from contamination with phenol and sodium chloride. Contaminants can cause cytotoxicity, and salt will interfere with lipid complexing thereby decreasing transfection efficiency.

3. Day 3

Remove medium containing the DNA- Lipofectamine™ 2000 complexes and replace with 10 ml complete culture medium (no antibiotics). Incubate at 37°C in a humidified 5% CO_2 incubator.

4. Day 4 – 5

4.1 Collect virus-containing supernatants 48 hours post-transfection into to a 15 ml sterile, capped, conical tube. Collection of virus-containing supernatants can also be done 72 hours post-transfection, however minimal differences in viral yield are observed whether supernatants are collected at either 48 or 72 hours post-transfection.

Caution: At this stage, follow the guidelines for working with BL-2 organisms.

4.2 Centrifuge virus-containing supernatants at 1600 g for 15 minutes at 4°C to pellet debris, followed by supernatant filtration through a Millex-HV 0.45 µm or equivalent PVDF filter. This step prevents contamination of packaging cells (retroviral producers) into the cell line to be transduced.

Caution: At this stage, follow the guidelines for working with BL-2 organisms.

Steps 5 to 10 are a brief protocol of YFP-Parkin retroviral transduction in human primary fibroblasts. The procedure is similar for other mammalian cell lines.

Timing: 15-17 days

Critical: Transduction protocols are performed following BL-2 procedures.

Critical: When using human primary cells, use cells at a lower PD in order to avoid producing a cell line that by the end of the transduction process is senescent or approaching senescence. To calculate the PD, cells should be trypsinized and counted for total number of cells calculation. The following formula should then be applied: PD=X ± (Ln(n2/n1)/Ln2, where X is the number of the accumulated PD; n2 is the total number of cells obtained and n1 is the number of cells initially seeded in the plate.

5. Day 1

Seed 2 x 10^5 cells/well into a 6-well plate (cells should be 50-60% confluent at the time of transduction). Incubate cells at 37°C overnight in a humidified 5% CO_2 incubator.

Critical: Primary human fibroblasts do not form colonies when proliferating and therefore do not allow for viral titering, which permits optimization of viral transduction volumes for efficient transductions. Furthermore, efficiency of transfection and viral particles generation can vary from experiment to experiment and with the size of the plasmid DNA. We advise performing transduction with several viral-supernatant volumes. Example: seed 3 wells in a 6-well plate: 1) for 1 mL viral transduction; 2) for 2 mL viral transduction and 3) for non-transduced control (mock).

6. Day 2

Add the purified media with 10 µg/ml polybrene to cells at 50-60% cell confluency. Incubate at 37°C overnight in a humidified 5% CO_2 incubator. Note that this step should be done immediately after step 4.2, thus seeding the cells for transduction (step 5)

should be performed the day before to viral collection. Alternatively, viral particles can be frozen at -80°C after collection and purification (note that in some cases transduction efficiency can be affected if using viral particles that were previously frozen; avoid freeze/thaw cycles).

7. Day 3

Remove the media containing viral particles and refresh the media with 2 ml of complete culture medium. Incubate at 37°C overnight in a humidified 5% CO_2 incubator.

Caution: Viral particle containing medium should be disposed according to BL-2 safety procedures.

8. Day 4

Remove the medium and wash the cells once with PBS. Trypsinize the cells and re-plate all cells (eachwell of a 6-well plate) into one 10 cm plate containing complete culture medium. Incubate at 37°C overnight in a humidified 5% CO_2 incubator.

Caution: Medium containing residual viral particles should be disposed according to BL-2 safety procedures.

9. Day 5

For Zeocin™ selection, add 10 mL complete culture medium with 250 µM Zeocin™ to select for stably transduced cells. Human primary fibroblasts should be at 40-50% cell confluency when adding Zeocin™ for maximum selection efficiency. Replace medium with fresh medium containing 250 µM Zeocin™ every 3-4 days.

Note: If using a different cell line, prior to this step a survival curve with different Zeocin™ concentrations should be performed on non-transduced cells to select for the lowest dose of Zeocin™ that causes 100% cell death.

10. Day 15-17

After 10-12 days of selection (day 14-16), stably YFP-Parkin expressing fibroblasts should have repopulated the transduced dish and no live cells should be observed in the

mock dish. Remove the medium and replace with complete media. During this period, efficiently transduced cells may need to be passaged into bigger flasks as they reach confluency.

Critical: Store Zeocin™ at –20°C and thaw on ice before use. Zeocin™ is light sensitive. Store the drug, and medium containing drug, in the dark at 4°C. Culture medium containing Zeocin™ may be stored at 4°C protected from exposure to light for up to 1 month.

Induction of widespread mitophagy

Timing: 4 days, up to 6 days

In this protocol, we describe general conditions for the induction of widespread mitophagy in primary cells. Despite following a similar protocol, primary and immortalised cells can differ in a few aspects of the protocol; below we describe the details regarding these differences.

Critical: Cell density prior and during induction of widespread mitophagy can impact on the success of the protocol.

11. Day 1

11.1 Seed 1.5x 10^6 human primary fibroblasts (MRC5 or IMR90) (20<PD<25) into 10 cm culture dishes in 10 ml of complete culture medium. Incubate cells at 37°C overnight in a humidified 5% CO_2 incubator.

11.2 Seed cells for mitochondria depletion QC assays: 5x10^5 cells/well in 6-well plates, for mitochondrial protein expression analysis by western blot or mtDNA copy number by qPCR, or 4x10^4 cells/well in coverslips in 12-well plates, for mitochondrial protein expression analysis by immunofluorescence staining, and incubate at 37°C overnight in a humidified 5% CO_2 incubator.

Note: Mitochondria depletion QC assays should be performed immediately after removal of the mitophagy inducer drug. Nevertheless, if the main experiment requires culturing cells for longer (e.g. 2 days after removal of the mitochondria uncoupler drug), then extra cells should be seeded and harvested, at the same time as the cells in the main experiment, for QC assays.

Critical: Cells should be 70% confluent the next day. The number of cells seeded is dependent on the PD value, with advancing PDs cells start to become bigger (pre-senescent), which impacts on cell culture density (e.g. lower PD cells require to be

seeded in higher density). It is, therefore, critical to adjust the number of cells to the PD value.

Note: Other cell lines, particularly immortalised cells, may require different cell culture density when applying this protocol (e.g. HeLa cells should be at 10-20% confluency prior induction of mitophagy for optimal efficiency of the protocol).

12. Day 2 – 3

Treat cells every 24 hours, during 48 hours, with 12.5 μM CCCP in complete medium.
Note: Unlike primary cells, Immortalised cells (e.g. KP-4, HeLa, 3T3 and SVEC cells) are treated every 12 hours, during 48 hours, with 12.5 μM CCCP in complete medium. Cells can also be treated with 1 uM antimycin A and 1 uM oligomycin A in complete culture medium. If using other cell lines that are not mentioned in this protocol, the mitochondrial uncoupler dose and treatment duration may need to be adjusted.

Critical: CCCP, antimycin A and oligomycin A are not stable in the media for longer periods of time and therefore need to be refreshed at least every 24 hours.

13. Day 4

Most cells lines tested have undergone extensive mitophagy at this point. At this stage, harvest cells for mitochondria depletion QC assays and proceed with the main experiment.

13.1 *Cells for mitochondrial depletion QC assays*. Remove the medium containing CCCP and rinse cells once in pre-warmed PBS. Harvest cells for mitochondria depletion QC assays according to a pre-determined method (see below).

13.2 *Cells to proceed with the experiment*. Remove the medium containing CCCP and rinse cells once in pre-warmed PBS. Refresh the medium with complete medium and incubate cells at 37°C in a humidified 5% CO_2 incubator, for the duration of the experiment (up to 2 days post mitochondria depletion).

Long-term culture of mitochondria-depleted cells

Timing: 4 days, up to 30 days

Absence of mitochondria will impact on the function of the mitochondrial enzyme dihydroorotate dehydrogenase (DHODH) which is involved in pyrimidine synthesis (King and Attardi 1996). One way to overcome this is through the addition of uridine to the cell culture media. We have found that primary fibroblasts positively respond to the addition of uridine to the cell culture medium, allowing viability in culture for longer periods of time. Below, we describe how to culture mitochondria-depleted primary cells for a long period (up to 15 days in proliferating cells and up to 30 days in permanently arrested (senescent) cells).

14. Day 1

    Seed $1.5x10^6$ human primary fibroblasts (20<PD<25) into a 10 cm dish in complete media and allow cells to adhere at 37°C overnight in a humidified 5% CO_2 incubator. Cells should be 70-90% confluent the next day. At the same time, seed cells for mitochondria depletion QC assays as described previously.

    Critical: It is critical that cells are 70 to 90% confluent prior treatment with the mitochondria uncoupler. Cell density will depend on the duration of the experiment: the longer the experiment the higher the cell density required prior treatment, as mitochondria-depleted cells tend to become smaller with time, compromising optimal cell density/contact and viability.

15. Day 2 - 3

    Treat cells every 24 hours, over a 48 hour period, with 12.5 μM CCCP in complete medium. Cells can also be treated with 1 uM antimycin A and 1 uM oligomycin A in complete culture medium.

    Critical: CCCP, antimycin A and oligomycin A are not stable in the media on cells for longer periods of time and therefore need to be refreshed at least every 24 hours. Different primary cells may need adjusting drug refreshment periods.

16. Day 4

    Remove medium containing CCCP and replace with complete medium with additional 50ug/ml uridine. Incubate cells at 37°C in a humidified 5% CO_2 incubator. At this point cells should de devoid of mitochondria, collect cells for mitochondria

depletion QC assays, as described previously.

17. Day 5-up to 15

    Refresh medium supplemented with 50ug/ml uridine every 2 days until the end of your experiment. By the end of the experiment harvest cells for the main experiments and cells for mitochondria depletion QC assays as described previously.

    Critical: In long-term experiments, it is critical for cell fitness and viability that cells are cultured in medium supplemented with uridine, as it will partially overcome the limiting pyrimidine levels in mitochondria-depleted cells. Secondly, mitochondrial depletion should also be assessed at long-term post-depletion, to ensure viable cells are not simply the ones that escaped initial depletion.

Readouts of widespread mitophagy

We suggest the following conventional methods for determination of widespread mitochondria: western blotting (see Figure 2A & G), immunofluorescence staining for specific mitochondrial proteins followed by microscopy and/or flow cytometry analysis (see Figure 2D & E) and qPCR for mtDNA copy number (see Figure 2E). Antibodies used for Western blotting and Immmunofluorescence and primers for mtDNA copy number assays are described in the Material section.
Furthermore, we can detect clearance of mitochondria by live cell imaging of YFP-Parkin cells expressing a mitochondrial targeted protein (for instance mito-dsRRed) (See video Figure 1). Briefly, for live-cell imaging, plate YFP-Parkin expressing cells, transiently expressing mitochondrial targeted dsReD (mito-dsRed), on 35 mm glass-bottom dishes and image using a long-term time lapse Nikon TE2000 microscope. Prior to imaging, treat cells with antimycin and oligomycin using the protocol described above.

Troubleshooting

Troubleshooting advice can be found in Table 4.

Timing

Generation of Parkin encoding retrovirus, 4 – 5 days. Retroviral transduction and selection of Parkin expressing cells, 15 – 17 days. Induction of widespread mitophagy, 4 – 5 days. Long

term culture of mitochondria-depleted cells, up to 30 days. QC analysis of mitochondrial depletion, 2 – 3 days.

Anticipated results

In Figure 2, we show different experiments that validate the complete removal of mitochondria using the widespread mitophagy protocol. In panel A, KP-4 YFP-Parkin cells were treated with A/O for 48 hours and then immunoblotted with Mitoprofile. In panels B and C the same cells were immunostained for COXIV and TIMM44 and no mitochondria were observed. In panel D, we confirm mitochondrial depletion by using FACS for the same proteins as in B and C. In panel F we illustrate the procedure for clearance of mitochondria followed by long-term culture of cells (only applicable to human primary fibroblasts). In panel G we show expected western blotting results for several mitochondrial proteins: NDUFB8, SDHA, UQCRC2 and TOMM20 at day 4 and 16 after removal of CCCP. These results demonstrate that the initial CCCP treatment is sufficient for clearance of mitochondria and no repopulation occurs at later stages.

Figure legends

Figure 1. Overview of enforced mitophagy protocol

Cells stably expressing YFP-Parkin are treated with CCCP or a combination of Antimycin A and Oligomycin A for up to 48 h. This disrupts the mitochondrial inner membrane potential (Δψm), leading to PINK1 accumulation on the outer mitochondrial membrane and Parkin activation and recruitment at the mitochondria. Here, Parkin ubiquitinates a multitude of mitochondrial proteins that serves as signal for autophagosome engulfment of damaged mitochondria. Ultimately, all mitochondria are removed via mitophagy.

Figure 2. Validating Parkin-mediated widespread mitophagy

A. KP-4 cells stably expressing YFP-Parkin were treated with antimycin A and oligomycin A (AO) for 48 hours and then immune-blotted with a cocktail of antibodies recognizing various mitochondrial proteins. B-C. Following induction of mitophagy, KP-4 YFP-Parkin cells were immuno-stained for the mitochondrial proteins COXIV (B) and TIMM44 (C). Scale bar is 50 µm. D. Flow cytometry analysis showing reduced levels of COXIV and TIMM44 in AO-treated KP-4 cells expressing YFP-Parkin. E. mtDNA quantitative PCR on KP-4 YFP-Parkin cells after induction of mitophagy. A representative experiment (out of two) is shown with error bars representing standard deviation for three technical replicates. F. Workflow of widespread

mitophagy and long-term culture of human primary fibroblast with depleted mitochondria. G. Western blot analysis for mitochondrial content in primary fibroblasts 4 and 16 days after CCCP release.

Author contribution statements

C.C-M.,G.I., S.T. and J.P. developed the protocol. C.C-M.,G.I., S.T. and J.P designed the experiments described herein. G.I. and C.C-M. performed the experiments, interpreted the results and prepared figures. C.C-M.,G.I., S.T. and J.P. wrote the manuscript.

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Competing financial interests

The authors declare that they have no competing financial interests

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Table 1 | Cell lines for the generation of a stable YFP-Parkin-expressing cell line

Cell line	Species	Source	Cell type	Application
Phoenix Amphotropic	Human	ATCC (Cat. Number CRL-3213)	Immortalised	Viral particles generation
MRC5 Fibroblasts	Human	ECACC, Salisbury, UK (Cat. Number 05011802)	Primary	Viral transduction and generation of a stable YFP-PARKIN-expressing cell line
IMR-90 Fibroblasts	Human	ECACC, Salisbury, UK (Cat. Number 85020204)	Primary	Viral transduction and generation of a stable YFP-PARKIN-expressing cell line
KP-4	Human	RIKEN	Immortalised	Viral transduction and generation of a stable YFP-PARKIN-expressing cell line
U2OS	Human	ATCC (Cat. Number HTB-96)	Immortalised	Viral transduction and generation of a stable YFP-PARKIN-expressing cell line
MCF-7	Human	ATCC (Cat. Number HTB-22)	Immortalised	Viral transduction and generation of a stable YFP-PARKIN-expressing cell line
HeLa	Human	ATCC (Cat. Number CCL-2)	Immortalised	Viral transduction and generation of a stable YFP-PARKIN-

				expressing cell line
3T3-SA	Mouse	ATCC (Cat. Number CCL-92)	Immortalised	Viral transduction and generation of a stable YFP-PARKIN-expressing cell line
NIH-3T3	Mouse	ATCC (Cat. Number CRL-1658)	Immortalised	Viral transduction and generation of a stable YFP-PARKIN-expressing cell line
SVEC	Mouse	ATCC (Cat. Number CRL-2167)	Immortalised	Viral transduction and generation of a stable YFP-PARKIN-expressing cell line

Table 2 | Antibodies for widespread mitophagy QC assays

Primary Antibodies

Antibody	Species	Host	Reference/ Manufacturer	WB (dilution)	IMF (dilution)
MitoProfile Membrane Integrity antibody cocktail	Human Mouse Rat Cow	Mouse monoclonal	ab110414/ Abcam	1:1000	_____
TIMM44	Human Mouse	Rabbit monoclonal	ab194829/ Abcam	---------- -	1:200
COXIV	Human Mouse Rat Monkey	Mouse monoclonal	11967/ Cell Signaling	---------- -	1:200
SDHA	Human Mouse	Rabbit monoclonal	#11998/ Cell Signaling	1:1000	1:100
NDUFB8	Human Mouse	Mouse monoclonal	ab110242 - Abcam	1:1000	_____
UQCRC2	Human Mouse	Mouse monoclonal	ab14745 - Abcam	1:1000	_____
TOMM20	Human Mice	Mouse monoclonal	ab56783 - Abcam	1:1000	_____

GAPDH	Human Mouse	Rabbit monoclonal	#5174/ Cell sigalling	1:5000	_____
α-tubulin	Human Mouse rat	Mouse monoclonal	T6074/ Sigma-Aldrich	1:5000	_____
actin	Mammals Yeast Fungi	Mouse monoclonal	8691001/ MP Biomedicals	1:5000	_____

Secondary Antibodies

Secondary antibody	**Reference/ Manufacturer**	**WB (dilution)**	**IMF (dilution)**
Anti-rabbit Fluorescein-conjugated secondary antibody AlexaFluor 647	A21244/ Molecular Probes	_____	1:1000
Goat anti-rabbit IgG -HRP conjugated	A0545/ Sigma-Aldrich	1:5000	_____
Rabbit anti-mouse IgG -HRP conjugated	A2554/ Sigma-Aldrich	1:5000	_____

Table 3 | Primers for mtDNA qPRC

Gene		Primer Forward	Primer Reverse
mtDNA	*MTRNR2*	CGAAAGGACAAGAGAAATAAGG	
mtDNA	*MTTL1*		CTGTAAAGTTTTAAGTTTTATGCG
nuclear	*β-Globin*	CAACTTCATCCACGTTCACC	GAAGAGCCAAGGACAGGTAC

Table 4 | Troubleshooting

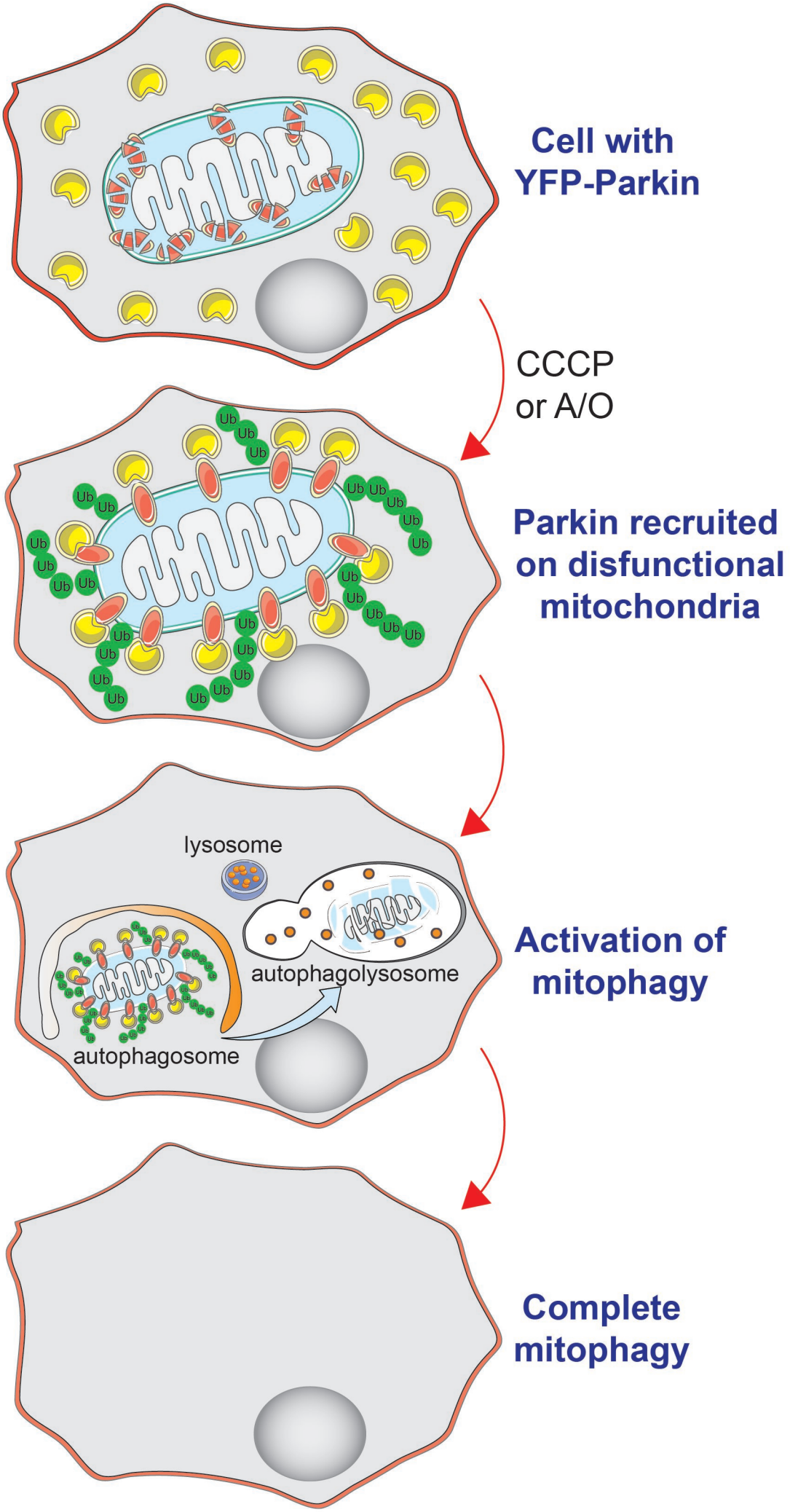
Step	Problem	Possible reason	Possible solution
1-10	***None or few cells expressing YFP-Parkin***	*Inefficient viral transfection that can be a result of:*	
		a) Loss of viral packaging plasmid in the Phoenix™ Amphotropic cells during replication.	It is important that cells are previously selected with the respective selecting antibiotics (Hygromycin B and Diphteria toxin).
		b) Suboptimal cell density. If cell density at the time of transfection is suboptimal (low cell density) cells may not be in the optimal growth phase for transfection. This can result in insufficient uptake of plasmid DNA-Lipofectamin complexes into the cells.	Phoenix™ Amphotropic cells should be 90-95% confluent by the time of transfection.

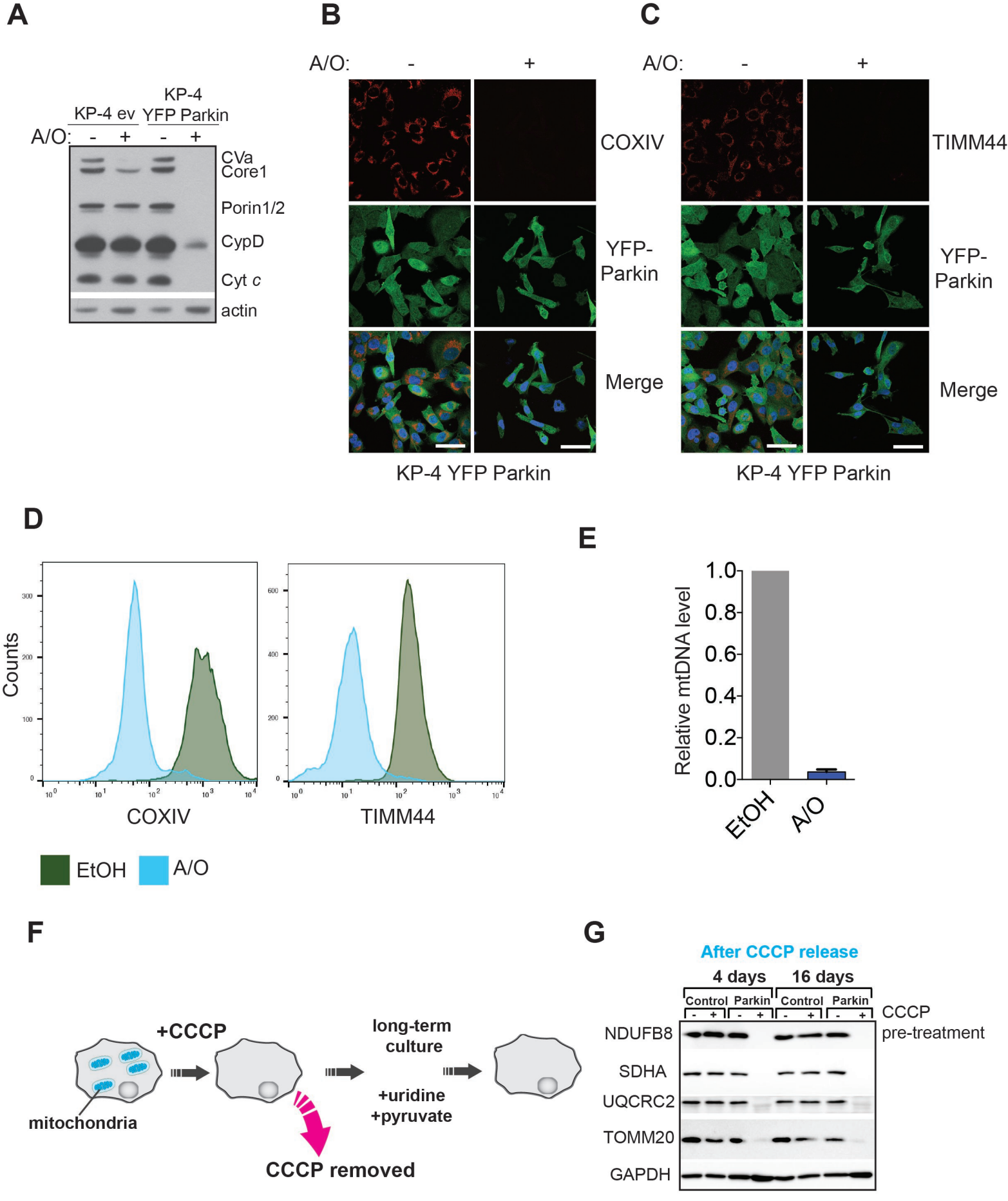
Inefficient viral transduction that can be a result of:	
a) Low viral particles being generated by the packaging cells.	See above for possible solutions.
b) Suboptimal concentration of polybrene during the transduction process.	Determine the concentration of polybrene to be used in the cell line of interest with minor toxic effects. For MRC5 fibroblast, 10µg/ml is the optimal concentration of polybrene for an efficient transduction. Note that some cell lines are particularly sensitive to polybrene, in this case do not use polybrene.
c) Excessive antibiotic used for selection.	Determine the antibiotic sensitivity of the cell line of interest by performing a survival curve. Use the minimum antibiotic required

			to kill non-transduced cells. For the cell lines described in this protocol, 250 µM of Zeocin™ is the optimal antibiotic concentration.
12	***Incomplete mitophagy***	*Loss of CCCP efficiency*	CCCP is photosensitive. It is critical to aliquot very small volumes (e.g. 10 µl) of CCCP, store it at -20°C and protect it from light in order to maintain its efficiency.
		Suboptimal mitochondrial membrane uncoupling treatment	Perform a drug concentration and treatment duration (including drug refreshment periodicity) testing, if using a different cell line to the ones described in this protocol.
		Suboptimal cell density. Cell density, prior and during mitochondria membrane uncoupling, is a major determinant of cell viability upon complete mitophagy. Low cell confluency, prior and during uncoupling, may result in cell death.	Primary fibroblasts require 70-90% cell density, prior and during treatment, for optimal cell viability post treatment. However, other cells lines, particularly immortalized lines (e.g. HeLa cells) require lower cell confluency using this protocol. Determine the optimal cell density for the cell line of interest is crucial

			for the success of the protocol. Optional: A possibility to avoid cell death is the treatment with caspase inhibitors.
12	***High rate of cell death during mitochondria uncoupling***	*Suboptimal mitochondrial membrane uncoupling treatment.* CCCP can be toxic to cells if used in inappropriate doses and treatment duration and periodicity.	For the cell lines described in this protocol, 12.5 µM of CCCP is the optimal dose promoting complete mitophagy, with minimal cell toxicity or off target effects. However, if using different cell lines to the ones described in this protocol, perform a CCCP concentration dependent survival curve and determine the optimal drug dose and treatment duration and periodicty.
13	***High rate of cell death shortly post complete mitophagy***	*Suboptimal cell density.*	See above (step 12)
17	***Premature loss of cell viability in long-term***	*Suboptimal cell density*	For primary fibroblasts, cells should be 70% confluent prior mitochondria

experiments		membrane uncoupling treatment and 90% confluent after re-seeding cells for experiments post-induction of complete mitophagy.
	Suboptimal concentration of uridine. In long-term experiments, it is critical for cell fitness and viability that cells are cultured in medium supplemented with uridine to overcome the limiting pyrimidine levels in mitochondria-depleted cells.	For primary fibroblasts, supplement the cell culture medium with 50 mg/ml of uridine. Other cells lines may have different uridine requirements, therefore determining the uridine concentration for the cell line of interest is fundamental for extension on cell viability in mitochondria-depleted cell.


Cell with YFP-Parkin
CCCP or A/O
Parkin recruited on disfunctional mitochondria
lysosome
autophagolysosome
autophagosome
Activation of mitophagy
Complete mitophagy
PARKIN
degraded PINK1
Ubiquitin chains
mitochondria
stabilized PINK1
Cathepsins


A
KP-4 ev
KP-4 YFP Parkin
A/O: - + - +
CVa Core1
Porin1/2
CypD
Cyt c
actin
B
A/O: - +
COXIV
YFP-Parkin
Merge
KP-4 YFP Parkin
C
A/O: - +
TIMM44
YFP-Parkin
Merge
KP-4 YFP Parkin
D
Counts
COXIV
TIMM44
EtOH
A/O
E
Relative mtDNA level
1.0
0.8
0.6
0.4
0.2
0.0
EtOH
A/O
F
+CCCP
mitochondria
long-term culture
+uridine
+pyruvate
CCCP removed
G
After CCCP release
4 days
16 days
Control
Parkin
Control
Parkin
CCCP pre-treatment
NDUFB8
SDHA
UQCRC2
TOMM20
GAPDH