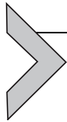

CHAPTER THREE

Monitoring Mitophagy in Mammalian Cells

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Contents

1. Introduction 40
2. Transmission Electron Microscopy 40
3. Western Blot Analysis of Mitophagy 42
4. Fluorescence Methods for Analyzing Mitophagy 45
5. Analyzing Mitophagy Using Mitochondria Mass 47
6. Mitochondria-Targeting Probes for Mitophagy Assay 47
   6.1 MitoTimer for mitochondria turnover 47
   6.2 Quantitative analysis of mitophagy by mitochondrially targeted fluorescent probe 48
7. Mitophagy Inducer and Inhibitors 49
8. Future Perspectives 51
Acknowledgments 52
References 52

Abstract

It is essential for a cell to maintain a proper mitochondrial quality and quantity for normal cellular functions. Damaged or unwanted mitochondria can be selectively removed through mitophagy. Mitophagy research has attracted great attention from life sciences and biomedical fields, it is thus important for the community to properly measure mitophagy. Here, we will focus on the current techniques that have been used to monitor mitophagy in mammalian cells, including morphological and biochemical parameters for monitoring the occurrence of mitophagy.

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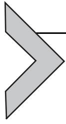
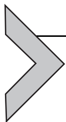


1. INTRODUCTION

Mitochondria are vital organelles that can control cell life and death. It is thus essential for a cell to maintain a proper mitochondrial quality and quantity for normal cellular functions (Chan, 2006; Karbowski & Youle, 2003; Schon & Przedborski, 2011; Suen, Norris, & Youle, 2008; Youle & Narendra, 2011). Even under basal conditions, mitochondria undergo continuous cycles of fusion and fission and of biogenesis and degradation that serve to produce daughter mitochondria and remove unwanted or defective mitochondria. It is now clear that mitochondria can be selectively removed through mitochondrial autophagy, or mitophagy, although mitochondria are also degraded during nonselective bulk cytoplasmic degradation through general autophagy (Feng, Liu, Zhu, & Chen, 2013). Both receptor and nonreceptor-mediated mitophagy have been described and extensively reviewed (Ding & Yin, 2012; Feng et al., 2013; Liu, Sakakibara, Chen, & Okamoto, 2014; Matsuda & Tanaka, 2010; Scarffe, Stevens, Dawson, & Dawson, 2014; Stolz, Ernst, & Dikic, 2014; Winklhofer, 2014; Zhu, Dagda, & Chu, 2011). Given the significant progress and the explosion of information in this exciting field, it is important for the community to properly measure mitophagy. Here, we will discuss the current techniques that have been used to monitor mitophagy in mammalian cells. As mitophagy is a specific and selective form of autophagy, it is important to measure both the involvements of general autophagy and the mitochondria-specific phenotypes. Additionally, as with those of autophagy, both morphological and biochemical parameters are required for monitoring the occurrence of mitophagy.



2. TRANSMISSION ELECTRON MICROSCOPY

The morphological hallmark of general autophagy is the formation of double-membrane autophagosomes that contain cytoplasmic material and/or organelles including mitochondria for subsequent degradation. The autophagosomes will fuse with the lysosomes to form matured autolysosomes, which become one membrane structure. To detect both early autophagosomes and late autolysosomes, transmission electron microscopy (TEM) remains the best and most powerful approach. Similarly, the ultrastructural evaluation of mitophagy using TEM is a direct method for confirming mitochondrial autophagy or clearance. The early stage of

mitophagy (autophagosome with engulfed mitochondria) can be easily detected through the identification of unique mitochondrial structures such as the cristae. The late stage of mitophagy may be reflected by the single-membrane autolysosomes with residual mitochondria on the basis of their similar electron density with mitochondria (Klionsky et al., 2012; see Fig. 3.1). Because mitochondria are morphologically altered during the progression of mitophagy, it would be better to perform immunogold-labeled EM for specific mitochondrial markers, such as TOM20, TIM23, or VDAC1 (Ding et al., 2012; Liu et al., 2012), to confirm the nature of the mitochondria in the late stage of mitophagy. It should be aware that TEM could sometimes be problematic due to misinterpretations mostly deriving from methodological artifacts. It is thus important to take proper care of the sample to be analyzed right from the beginning of the preparation and the fixation. The fixation of *in vitro* samples is relatively straightforward (see protocol below). However, the fixation of tissue samples requires care to avoid problems of nonrepresentative or uninformative tissue sections. Fresh samples should be handled in a timely manner to avoid changes in cellular structures that may occur during the manipulations; the samples should be fixed immediately with freshly prepared fixatives. For *in vitro* cultured cells, the cells can grow in plastic chips placed into the culture medium, and the fixation and sectioning can be carried out without trypsin digestion to keep the cells intact during TEM. For fresh tissue samples, perfusion fixation should be used when possible. One should be aware that autophagosomal maturation and its fusion with lysosomes are a relatively rapid process, and it may be difficult to recognize mitochondrial morphology within autophagosomes. The application of autophagic inhibitor Bafilomycin A1

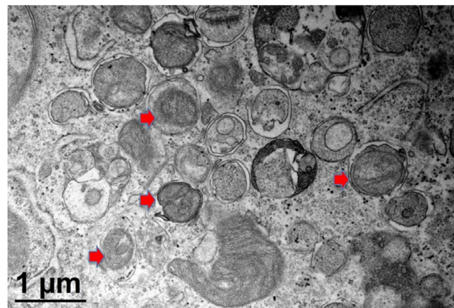
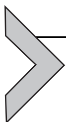


Figure 3.1 Analysis of mitophagy via electron microscopy. HeLa cells were treated with carbonyl- cyanide p-trifluoromethoxyphenylhydrazone (FCCP) for 2 h, and the samples were analyzed using electron microscopy. Arrows indicate that mitochondria are enclosed within autophagosomes.

(BA1), an inhibitor of vacuolar-type $H^{(+)}$-ATPase and autophagosome–lysosome fusion, prior to sampling may prevent autophagosome maturation and traps newly formed autophagosomes. This may facilitate the identification of cargo, such as mitochondria, as well as providing comparative information on the rates of autophagy progression.

Details

1. Cells were cultured in proper medium containing 10% fetal calf serum (FBS) (HyClone) and 0.1 mg/mL penicillin–streptomycin at 37 °C under 5% CO_2. When the cell density was 60–80%, the cells were then placed under the conditions that could induce mitophagy. For example, hypoxia is a typical mitophagy inducer, and hypoxic conditions can be achieved by flushing the cells with a preanalyzed gas mixture of 1% O_2, 5% CO_2, and 94% N_2 in a hypoxia chamber (Billups-Rothenberg Inc.). FCCP and other mitochondrial toxins can also be employed to induce mitophagy. BA1 (10–20 n*M*) was added 2–4 h prior to sampling for easy detecting autophagosomal structures.
2. The cells are rinsed 3 × with phosphate buffer saline (PBS) and fixed for at least 60 min with freshly prepared 2.5% glutaraldehyde at room temperature or overnight at 4 °C.
3. After fixation, the cell monolayers are washed 3 × in PBS and then post-fixed in aqueous 2% OsO4 for 1 h.
4. The cells were then dehydrated with sequential washes in 50%, 70%, 90%, 95%, and 100% ethanol and then embedded in PolyBed 812 epoxy resin.
5. Ultrathin (60 nm) sections are collected on copper grids and stained with 2% uranyl acetate in 50% methanol for 10 min, followed by incubation in 1% lead citrate for 7 min.
6. The sections are photographed using a transmission electron microscope at 80 kV.



3. WESTERN BLOT ANALYSIS OF MITOPHAGY

Mitophagy can be monitored biochemically through the degradation of mitochondrial proteins together with the appearance of autophagy markers such as LC3-II (microtubule-associated protein 1 light chain 3) and degradation of p62. This can be achieved by analyzing the mitochondrial inner-/inter-/outer-membrane proteins or mitochondrial matrix proteins in addition to the autophagy biochemical hallmarks. The LC3 protein is a ubiquitin-like protein that can be conjugated to phosphatidylethanolamine

(PE). LC3 is initially synthesized in an unprocessed form, proLC3, which is converted into a proteolytically processed form lacking amino acids from the C terminus, LC3-I, and is finally modified into the PE-conjugated form, LC3-II. The total levels of LC3 maintain state balance, and in a stress condition, there may be increases in the conversion of LC3-I to LC3-II; (see Fig. 3.2). Thus, Western blotting can be easily used to monitor changes in LC3 amounts to mitophagy and BA1 inhibited LC3 flux (Chen et al., 2014). A Western blotting analysis of the levels of mitochondrial proteins from different mitochondrial subcompartments is valuable for validating the data from EM studies. However, more recent studies suggest that most outer mitochondrial membrane proteins are degraded by the ubiquitin proteasome system, whereas the matrix proteins are degraded through Adenosine Triphosphate (ATP)-dependent protease in addition to autophagy (Yoshii, Kishi, Ishihara, & Mizushima, 2011). Therefore, a simultaneous analysis for outer- and inner-membrane mitochondrial proteins and matrix proteins, such as TOM20, TIM23, CypD, and HSP60, would be more appropriate for monitoring mitophagy. In our opinion, VDAC1 may not be a good mitophagy biochemical marker protein because it is induced by many different types of stresses. For general autophagy, an autophagy inhibitor, such as 3-MA or BA1, will be supplemented to the cells to block the mitochondrial protein degradation process. The protein levels of *Atg* genes may not change significantly during mitophagy. However, the knockdown of ATG5 or ATG7 is normally able to prevent mitochondrial protein degradation, whereas the knockdown of Beclin1 may fail to prevent mitophagy in some cases.

The degradation of p62, or sequestosome-1, was widely used as a biochemical marker for general autophagy. p62 was reported to act as a cargo receptor for ubiquitinated protein aggregates that are intended for selective

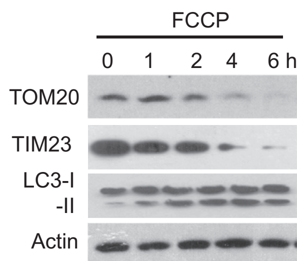


Figure 3.2 FCCP induces mitophagy. HeLa cells were treated with 10 μ*M* FCCP for the indicated times. Mitochondrial inner membrane protein TOM20, outer-membrane protein TIM23, and LC3 were detected via Western blotting.

autophagy (Geisler et al., 2010; Kirkin, Lamark, Johansen, & Dikic, 2009; Kirkin, McEwan, Novak, & Dikic, 2009; Kraft, Peter, & Hofmann, 2010; Lamark, Kirkin, Dikic, & Johansen, 2009). It was found that p62 translocates onto mitochondria via an interaction with ubiquitin, which is significantly increased in response to mitochondrial stresses, which could theoretically recruit LC3 and autophagosomes to facilitate subsequent selective autophagy. p62 was also found to bind other critical signaling intermediates such as tumor necrosis factor (TNF) receptor-associated factor 6, which is essential for the regulation of nuclear factor-kappa B (NF-κB) during osteoclastogenesis and bone homeostasis as well as in cancer (Mathew et al., 2009; Moscat & Diaz-Meco, 2009; Sanz, Diaz-Meco, Nakano, & Moscat, 2000; Yu et al., 2009). Whether ubiquitin/p62/LC3 complexing is sufficient for Parkin-mediated mitophagy remains a subject of debate and requires further investigation. It could be a useful marker for mitophagy, and care should be taken to interpret the data properly because it is such a multifunctional protein.

Details

1. Cells were treated with desirable conditions such as hypoxia and FCCP (all cells must be replaced with fresh medium or medium supplement with mitophagy inducers during the treatments, and in old medium, nutrients are utilized, which causes energy deprivation and general autophagy). The cells are then treated with BA1 (5–10 n*M*) for 2–4 h prior to sample collection. Cells were scraped and immediately washed by PBS once and were then kept on ice.
2. Cells were lysed in lysis buffer for 40 min on ice (20 m*M* Tris, pH 7.4, 137 m*M* NaCl, 2 m*M* ethylene diamine tetraacetic acid (EDTA), 10% glycerol, 1% NP-40, protease and phosphatase inhibitors). After centrifugation at $12{,}000 \times g$ for 15 min, the supernatant was boiled in sample buffer (4% sodium dodecyl sulfate (SDS), 20 m*M* Tris, pH 6.8, 20% glycerol, 0.1% bromophenolblue, 200 m*M* β-mercaptoethanol) for 5 min.
3. Equivalent quantities of protein (10–20 μg) were subjected to SDS–polyacrylamide gelelectrophoresis (PAGE) and then transferred to nitrocellulose membranes.
4. The membranes were probed with the indicated primary antibodies (TOM20-BD612278, TIM23-BD611222, LC3-Sigma L7543/MBL PM036/M186, p62-MBL PM045, actin sigmaA5441), followed by the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (KPL, Gaithersburg, MD).
5. Immunoreactive bands were visualized with a chemiluminescence kit (Millipore).

4. FLUORESCENCE METHODS FOR ANALYZING MITOPHAGY

Fluorescence microscopy using green fluorescent protein (GFP)-LC3 as a marker of autophagy can be used to monitor mitophagy via its colocalization with mitochondria markers in cells (Chen et al., 2014; Liu et al., 2012). The GFP-LC3 plasmid can be co-transfected with mitochondrial localized DsRed (Mito-DsRed) into cells, or mitochondria can be stained with Mitotracker or immunostained with anti-TOM20, Tim23, voltage-dependent anion-selective channel protein 1 (VADC1), or Cytochrome C antibodies. A straightforward colocalization of LC3 (green) and mitochondria (red) displays a yellow punctate form in merged images. Sometimes, the colocalization with LC3 and mitochondria do not always appear yellow due to differences in the intensity of staining. A high-resolution confocal microscope can clearly show that the mitochondria (red) are enclosed in GFP-LC3-labeled autophagosome (see Figs. 3.3 and 3.4). In addition to these, the quantification of the average number of GFP-LC3 puncta or the surface areas colocalized with mitochondria per cell was performed by counting approximately 200 cells (Chen et al., 2014; Liu et al., 2012) in three separate experiments, respectively. In addition to LC3, other autophagy players, such as Unc-51 like autophagy activating kinase 1 (ULK1) and p62, were found to translocate onto mitochondria. ULK1 plays a more specific role in mitophagy. A *ulk1*-knockout mouse model

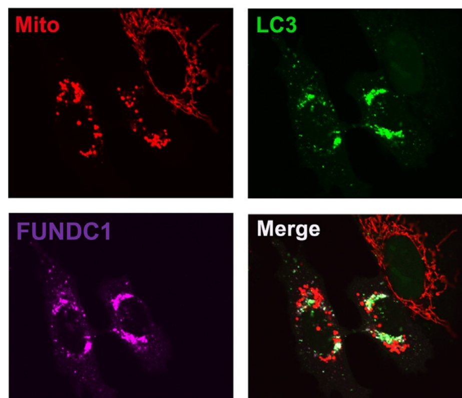


Figure 3.3 Analysis of mitophagy via fluorescence microscopy. HeLa cells were ectopically expressed with FUNDC1-myc together with GFP-LC3 for 24 h; the cells were then fixed and immunostained with Myc (purple) and TOM20 (red).

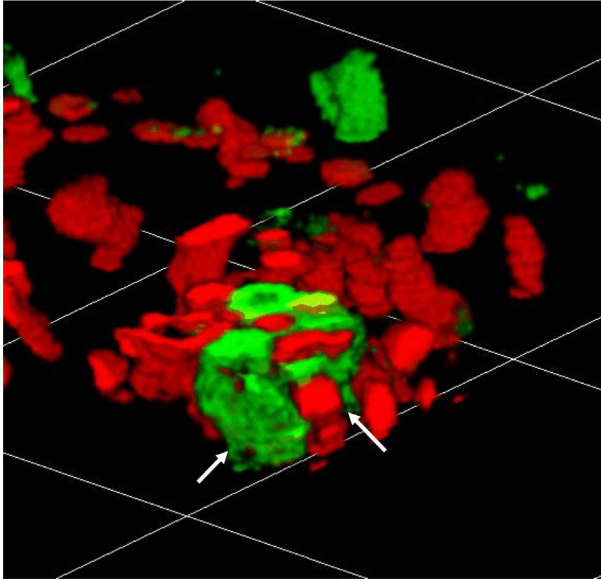
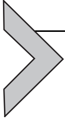


Figure 3.4 Analysis of mitophagy via high-resolution confocal microscopy. HeLa cells were transfected with FUNDC1 (FUN14 domain containing 1)-myc together with GFP-LC3 for 24 h; the cells were then fixed and immunostained with TOM20 (red). The picture was acquired using 3D methods. Arrows indicate that mitochondria (red) are enclosed within autophagosomes (green).

demonstrated defects in the autophagic clearance of mitochondria during erythroid maturation (Kundu et al., 2008). ULK1 is recruited to fragmented mitochondria in response to hypoxia or FCCP (Itakura, Kishi-Itakura, Koyama-Honda, & Mizushima, 2012). The translocated ULK1 interacts with its substrate FUNDC1 to phosphorylate FUNDC1 (Wu et al., 2014).
Details

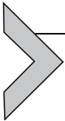
1. Cells were grown on glass coverslips. Following treatment, the cells were washed 3 × with PBS and fixed with freshly prepared 4% formaldehyde at 37 °C for 30 min.
2. Antigen accessibility was increased via treatment with 0.2% Triton X-100 for 10 min on ice. The cells were then incubated with primary antibodies for 2 h, and, after washing 5 × with PBS, they were stained with a secondary antibody for a further 1 h (donkey anti-mouse Alex Flour 555 Invitrogen-A31570, donkey anti-Rabbit Alex Flour 555, Invitrogen-A31572, donkey anti-goat-Cy5 Invitrogen-A21082, donkey anti-mouse 488 Invitrogen-A21202).

3. Cell images were captured with a confocal microscope or high-resolution confocal microscope. The area and number of LC3 aggregates were measured using ImageJ software by counting approximately 200 cells.



5. ANALYZING MITOPHAGY USING MITOCHONDRIA MASS

The simplest method of monitoring the amount of remaining mitochondria is through analyzing the mitochondrial mass. This method can be used to determine the efficiency of the degradation of dysfunctional, aged, or impaired mitochondria. Mitochondrial mass can either be measured using an fluorescence-activated cell sorting (FACS) technique using Nonyl acridine orange (NAO, Invitrogen A1372) or, on a single cell, via immunocytochemistry using MitoTracker Green FM (Invitrogen M7514) and mitochondrial marker protein antibodies (see above). EM can also be used to verify the loss of entire mitochondria, and polymerase chain reaction (PCR) (or fluorescence microscopy) can be used to quantify mitochondrial DNA, such as 16S rRNA, cytochrome c oxidase subunit I (COX1), CYTB (Cytochrome b, Complex III subunit), or the nuclear DNA of hexokinase, H19 or beta-1 globin. The mitochondrial-to-nuclear DNA ratio in each sample can consequently be calculated by dividing the value of mitochondria DNA by that of nuclear DNA (Lagouge et al., 2006).



6. MITOCHONDRIA-TARGETING PROBES FOR MITOPHAGY ASSAY

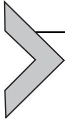
6.1. MitoTimer for mitochondria turnover

Mitochondrial biogenesis and mitophagy are processes that determine mitochondrial turnover, along with fusion and fission. Recently, Roberta A. Gottlieb's group developed a new probe called MitoTimer for detecting mitochondria turnover. MitoTimer is a mutant of the red fluorescent protein, DsRed, in which fluorescence shifts irreversibly from green to red as the protein is oxidized. They targeted the probe to the mitochondrial matrix with a mitochondrial-targeting sequence (coined "MitoTimer"). MitoTimer is a novel fluorescent reporter protein that can continuously monitor mitochondrial dynamics within cells and is of particular value where mitophagy is closely matched with biogenesis. MitoTimer was used to analysis the multiple parameters of mitochondrial health under both physiological and pathological conditions and will be highly useful tool for future research of mitochondrial function (Ferree et al., 2013; Hernandez et al., 2013; Laker et al., 2014).

6.2. Quantitative analysis of mitophagy by mitochondrially targeted fluorescent probe

To quantitatively detect mitophagy in yeast, the Klionsky group reported a simple method to monitor mitophagy using the Om45-GFP processing assay (Kanki, Kang, & Klionsky, 2009). Om45 is a mitochondrial outer-membrane protein, and a GFP-tagged Om45 (GFP at the C terminus of Om45) is correctly localized on mitochondria. Following the uptake of mitochondria into the vacuole, Om45-GFP is degraded, releasing the intact form of GFP, which can be detected via immunoblotting. Thus, mitophagy can be monitored and quantified according to the appearance and intensity of free GFP via immunoblot. A similar GFP-tagged mitochondrial protein-processing assay was also developed in mammalian cells. Yoshii et al. developed a flow cytometry-based assay using cell lines that stably express GFP proteins fused to either the mitochondrial matrix (subunit 9 of F0-ATPase, Su9-GFP) or an outer mitochondrial protein (GFP-Omp25; Yoshii et al., 2011). They found that the Su9-GFP and GFP-Omp25 signals were largely unaffected in Parkin-untransfected mouse embryonic fibroblasts (MEFs) but significantly decreased in Parkin-transfected wild-type MEFs in a time-dependent manner following carbonyl cyanide m-chlorophenylhydrazone (CCCP) treatment, which is inhibited in the absence of FIP200 or ATG5 (Yoshii et al., 2011). These studies confirmed that the degradation of matrix proteins can be used as a quantitative approach to monitor mitophagy in mammalian cells. Tandem mCherry–GFP tags were attached to the outer mitochondrial membrane localization signal of the protein FIS1 (residues 101–152) and were used to monitor the lysosomal delivery by the color change during mitophagy (Allen, Toth, James, & Ganley, 2013). Under normal conditions, mitochondria displayed both red and green fluoresce. Upon mitophagy, the mitochondria were delivered to lysosomes in which the low pH quenches the GFP signal but not mCherry (Allen et al., 2013). Recently, Miyawaki's group developed a sensitive and quantitative technique using Mitochondria-targeted Keima (mt-Keima), a ratio-metric pH-sensitive fluorescent protein that is targeted into the mitochondrial matrix. A low-ratio mt-Keima-derived fluorescence (543 nm/458 nm) indicates a neutral environment, whereas a high-ratio fluorescence indicates an acidic pH[18]. Thus, mt-Keima enables the differential imaging of mitochondria in the cytoplasm and mitochondria in acidic lysosomes. Because mt-Keima is resistant to lysosomal proteases, it enables measurements of the cumulative lysosomal delivery of mitochondria over

time (Katayama, Kogure, Mizushima, Yoshimori, & Miyawaki, 2011). The time-lapse imaging of mt-Keima and GFP–Parkin was used to simultaneously observe Parkin recruitment to and the autophagic degradation of mitochondria after membrane depolarization. This method was recently employed to determine whether USP30 opposes Parkin-mediated mitophagy (Bingol et al., 2014).



7. MITOPHAGY INDUCER AND INHIBITORS

An increasing number of inducers are used to trigger mitophagy. These include reactive oxygen species (ROS), mitochondrial toxins (FCCP/CCCP, Rotenone, Antimycin A, Valmycin, Oligomycin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), and 6-Hydroxydopamine), hypoxia, ceramides, and selenite. It appears that the perturbation of mitochondrial ATP production and ROS generation can lead to defects in mitochondria and the activation of mitophagy.

It has been demonstrated that increased intracellular ROS can activate both general autophagy and mitophagy. The specific induction of mitochondrial ROS was also found to induce mitophagy. The *in situ* induction of ROS was performed using a genetically encoded mitochondrial-targeted photosensitizer, KillerRed, which is targeted to mitochondria (mtKR) to examine the effect of a short burst of ROS in the mitochondria (Wang, Nartiss, Steipe, McQuibban, & Kim, 2012). KillerRed is a homolog of GFP that produces 1000-fold more ROS than GFP upon photoactivation with 561 nm light (Roy, Carpentier, Bourgeois, & Field, 2010). KillerRed can be readily expressed in cultured cells and can be targeted to mitochondria by attaching two mitochondrial-targeting sequences to the N-terminus of KillerRed (mtKR; Bulina et al., 2006). Using live-cell imaging, it was demonstrated that the photoactivation of mtKR produces ROS to directly cause mitochondrial damage, which leads to Parkin-dependent mitophagy (Wang et al., 2012). The overexpression of the mitochondrial antioxidant protein superoxide dismutase 2 suppresses the mitophagy induced by ROS. Studies have demonstrated that ROS can mediate the selective mitophagy induced by 6-hydroxydopamine (6-OHDA), rotenone, and staurosporine in neuron cells, which can induce the externalization of cardiolipin for initiating mitophagy (Chu et al., 2013).

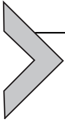
Mitochondria are the central organelle that utilize oxygen for energy production and are highly sensitive to cellular oxygen levels. It is not

surprising that hypoxia induces the mitophagy mediated by the FUNDC1 or BNIP3L/NIX pathway. Specifically, we found that FUNDC1, a new mitophagy receptor localized at the outer mitochondrial membrane protein, has a typical LIR (LC3 interaction region) motif that specifically interacts with LC3 for subsequent mitophagy. Under normal conditions, FUNDC1 is highly phosphorylated by Src kinase and CK2 at distinct sites. In response to hypoxia, Src kinase and CK2 are inactivated and a phosphatase, such as phosphoglycerate mutase family member 5 (PGAM5), is activated to dephosphorylate FUNDC1, which then have significantly higher affinity for LC3 binding and mitophagy. NIX/BNIP3L and BNIP3 are also responsive to hypoxia and are phosphorylated under stress conditions. We have thus proposed that monitoring the phosphorylation status of mitophagy receptors such as FUNDC1, NIX/BNIP3L, and BNIP3, by specific phosphor-antibodies will be useful for quantitatively assaying mitophagy levels. Both wild-type FUNDC1 and S13A mutants can induce mitophagy in the absence of Parkin (Chen et al., 2014; Liu et al., 2012).

Many mitochondrial complex inhibitors have been employed to induce mitophagy in cells. Complex I inhibitor, Rotenone (Chu et al., 2013); the complex III inhibitor, Antimycin A (McLelland, Soubannier, Chen, McBride, & Fon, 2014); a potassium ionophore, Valinomycin; and the ATP synthase inhibitor, Oligomycin A can induce mitophagy in a PINK1/Parkin-dependent fashion (Bhatia-Kissova & Camougrand, 2013; Yamano, Fogel, Wang, van der Bliek, & Youle, 2014). CCCP/FCCP is a lipid-soluble weak acid and a potent mitochondrial uncoupling agent that increases the proton permeability across the mitochondrial inner membranes, thus dissipating the transmembrane potential and depolarizing the mitochondria. CCCP/FCCP could induce the lysosomal removal of depolarized mitochondria, which was considered to be mediated by mitophagy. CCCP/FCCP (10–20 μ*M*) was used as an inducer in Parkin/PINK1-, NIX/BNIP3L-, and FUNDC1-mediated mitophagy when the cells were treated for 0.5–24 h (Chen et al., 2014; Ding et al., 2010; Liu et al., 2012; Narendra, Tanaka, Suen, & Youle, 2008). NIX/BNIP3L was found to be involved in the FCCP-induced autophagic degradation of mitochondria (Sandoval et al., 2008). MEF cells with NIX deficiency had a lower ROS level. ROS and the mitophagy receptor NIX are important in the induction and initiation of mitophagy by enhancing the translocation of Parkin onto the damaged mitochondria (Ding et al., 2010). Increased numbers of depolarized mitochondria and mitophagy were observed in fibroblasts derived from patients who were deficient in

coenzyme (CoQ), a small lipophilic molecule critical for the transport of electrons from complexes I and II to complex III in the mitochondrial respiratory chain. Interestingly, CoQ supplementation or cyclosporin A (CsA) treatment attenuated mitophagy in these cells, suggesting that these mitochondrial defects are specifically induced by CoQ deficiency and that MPT can regulate this type of mitochondrial damage as well as mitophagy (Cotan et al., 2011; Rodriguez-Hernandez et al., 2009).

Ceramide is a bioactive sphingolipid that mediates cell death (Ogretmen & Hannun, 2004; Salazar et al., 2009). The role for ceramide and other sphingolipids in general autophagy has been widely reported. Recent studies have demonstrated that C_{18}-ceramide induces lethal autophagy via the selective targeting of mitochondria through a direct interaction between ceramide and LC3B-II on mitochondrial membranes (Jiang & Ogretmen, 2013; Sentelle et al., 2012). Accordingly, CerS1/C_{18}-ceramide was necessary and sufficient for the induction of lethal mitophagy and tumor suppression *in vivo* (Sentelle et al., 2012).



8. FUTURE PERSPECTIVES

Mitophagy is a highly complex and dynamic cellular process that regulates both mitochondrial quality and quantity. Both morphological and biochemical hallmarks have been described, and the use of combinatory approaches to measure mitophagy is strongly recommended. No single parameter was sufficient to document mitophagy. Despite the significant recent advances in our knowledge of selective mitophagy, reliable quantitative assays to monitor mitophagy still need to be developed. Additionally, monitoring mitophagy *in vivo* remains a challenge. Given that many of the mitophagy players, such as Parkin, p62, ULK1, and mitophagy receptors, including FUNDC1, Bcl-2/E1B19kDa-interactingprotein3-like (NIX/BNIP3L), BCL2/adenovirus E1B 19kDa interacting protein 3 (BNIP3), and ATG32 are phosphorylated in response to many distinct mitophagy stimuli, we recently suggested that the phosphorylation status of these mitophagy players could be used for the quantitative approach and can be applied *in vivo* (Chen et al., 2014). This requires developing specific antibodies to detect the specific phosphorylation sites. It is also important to identify the kinases and phosphatases that are responsible for regulating mitophagy.

As described above, ceramide and many compounds, including mitochondrial toxins, are used to induce mitophagy. These compounds are useful

for studying mitophagy in cultured cells. It should be noted that these compounds may have off-target effects in addition to mitophagy. More specific mitochondria-targeting compounds may be useful in the study of mitophagy. Compounds that target to mitophagy receptors or regulators are important for the study of mitophagy and for drug development. When possible, physiologically relevant stimuli, such as hypoxia, are recommended. Additionally, an expanding number of players have been reported to be involved in mitophagy. Future studies should address whether there are common or converging pathway(s) to mediate mitophagy in response to distinct mitochondrial stresses. Better qualitative and quantitative methods for assaying mitophagy may increase understanding of molecular mitophagy and possible drug development.

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