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JOURNAL TITLE: Nature Chemical Biology

PUBLICATION DATE: 19 January 2017

PUBLISHER: Nature Publishing Group

DOI: 10.1038/nchembio.2287

The Pharmacological Regulation of Cellular Mitophagy

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Abstract

Small molecules are pharmacological tools of considerable value in dissecting complex biological processes and identifying potential therapeutic interventions. Recently, the cellular quality control process of mitophagy attracted considerable research interest, however, the limited availability of suitable chemical probes restricted our understanding of the molecular mechanisms involved.

Current approaches to initiate mitophagy include acute dissipation of the mitochondrial membrane potential ($\Delta\Psi_m$) by mitochondrial uncouplers (*e.g.* FCCP/CCCP), or the use of Antimycin A/Oligomycin to impair respiration. Both approaches impair mitochondrial homeostasis, and therefore limit the scope for dissection of subtle, bio-energy related regulatory phenomena.

Recently, novel mitophagy activators acting independently of the respiration collapse have been reported, offering new opportunities to understand the process and potential for therapeutic exploitation.

We have therefore summarized the current status of mitophagy modulators and analyzed the available chemical tools, commenting on their advantages, limitations and current applications.

Key words: mitophagy, FCCP, SIRT1, p53, Nrf2, PMI

Introduction

Mitochondrial autophagy, known as mitophagy, governs the elimination of dysfunctional or superfluous mitochondria and is therefore fundamental to both mitochondrial and cellular homeostasis.[1] Impaired mitophagy may lead to an accumulation of damaged organelles within cells, underlying the pathogenesis of a number of chronic conditions including cancer, cardiovascular and liver diseases and neurodegeneration, particularly Parkinson's disease (PD).[2]

Mitophagy operates via a number of distinct but interconnected mechanisms that can be generally classified as ubiquitin (Ub) dependent and independent pathways.[3] Ub-driven mitophagy relies on the ubiquitination of mitochondrial surface proteins for the recognition of damaged organelles by the autophagic machinery. The PINK1-Parkin pathway for activation and execution of the process is currently the most studied mechanism of mitochondrial priming (**Figure 1b**). Dissipation of the mitochondrial membrane potential ($\Delta\Psi_m$) leads to the stabilization of the Ser/Thr kinase PINK1 (PTEN induced putative kinase 1), thus facilitating its accumulation on the outer mitochondrial membrane (OMM).[4] PINK1 activates the E3 Ub ligase Parkin via mechanism involving the phosphorylation of both Parkin and its substrate Ub at Ser^{65}.[5,6] Once localized on mitochondria, Parkin acts in concert with PINK1 to amplify the initial signal by decorating mitochondria with Ub chains, which are sequentially phosphorylated by PINK1 (**Figure 1c**).[6] Besides Parkin, other Ub ligases, such as SMURF1[7] and Gp78[8], have been reported to contribute to the mitochondrial priming. The accumulation of $pSer^{65}$-Ub chains on the OMM triggers the recruitment of the autophagy receptors optineurin (OPTN) and nuclear dot protein 52 (NDP52), which promote the biogenesis of phagophores in close proximity to mitochondria by recruiting the autophagy-initiating factors ULK1 (unc-51 like autophagy activating kinase 1), DFCP1 (Double FYVE-domain containing protein 1) and WIPI1 (WD repeat domain, phosphoinositide interacting 1).[6] Moreover, OPTN and NDP52 act as molecular adaptors anchoring Ub-labelled mitochondria into autophagosomes by interacting directly with LC3 through their LC3-interacting regions (LIR) (**Figure1b, c**).

In addition to Ub-driven mitophagy, the OMM-localized mitophagy receptors BNIP3 (BCL2/adenovirus E1B 19 kDa protein-interacting protein 3),[10] NIX1/BNIP3L,[9] and FUNDC1 (FUN14 domain containing 1)[11] target mitochondria to autophagosomes independently of mitochondrial ubiquitination by interacting directly with LC3 and GABARAP ($GABA_A$ receptor associated protein) through typical or atypical LIR motifs (**Figure 2a**). NIX1 was originally identified as a key mediator of mitochondrial removal

during erythrocyte maturation,[12] but recently shown to be involved in $\Delta\Psi_m$ loss-induced mitophagy.[9]

The expression of both NIX1 and the related BNIP3 is partially under the control of HIF-1 (hypoxia-inducible factor 1) and their up-regulation during hypoxia promotes the clearance of damaged mitochondria.[14,15] BNIP3 along with FUNDC-1 are considered the main mediators of hypoxia-induced mitophagy.[11,14] In contrast to NIX1 and BNIP3, the expression of FUNDC1 is not altered significantly during hypoxic or mitochondrial depolarization events, but instead its activity relies heavily on post-translational modifications.[16] Under basal conditions, the interaction between LC3 and FUNDC1 is suppressed by the phosphorylation of the latter at multiple sites that are located close to its LIR domain. Upon hypoxia or $\Delta\Psi_m$ collapse however, the dephosphorylation of FUNDC1 at Ser^{13} by the Ser/Thr phosphatase PGAM5 (Phosphoglycerate mutase family member 5) enhances its binding affinity for LC3 and facilitates the anchoring of mitochondria into autophagosomes (**Figure 2b**).[17]

Notably, the progress in understanding the molecular hierarchy governing mitophagy achieved with the aid of only a limited number of chemical tools. In particular, the conventional approach to trigger mitophagy *in vitro* resides in the chemically-induced collapse of $\Delta\Psi_m$ and subsequent stabilization of PINK1. The vast majority of mitophagy inducers identified to date are in essence mitochondrial toxins that initiate a mitophagic response by inhibiting mitochondrial respiration. Despite their limitations and plethora of off-target effects, such agents are widely used in regulatory and mechanistic cell biology studies due to the lack of more suitable chemical tools.

There is therefore a need to devise novel pharmacological approaches to manipulate the process and develop agents to activate mitophagy without perturbing the organelle as dramatically as most current inducers do. Besides their potential therapeutic utility in conditions associated with mitophagy defects, such agents would also serve as invaluable chemical probes to corroborate the core pathways of the process and unveil regulatory mechanisms and events that occur independently from the respiratory collapse. To delineate the current state of the field and highlight recent advances, we provide a critical appraisal of the chemical tools currently used to modulate mitophagy in addition to focusing on the most recent and promising approaches that pave the way for a better understanding of the process and leave hope for its future therapeutic exploitation.

H^+ Ionophores

Amongst the inducers of mitophagy identified to date, proton (H^+) ionophores, hereafter referred to as protonophores, are employed widely in cell biology studies. Protonophores are weakly acidic lipophilic compounds capable of transporting H^+ across the inner mitochondrial membrane (IMM), thereby depleting the electrochemical proton gradient and uncoupling oxidative phosphorylation from the electron transport chain (ETC).[18] As a result of the extensive H^+ leak mediated by such agents, mitochondria are targeted for degradation, while the rate of oxygen consumption is increased in an effort to restore the diminished ATP generation, making protonophores valuable tools for the study of both mitochondrial autophagy and bioenergetics.

The phenylhydrazones carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP) and carbonyl cyanide-*p*-(trifluoromethoxy)phenylhydrazone (FCCP) and to a lesser extent the 2,4-dinitrophenol (DNP) have been extensively employed to probe the underlying mechanisms of mitophagy and characterize the biological pathways involved in this process (**Table 1**).[6,19–21] CCCP in particular was one of the first agents reported to induce mitophagy and was successfully applied to unveil the involvement of the PD-associated protein Parkin in the regulation of this process.[20]

Despite their widespread use, protonophores have several limitations as mitophagy-stimulating chemical probes (**Figure 1a**). In particular, their effects on mitochondria do not resemble naturally occurring conditions, being largely detrimental and leading eventually to mitochondrial failure.[22] Furthermore, they affect the entire mitochondrial population rather than a specific subgroup and can even lead to the complete elimination of mitochondria from cells after longer treatments.[20] Moreover, traditional protonophores are not specific for mitochondria and have protonophoric activity on other membranes, particularly the plasma membrane.[23] As a result, they mediate a variety of off-target effects, including disruption of the microtubule cytoskeleton[24], inhibition of lysosomal activity[25] and activation of ion channels,[26] which leads to relatively high levels of toxicity and a narrow therapeutic window. Notably, FCCP is cytotoxic, even at concentrations that are not high enough to dissipate $\Delta\Psi_m$ and thus induce mitophagy.[27]

Recently, a second generation protonophore, which is equally potent but less cytotoxic than FCCP, was reported.[28] This novel compound, named BAM15, can enhance the rate of mitochondrial respiration in both cells and isolated mitochondria and exert protective effects against renal ischemia-reperfusion damage *in vivo*. In contrast to

FCCP, BAM15 exhibits high selectivity for the IMM and does not depolarize the plasma membrane or cause mitochondrial failure at high concentrations. However, its mitophagy-inducing potential is not fully evaluated yet and further work is therefore required to assess its suitability in this context.

Mitochondrial toxins

Similarly to protonophores, various toxins and respiration inhibitors activate PINK1-mediated mitophagy following dissipation of the $\Delta\Psi_m$ (**Figure 1a**). A representative example is valinomycin, a highly specific potassium (K^+) ionophore that owing to its high lipophilicity, is able to transport K^+ ions across the IMM by masking their charge through a reversible interaction (**Table 1**).[29] The resulting accumulation of K^+ inside the mitochondrion eliminates the electrical potential and activates the PINK1-Parkin pathway without altering the pH gradient (**Figure 1a, b**).

In addition to valinomycin, the ionophore salinomycin has been reported to induce mitophagy by interfering with mitochondrial K^+ homeostasis (**Table 1**).[30] However, its mode of action is distinct to that of valinomycin and instead of depolarizing the IMM, salinomycin induces its transient hyperpolarization.[30,31] In contrast to valinomycin, salinomycin increases the efflux of K^+ from the mitochondrion by catalyzing their electroneutral exchange against protons and subsequently modifies the pH gradient across the IMM.[31] The acidification of the mitochondrial matrix impairs mitochondrial respiration and thus, limits the generation of ATP, eventually leading to mitochondrial failure and activation of the mitochondrial removal process.[30,31]

Another example is the antibiotic antimycin A that elicits a mitochondria-targeted autophagic response by inhibiting the respiratory complex III and leading to increased levels of reactive oxygen species (ROS) (**Table 1**).[32] Compared to protonophores and other mitochondrial toxins, antimycin A causes a relatively limited decrease in $\Delta\Psi_m$ that is rapidly balanced by the reverse hydrolysis activity of the F_1Fo-ATPsynthase.[33] For that reason, it is often used in combination with oligomycin (**Table 1**), which prevents this compensatory mechanism by inhibiting the F_1Fo-ATPsynthase and thus facilitates a more intense depolarization.[6] The activation of mitophagy by antimycin A and oligomycin is generally considered more relevant to naturally-occurring conditions, since both inhibitors are relatively selective for their targets and mediate fewer toxic effects, resulting in subtler mitochondrial damage.[6,29]

Similarly to antimycin A, a diverse range of chemicals, including sodium selenite, diquat, retigeric acid B and paraquat have been reported to trigger mitophagy in response to elevated levels of oxidative stress (**Table 1**).[4,20,35–37] Of particular interest

is sodium selenite, which induces a lethal form of mitophagy in human glioma cells, but not astrocytes.[36] Mechanistically, sodium selenite appears to stimulate mitophagy by promoting a ROS-dependent activation of the E3 ubiquitin ligase MUL1 (mitochondrial E3 ubiquitin protein ligase 1), which in turn recruits ULK1 (Unc-51 Like Autophagy Activating Kinase 1) to mitochondria, thus facilitating their autophagic degradation.[38] More recently, it was shown to up-regulate the expression of ceramide synthase 1, which controls the biosynthesis of the sphingolipid C_{18}-ceramide that in turn promotes lethal mitophagy by inhibiting mitochondrial respiration and recruiting LC3 via a direct interaction.[39]

Parkinsonian toxins

The pesticide paraquat, which is another well-studied mitochondrial toxin, activates mitophagy via excessive complex I-dependent superoxide generation.[4,20] Similarly to other toxins, paraquat-induced mitophagy is initiated by mitochondrial depolarization and operates via the PINK1-Parkin pathway. Paraquat belongs to a group of neurotoxins, which are known to induce parkinsonian symptoms and are often used to model Parkinson's disease both *in vitro* and *in vivo*.

Like paraquat, other parkinsonian toxins, including rotenone, MPP^+ (1-methyl-4-phenylpyridinium) –the metabolically active form of MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)– and 6-OHDA (6-hydroxyldopamine) stimulate mitophagy, although by a different mode of action (**Table 1**).[40–42] Rotenone in particular causes a relatively mild mitochondrial depolarization, which is inadequate to induce PINK1-mediated mitophagy in primary neurons or neuroblastoma cells.[41] Rotenone, as well as 6-OHDA (and so does Staurosporine) activate the process by promoting the externalization of cardiolipin, which recruits the autophagic machinery via a direct interaction with LC3 (**Figure 2c**). Moreover, 6-OHDA and MPP^+ were both found to promote the phosphorylation and mitochondrial accumulation of ERK2 (extracellular signal-regulated protein kinase 2), an event that drives mitochondria into autophagosomes and facilitates their degradation.[40,43]

The identification of divergent mitophagic pathways in neurons, which are exploited by the parkinsonian toxins and others, such as the apoptotic agent Staurosporin, suggest that the PINK1-Parkin pathway might be not the primary mechanism of neuronal mitochondrial quality control.[41] In fact, neurons exhibit a poor response to FCCP/CCCP-induced dissipation of $\Delta\Psi_m$ compared to HeLa and other non-neuronal cell lines, as evidenced by the delayed translocation of Parkin.[44] Parkinsonian toxins

may therefore represent preferential chemical tools to stimulate mitophagy in neurons and investigate the underlying regulatory mechanisms.

Modulators of PINK1-Parkin activity

Ample evidence from the literature suggests that the integrity of the PINK1-Parkin pathway is essential for mitochondrial recycling and function.[45] Mutations in PINK1 and Parkin that affect their catalytic activities can lead to impaired mitophagy and underlie rare forms of early-onset PD. Despite the recent advances and scientific excitement around this pathway, efforts to pharmacologically modulate it remain limited.

An unconventional but promising strategy to enhance PINK1-mediated mitophagy is based on the identification of N^6-furfuryl ATP (kinetin triphosphate, KTP) as a neo-substrate that exhibits higher affinity for PINK1 compared to the native substrate, ATP (**Figure 3a**).[46] KTP was shown to augment the kinase activity of both $PINK1^{wt}$ and $PINK1^{G309D}$, a Parkinson's disease-associated mutant with severely impaired kinase activity. Interestingly, kinetin (N^6-furfuryl adenine), a cell permeable precursor of KTP, was able to enhance the PINK1-mediated phosphorylation of Parkin, accelerating its mitochondrial recruitment and the subsequent initiation of mitophagy after CCCP-induced mitochondrial insult (**Table 1**).[46] Although still at an early stage, preliminary results suggest that this approach has great potential to restore mitophagy in conditions associated with loss-of-function PINK1 mutations. However, kinetin does not affect the stabilization of PINK1 under resting conditions and thus, an external stimulus, such as CCCP, is required to initiate mitophagy, which restricts its application as a mitophagy-stimulating chemical tool.

An alternative and rather interesting approach to augment the activity of the PINK1-Parkin pathway resides in the inhibition of the tumor suppressor p53, which regulates the expression levels of *PARK2*.[47] On the other hand, Parkin represses the transcription of *p53* by physically interacting with its promoter region[48,49] – although this appears to depend on cell or tissue type.[47] This suggests that at least in some particular cell types a feedback loop between Parkin and p53 exists that may play a regulatory role in mitophagy. In support of this notion, p53 may suppress mitophagy by directly interacting with Parkin and halting its translocation to mitochondria, whilst pharmacological intervention with pifithrin-α, a small molecule inhibitor of p53, restored mitochondrial clearance in a p53-dependent manner (**Figure 3b**).[50] In a different study, pifithrin-α was shown to promote mitophagy and ameliorate mitochondrial dysfunction in type I and II mouse models of diabetes.[51]

Interestingly, elevated levels of p53 exist in the brains of animal models and human cases of PD, a condition strongly associated with impaired mitochondrial function and quality control,[52,53] while inhibition of p53 by pifithrin-α reduces dopaminergic neuron death and protects against MPTP neurotoxicity *in vivo*.[54] Moreover, $p53^{-/-}$ mice have higher levels of basal mitophagy compared to their wild type counterparts, leading to increased resistance against doxorubicin-induced mitochondrial injury.[50] However, p53 is a master regulator of several cellular processes and as a result, its pharmacological targeting may impinge diverse biological effects beyond the induction of mitophagy. Therefore, despite the emerging link between p53 and Parkin-mediated mitophagy, the specificity in targeting mitophagy via this approach is questionable.

In addition to p53 inhibitors, a diverse group of small molecule modulators of *PARK2* transcription was recently identified through high-throughput screening (HTS) of bioactive compounds using a Firefly/NanoLuc luciferase coincidence reporter system.[55] These include modulators of epigenetic mechanisms, such as histone deacetylase and bromodomain inhibitors, and small molecule inhibitors of cholesterol biosynthesis, such as mevastatin and fluvastatin.[56] Interestingly, the JNK inhibitor SR3306, another promising lead identified in the same study, seems to enhance mitochondrial quality control in a cell-based model of mitochondrial stress induced by the accumulation of misfolded ΔOTC (truncated ornithine transcarbamylase).

Recently, novel molecular routes to enhance PINK1-Parkin mitophagy without interfering directly with the pathway were described. A good example is the 18-kDa translocator protein (TSPO), which regulates mitophagy downstream of the PINK1-Parkin pathway via a ROS-sensitive mechanism.[21] In particular, TSPO blocks mitophagy following FCCP-induced mitochondrial insult by inhibiting mitochondrial ubiquitination, but not PINK1 stabilization or Parkin translocation. TSPO levels are elevated in pathological conditions associated with mitophagy defects, highlighting its pharmacological targeting as a suitable strategy to therapeutically activate mitophagy.[56] Though, the limited selectivity of the most widely used TSPO ligands may restrict the options for its targeting in this context,[56,57] the recent elucidation of TSPOs three-dimensional structure shall facilitate the design of improved chemicals to shed light on the protein's role in the modulation of mitophagy.[56]

The ubiquitin specific peptidase 30 (USP30) is a mitochondrion-localized deubiquitylase known for its role in the regulation of mitochondrial morphology.[58,59] USP30 antagonizes Parkin-driven mitophagy by removing poly-ubiquitin chains from damaged mitochondria and thus stalling mitochondrial priming. USP30 knockdown is sufficient to restore mitophagy in neuronal cells expressing a dysfunctional Parkin

mutant and rescue motor function and mitochondrial defects in fly models of PD devoid of a fully functional Pink1-Parkin pathway. Despite the promising results obtained by the manipulation of USP30 expression levels, no pharmacological proof of concept is reported to date and the potential of USP30 inhibitors as mitophagy-inducing molecules, such as the diterpenoid analogue 15-oxospiramilactone[60], remains to be elucidated.

In addition to USP30, other deubiquitinating enzymes, including USP8 and USP15 have recently been reported to oppose or even enhance Parkin-mediated mitochondrial priming.[61] This is not surprising given the pivotal role of Ub signaling in the regulation of mitophagy. However, deubiquitylases have diverse biological functions[62] and their pharmacological targeting by small molecule modulators could lead to non-specific events that are not directly related to the process of mitophagy thus limiting their use as mitophagy-stimulating chemical probes.

Iron chelators

The development of sensitive phenotypic assays for monitoring alterations in the levels of cellular mitophagy enabled the identification of several modulators of the process.[63,64] In particular, a chemical screen utilizing a tandem mCherry-GFP tag anchored on the OMM, yielded the iron chelator deferiprone (DFP) as a novel inducer of mitophagy (**Table 1** and **Figure 5b**).[63] DFP was shown to promote mitochondrial turnover via an iron depletion-dependent mechanism without causing a $\Delta\Psi_m$ collapse. Interestingly, DFP retains its activity in cells devoid of a fully functional PINK1-Parkin pathway and this has prompted its use as a chemical tool to assess Parkin-independent mitophagy in recent studies.[65,66]

Intriguingly, 1,10'-phenanthroline (Phen), another compound with siderophore-like properties, was identified as a "hit" in a chemical screen for mitophagy activators (**Table 1**).[64] In contrast to DFP, Phen mediates a collapse of $\Delta\Psi_m$, which is followed by a Drp1-dependent mitochondrial fragmentation and induction of mitophagy. Consistently with aspects of the latter study, Phen and the structurally distinct iron chelator, cicloprox olamine (**Table 1**), were reported to trigger mitophagy in response to mitochondrial depolarization in mammalian cells and nematodes.[67] However, Phen is unable to exert similar effects in neuronal-like SH-SY5Y cells, suggesting that its mitophagic activity might be cell type dependent.[68] In addition, the structurally similar siderophore 2'2-bipyridyl was shown to extend lifespan in *C. elegans* by inducing a mitophagic response that was dependent on PINK1 as well as PBR-1 and DCT-1, the

C. elegans homologues of Parkin and NIX1/BNIP3 respectively (**Table 1** and **Figure 5b**).[69]

Although pharmacologically induced iron depletion appears to stimulate mitophagy, further studies are required to elucidate the underlying mechanisms given the contradictory observations. With the exception of DFP, iron chelators trigger mitophagy by collapsing $\Delta\Psi_m$, which points towards mitochondrial toxicity. Nonetheless, a metabolic switch from oxidative phosphorylation to glycolysis is required for the induction of mitophagy by iron depletion,[63] which may indicate respiratory injury and thus, restrict the therapeutic application of such agents particularly in neurodegenerative conditions, as neurons rely heavily on oxidative phosphorylation.

SIRT1 activators

Nicotinamide (NAM) was recently reported to promote mitophagy without perturbing mitochondrial function or collapsing the $\Delta\Psi_m$ (**Table 1**).[70] On the contrary, primary human fibroblasts treated with NAM exhibit elevated levels of resting $\Delta\Psi_m$ – however this is accompanied by a fragmentation of the mitochondrial network.[71] The effects of NAM are attributed to the activation of SIRT1 (silent information regulator T1), an NAD^+-dependent deacetylase involved in the regulation of longevity.[71] By acting as its biosynthetic precursor, NAM increases the cellular availability of NAD^+, which in turn activates SIRT1 and promotes mitochondrial clearance.

Interestingly, other SIRT1 activators, including the natural products resveratrol and fisetin and the synthetic small molecule SRT1720 (**Table 1** and **Figure 5b**), reduce the mitochondrial network size to a similar extent as NAM, without increasing the $\Delta\Psi_m$, suggesting that the hyperpolarization induced by NAM is independent of its effects on SIRT1.[71] Mechanistically, SIRT1 could trigger mitochondrial turnover by increasing the deacetylation and subsequent activation of LC3[72] or activating the mitophagy regulator UCP2 (uncoupling protein 2)[73]. Additionally, SIRT1 has a pivotal role in cellular adaptation to hypoxic conditions by interacting with, and deacetylating, FoxO3 (forkhead box O3).[74] Activation of SIRT1 by calorie restriction up-regulates the FoxO3 downstream effector BNIP3 and promotes mitophagy in primary renal proximal tubular cells.[75] In this regard, it would be interesting clarify whether the regulation of mitophagy by SIRT1 operates exclusively through a receptor-based mechanism implying that the functional integrity of the PINK1-Parkin pathway would not be required.

In addition to NAD^+ precursors and SIRT1 agonists, agents that can indirectly modulate the activity of SIRT1 by inhibiting NAD^+-consuming enzymes, such as the poly(ADP-ribose) polymerase-1 (PARP-1), have the potential to stimulate mitophagy. A good

example is AZD2281/olaparib (**Table 1**), a PARP-1 inhibitor that induces mitophagy in breast cancer cells and decreases the mitochondrial network size in a cell-based model associated with impaired mitophagy.[73,76] Notably, hyperactivation of PARP-1 associates with impaired mitophagy and mitochondrial defects resulting from reduced NAD^+ cellular content and increased SIRT1 activity.[73] Genetic deletion or pharmacological inhibition of PARP-1 promotes an NAD^+-mediated activation of SIRT1 that results in enhanced mitochondrial quality and respiration.[77] Although the therapeutic effects of SIRT1 inducers in several *in vivo* disease models are well documented,[78,79] triggering mitophagy using this approach raises the recurring issue of specificity given the crucial role of the deacetylase in the regulation of closely related processes, such as general autophagy[80] and mitochondrial biogenesis.[81]

Nrf2 inducers and the anterograde regulation of cell mitophagy

The transcription factor nuclear factor E2-related factor 2 (Nrf2, Gene ID: 4780) has been proposed as an attractive target to enhance mitochondrial function and health. Genetic or pharmacological augmentation of Nrf2 activity has a number of beneficial effects on mitochondria.[82] Nrf2 orchestrates the expression of a battery of cytoprotective genes harboring antioxidant response elements (AREs) in their promoter regions, amongst which of particular relevance to general and selective forms of autophagy are p62/SQSTM1[83] and NDP52[84].

Despite the emerging understanding of the crosstalk between Nrf2 and mitochondria, its role in mitophagy until recently was unexplored. We recently reported the identification of a small molecule that activates Nrf2 by disrupting its regulatory protein-protein interaction (PPI) with Keap1 (Kelch-Like ECH-Associated Protein 1),[85] a redox-sensitive protein that acts as an adaptor for the Cullin 3/Rbx1 ubiquitin ligase complex, facilitating the ubiquitination of Nrf2 and its subsequent proteasomal degradation (**Figure 4**).[86] This compound, which we named p62/SQSTM1-mediated mitophagy inducer (PMI), increases the expression of p62/SQSTM1 and drives mitochondria into autophagy without disrupting the $\Delta\Psi_m$ (**Figure 5a**).[85] Nrf2 and p62/SQSTM1 are both indispensable to the mitophagic pathway exploited by PMI, as delivery of mitochondria to autophagosomes is abolished in $Nrf2^{-/-}$ or $p62^{-/-}$ cells. Interestingly, PMI is able to induce mitophagy in cells devoid of a fully functional PINK1-Parkin pathway (PINK1 KO or Parkin KD), which highlights its therapeutic potential in conditions associated with impaired PINK1 and Parkin activity.

Pharmacological activation of Nrf2 by PMI increments mitochondrial health as demonstrated by the elevated levels of resting $\Delta\Psi_m$ in treated cells, which is consistent

with the enhanced oxidative metabolism observed in Keap1-KO and Keap1-KD cells.[87] Moreover, PMI does not alter mitochondrial morphology and in contrast to common mitophagy inducers, the activation of mitophagy is not accompanied by mitochondrial swelling or fragmentation.[85]

PMI has a distinct mode of action compared to traditional Nrf2 inducers, such as the isothiocyanate sulforaphane (**Figure 4**).[85,88] Typical Nrf2 activators are electrophiles that react with sensor cysteines on Keap1, inducing an irreversible conformational change that halts the ubiquitination of Nrf2.[89] However, the vast majority of these compounds are capable of reacting with a range of cysteine-containing redox sensitive proteins, giving rise to a host of off-target effects.[86] The activation of general autophagy by sulforaphane is an example of great relevance as it occurs via a ROS-dependent mechanism and independently of its effects on Nrf2.[90] On the other hand, PMI promotes a targeted autophagic degradation of mitochondria without affecting general autophagy.[85] Notably, our preliminary results suggest that sulforaphane does not mediate similar effects on mitophagy, despite activating Nrf2 and up-regulating the expression of p62/SQSTM1. These differences further highlight the potential utility of PMI compared to sulforaphane and related electrophilic Nrf2 inducers and hint at the therapeutic potential of such compounds.

Pharmacological inhibitors of mitophagy

The most widely adopted protocol to block the process of mitophagy *in vitro* resides on the pharmacological inhibition of lysosomal acidification, using the V-ATPase (Vacuolar-type H^+-ATPase) inhibitor bafilomycin A1 or the lysomotropic agents chloroquine and hydroxychloroquine (for a review see [91]). In addition, compounds that suppress general autophagy by inhibiting the formation of autophagosomes have also been used to halt mitophagy, with the class III PI3K inhibitor 3-methyladenine being the most prominent example. Although these approaches are particularly effective at inhibiting mitophagy, they also affect other autophagic and lysosomal processes and are therefore largely unspecific.

Novel strategies to inhibit mitophagy in a more specific manner have been described, however their application to date has been limited. In particular, peptide inhibitors of receptor-mediated mitophagy that are based on the LIR of FUNDC1 were recently reported.[17] These include sequences that incorporate the Ser13 residue, whose phosphorylation status regulates the interaction of FUNDC1 with LC3 and thus its ability to target mitochondria to autophagosomes. The LIR-mimetic peptide ^{9}QDYESDDDSYEVLDLTE^{25} was shown to disrupt the binding of the mitophagy

receptor to LC3 with a K_d of 5.8 μM, which is 5-fold lower compared to that of the pSer13-containing analogue. Interestingly, conjugation of the unphosphorylated peptide inhibitor to a TAT-derived sequence yielded a cell-permeable probe that was able to suppress FCCP-induced mitophagy without affecting general autophagy, further supporting the potential of such molecules to modulate the process in a selective manner.

A different approach to inhibit mitophagy is based on the suppression of mitochondrial fragmentation, an event that has been associated with mitophagy.[92] Compounds that inhibit the activity of Drp1 (dynamin-1-related protein) are capable of halting mitochondrial division and potentially prevent the initiation of mitophagy under conditions of mitochondrial stress. The mitochondrial division inhibitor (mdivi) is an allosteric modulator of Drp1 that was identified through a phenotypic screening exercise in yeast cells.[93] Mdivi was shown to exert cardioprotective effects *in vivo* by blocking mitochondrial fission and at least in part by preventing abnormal mitophagy.[94] A different example of a Drp1 modulator is the cell-permeable peptide P110, which consists of the highly conserved Drp1 region 110 (^{49}DLLPRGT^{55}) conjugated to a cell-permeating TAT peptide-derived sequence.[95] Mechanistically, P110 appears to inhibit Drp1 by impairing its enzymatic activity and blocking its translocation to mitochondria by selectively disrupting its PPI with the OMM-localized protein Fis1. Interestingly, P110 does not inhibit mitochondrial fission under resting conditions, but instead prevents the abnormal mitochondrial fragmentation induced by several mitochondrial stressors, including FCCP and MPP^+. However, to our knowledge no studies describing the effects of P110 on mitophagy have been reported to date and its potential as a negative modulator of the process remains to be determined.

Concluding Remarks

Restoring the ability of cells to efficiently eliminate damaged or dysfunctional mitochondria is likely to be pivotal to the prevention of a wide range of diseases. Equally paramount is the need to identify novel chemical probes that can be used to understand the process of mitophagy, and correct defects. Notably, the increased understanding of the molecular determinants governing mitophagy has not been mirrored by the development of suitable chemical probes with potential therapeutic uses.

Both clinicians and scientists engaged in characterizing the mechanistic aspects of mitophagy will greatly benefit from improved pharmacological approaches to induce and control this mechanism of cellular quality control more selectively. From the recent

literature it is notable that the original protocols based upon acute mitochondrial depolarization induced by non-specific agents (*e.g*. FCCP/CCCP), have been gradually refined into "milder" approaches (*e.g*. Antimycin A/Oligomycin) aimed at promoting mitophagy in a more physiologically compliant manner. Although uncoupling agents have been instrumental in understanding how depolarization of the mitochondrion triggers regulatory pathways leading to removal of the disposable organelles, the need for alternative approaches that are translatable into an *in vivo* context is now a priority.

Specifically, the latest advancements in the field demonstrated that the process of mitophagy operates beyond the context of acute mitochondrial stress, such as during resting physiology, maintaining the integrity of the mitochondrial network and contributing to the preservation of cellular homeostasis. In particular, subtle changes in mitochondrial redox status[96] such as increases mitochondrial respiratory activity and energy production[97] were recently shown to activate mitophagy, adding another layer of complexity to the regulation of the process. Additionally, the accumulation of misfolded proteins can trigger PINK1-Parkin mediated mitophagy in energetically healthy mitochondria, suggesting that this pathway can also operate independently of respiratory collapse in response to other cues.[98] Mitophagy is therefore likely to be part of a finely tuned mechanism that integrates both anabolic and catabolic mechanisms catalyzing the events of mitohormesis.[99]

Triggering mitophagy through a pharmacologically-induced acute depolarization bears limitations, highlighting the need for alternative means to modulate the process and, eventually, exploit these for therapeutic purposes. The lack of mitophagy-specific phenotypic assays that are suitable for High Throughput Screening (HTS), hampers the identification of novel chemical tools to manipulate the process. In addition, given the potential of several mitochondrial stressors to activate mitophagy, positive HTS "hits" are likely to be agents that may exert disruptive effects on the organelle and thus, have limited therapeutic potential. On the other hand, the increased understanding of the signaling pathways involved in the regulation of mitophagy, shall offer opportunities for targeted pharmacological interventions through the development of rationally designed chemical probes, as illustrated in recently reported successful examples.[17]

Agents that modulate cellular adaptive responses to stressors or toxins have the potential to further progress our understanding of the underpinning pathways of mitophagy. Notably, stimulation of mitophagy is proven to occur in response to the activation of Nrf2, a transcription factor often referred to as the master regulator of the antioxidant response.[85] This highlights another scenario for mitophagy as a process

integrated and governed by pathways dictating cellular resilience to impaired physiology, therefore widening the prospects for subsequent pharmacological validation and intervention in this process.

We are therefore optimistic that the review of the pharmacological modulation of mitophagy provided here may draw attention to the need for new, specific modulators of mitophagy and facilitate discussion within the field about the best ways to identify and characterize the next generation of chemical probes and therapeutic agents that target this intriguing cellular process.

Financial Interest

N.D.G. and G.W. are shareholders in Keregen Therapeutics Ltd, an SME with a research interest in Nrf2 inducing compounds.

Acknowledgements

The research activities led by M.C. are supported by the following funders, which are gratefully acknowledged: Biotechnology and Biological Sciences Research Council [grant numbers BB/M010384/1 and BB/N007042/1]; the Medical Research Council [grant number G1100809/2], Bloomsbury Colleges Consortium PhD Studentship Scheme; The Petplan Charitable Trust; Umberto Veronesi Foundation; Marie Curie Actions and LAM-Bighi Grant Initiative. G.W. acknowledges Cancer Research UK (C9344/A10268), the BBSRC (BB/L01923X/1) and UCL School of Pharmacy for financial support.

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Legends to Figures

Figure 1. PINK1/Parkin-mediated mitophagy

The figure provides a schematic representation of the PINK1/Parkin pathway of mitophagy, activated by protonophores or mitochondrial toxins causing a collapse of the mitochondrial membrane potential ($\Delta\Psi_m$). Mitochondria collapsed in their $\Delta\Psi_m$ – depicted in red here and the other figures- accumulate PINK1 on their surface triggering the recruitment of the Ubiquitin Ligase Parkin. The latter amplifies the mitophagy signal generated by PINK1 ubiquitinating mitochondrial proteins and thereby providing more ubiquitin substrate for PINK1 to phosphorylate. In this model, the mitophagy receptors NDP52 and OPTN, promote the recruitment of ULK1, which drives autophagosomes initiation on the surface of mitochondria being disposed – depicted in brown here and throughout the illustrations. NDP52 and OPTN, following accumulation of PINK1 can drive the process independently from the recruitment of Parkin (B).

Figure 2. PINK1/Parkin-independent mechanisms of mitophagy

Autophagic removal of mitochondria with collapsed $\Delta\Psi_m$ may occur also via mechanisms other than the PINK1/Parkin pathway as proven during hypoxia which impairs the respiration of mitochondria flagging these for disposal. Panels A and B report the pathways involved in this process with A depicting the key steps of the mitophagy process mediated by the accumulation of NIX1 or BNIP3 on mitochondria to be removed –represented in red- and B the one coordinated by PGAM5 and FUNDC1: all leading to recruitment of autophagosomes via LC3 on mitochondria undergoing mitophagy –depicted in brown. The same happens when Cardiolipin as described in Panel C mediates mitophagy. The externalization of Cardiolipin on the mitochondrial surface triggers mitophagic response as it is demonstrated following inhibition of intracellular kinases via staurosporine or in presence of rotenone and 6-OHDA.

Figure 3. Activation of Ub-dependent mitophagy by modulators of the PINK1-Parkin pathway

The PINK1/Parkin way of mitophagy can be exploited by pharmacological tools acting on specific steps of the pathway. One of these consists in boosting the actual kinase activity of the accumulated PINK1 on mitochondria with collapsed $\Delta\Psi_m$ -depicted in red. Panel A reports specifically of kinetin (*N*-furfuryl adenine) which is a cell permeable precursor of the Kinetin Triphosphate (KTP): a neo-substrate for PINK1 showing higher

affinity compared to ATP. The increased KTP pools accelerate Parkin recruitment on mitochondria which already have their $\Delta\Psi_m$ collapsed -and therefore represented in red- subsequent initiation of mitophagy by improving the PINK1-mediated phosphorylation of Parkin. Panel B schematizes instead the pharmacological intervention with pifithrin-α, a small molecule inhibitor of p53, able to restore the clearance of mitochondria in a p53-dependent manner. Pifithrin-α operates exploiting the feedback loop between Parkin and p53 that regulates the expression levels of *PARK2* improving the process overall as done by Kinetin.

Figure 4. Chemical approaches to inhibit the Keap1-mediated degradation of Nrf2.

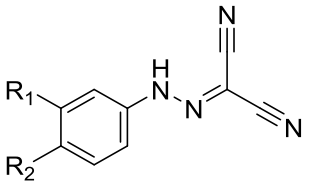
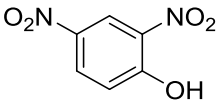
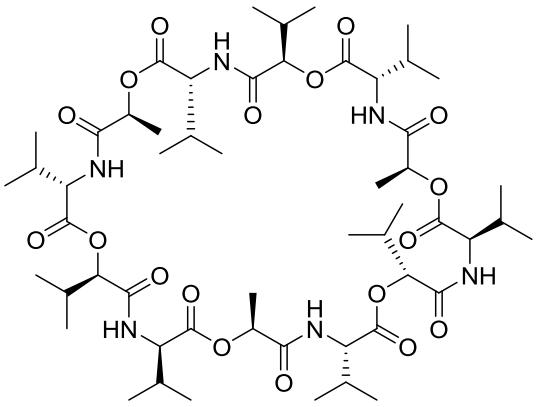
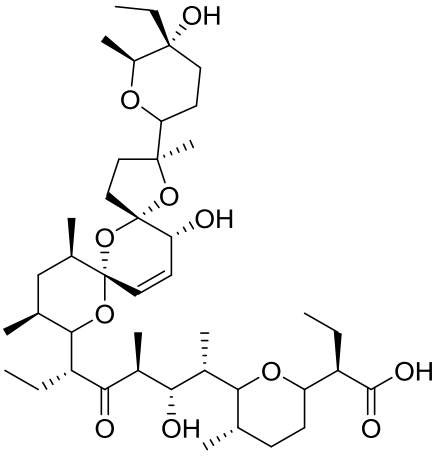
Model reporting indirect (sulforaphane) and direct (PMI) pharmacological strategies to inhibit the Keap1-mediated degradation of Nrf2 leading to the nuclear accumulation of the latter and the subsequent transcriptional activation of genes involved in the regulation of both mitochondrial function and autophagy. One of these is the sequestosome P62/SQSTM1 via which the activation of mitophay by the direct approach of PMI occurs.

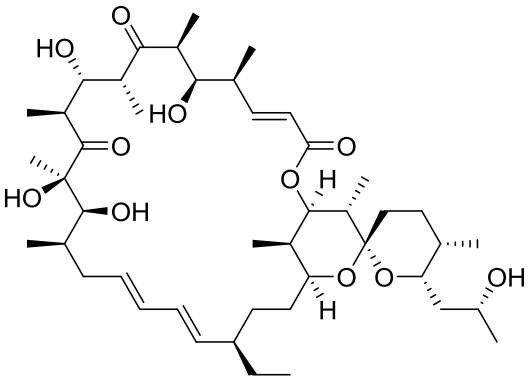
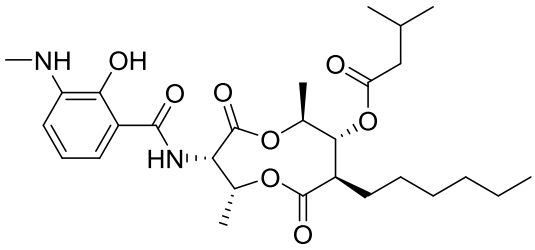
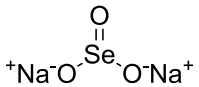
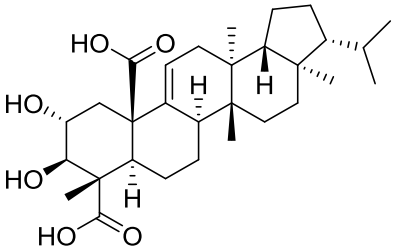
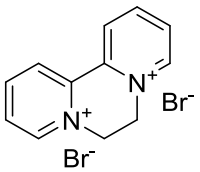
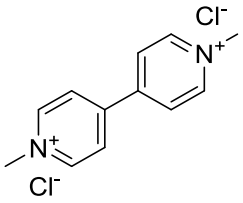
Figure 5. The model of Mitophagy induction via PMI, SIRT1 activators and iron chelators

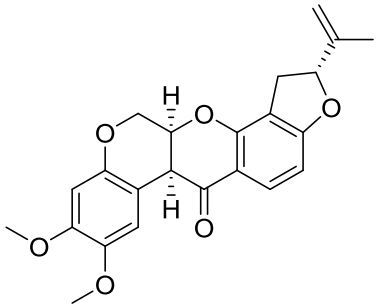
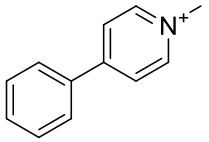
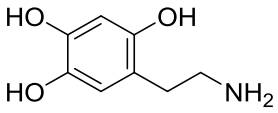
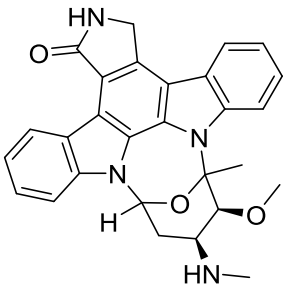
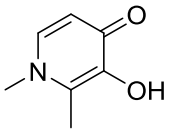
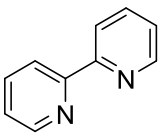
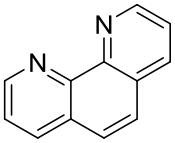
Panel A reports the cartoon representation of the mitophagy induction by PMI. PMI exploits ubiquitination mechanisms currently ill-defined. This approach promotes the autophagosomal targeting of mitochondria and their disposal. However it does so by leaving their $\Delta\Psi_m$ intact. Mitochondria are therefore depicted in brown but never in red implying that they are primed for mitophagic disposal without this intermediate step common to many processes of mitophagy. Panel B summarizes the induction of mitophagy via SIRT-1 activators that operate by increasing the $\Delta\Psi_m$ of mitochondria, which are therefore represented with a shining tone of green (i). The same panel reports also on the iron chelators, which apparently involve more than one mechanism to complete the autophagic removal of mitochondria (ii).

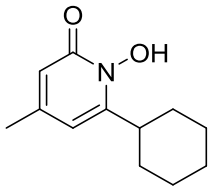
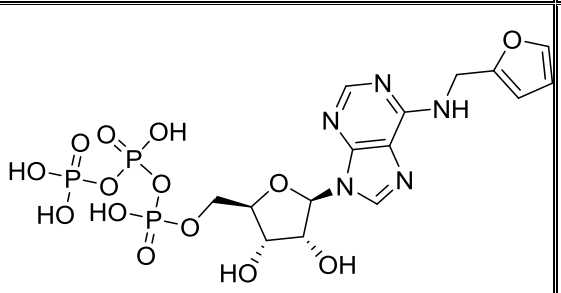
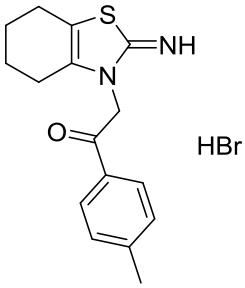
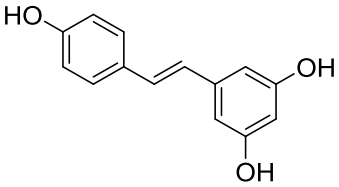
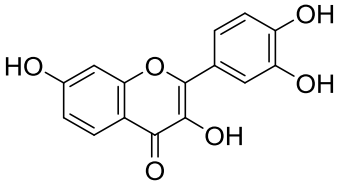
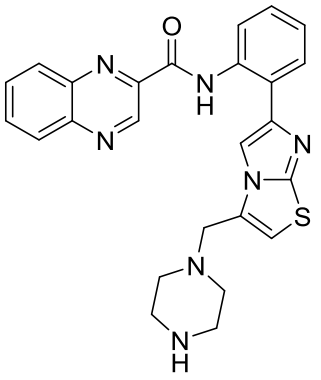
Table 1. List of the pharmacological agents inducing cellular mitophagy.

Pharmacological class	Mitophagy inducer	Chemical structure	Mechanism	Ref

Protonophores	CCCP and FCCP	 **FCCP**: R_1= H, R_2 = OCF_3 **CCCP**: R_1 = Cl, R_2 = H	Proton leak-induced loss of $\Delta\Psi_m$.	4,19–21,86
	DNP			100
K^+ ionophores	Valinomycin		$\Delta\Psi_m$ collapse due to K^+ influx.	4,64
	Salinomycin		K+/H+ antiporter activity leading to matrix acidification and mitochondrial failure.	30

Respiratory Complex III & ATP synthase inhibitor	Antimycin A & Oligomycin	 **Oligomycin A**  **Antimycin A**	Increased superoxide generation coupled with $\Delta\Psi_m$ loss.	6,64
Superoxide generator	Sodium selenite		MUL1 activation & ceramide synthase 1 up-regulation.	37,40
Apoptotic agent	Retigeric acid B		Mitochondrial damage and $\Delta\Psi_m$ loss.	38
Parkinsonian toxins	Diquat		Superoxide-induced mitochondrial damage.	4,36
	Paraquat			

	Rotenone		ROS accumulation and mitochondrial damage: Cardiolipin-dependent mitophagy.	42
	MPP+		ROS accumulation and mitochondrial damage: ERK1/2-dependent mitophagy.	42,43
	6-OHDA		ROS accumulation and mitochondrial damage: ERK1/2- and cardiolipin-dependent mitophagy.	41,42
Pan-kinase inhibitor	Staurosporin		Mitochondrial damage and $\Delta\Psi_m$ loss: activation of PINK1-Parkin pathway and cardiolipin-dependent mitophagy.	42
Iron chelators	Deferiprone (DFP)		Iron chelation-dependent and Parkin-independent mitophagy.	64,66
	2'2-Bipyridyl		Mitophagy dependent on PINK1, PDR-1 (Parkin) and DCT-1 (Nix).	70
	1,10'-Phenanthroline		Mitochondrial fragmentation and $\Delta\Psi_m$ loss.	65,68

	Cicloprox olamine		$\Delta\Psi_m$ loss	68
PINK1 enhancer	Kinetin triphosphate, KTP		Enhanced kinase activity of PINK1	47
p53 inhibitor	Pifithrin-α	 HBr	Inhibition of p53 and possible activation of Parkin.	51,52
SIRT1 agonists	Resveratrol		Activation of SIRT1.	72
	Fisetin			69,72
	SRT1720			72

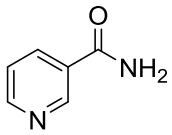
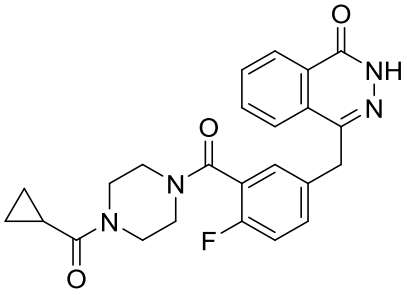
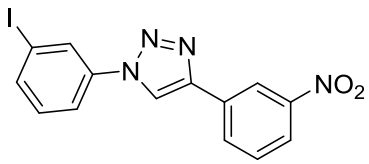
NAD^+ precursor	Nicotinamide (NAM)		NAD+ accumulation: activation of SIRT1.	69,71,72
PARP-1 inhibitor	AZD2281 (olaparib)		NAD^+ accumulation: activation of SIRT1.	74,77
Keap1 inhibitor	PMI		P62/SQSTM1-dependent mitophagy.	85

Figure 1.

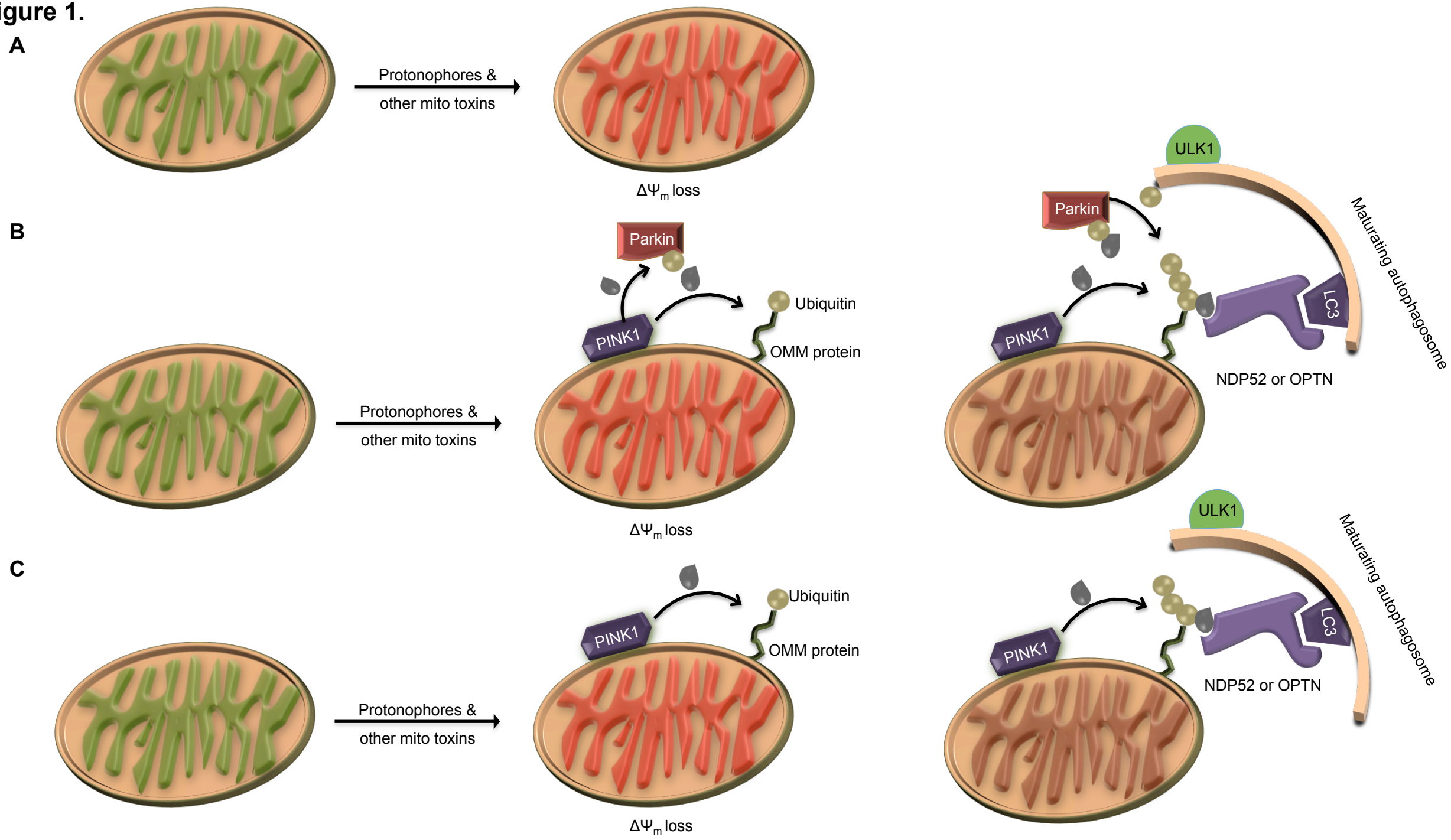

A
Protonophores &
other mito toxins
ΔΨm loss
B
Parkin
Ubiquitin
PINK1
OMM protein
Protonophores &
other mito toxins
ΔΨm loss
ULK1
Parkin
Maturating autophagosome
LC3
PINK1
NDP52 or OPTN
C
Ubiquitin
PINK1
OMM protein
Protonophores &
other mito toxins
ΔΨm loss
ULK1
Maturating autophagosome
LC3
PINK1
NDP52 or OPTN

Figure 2.

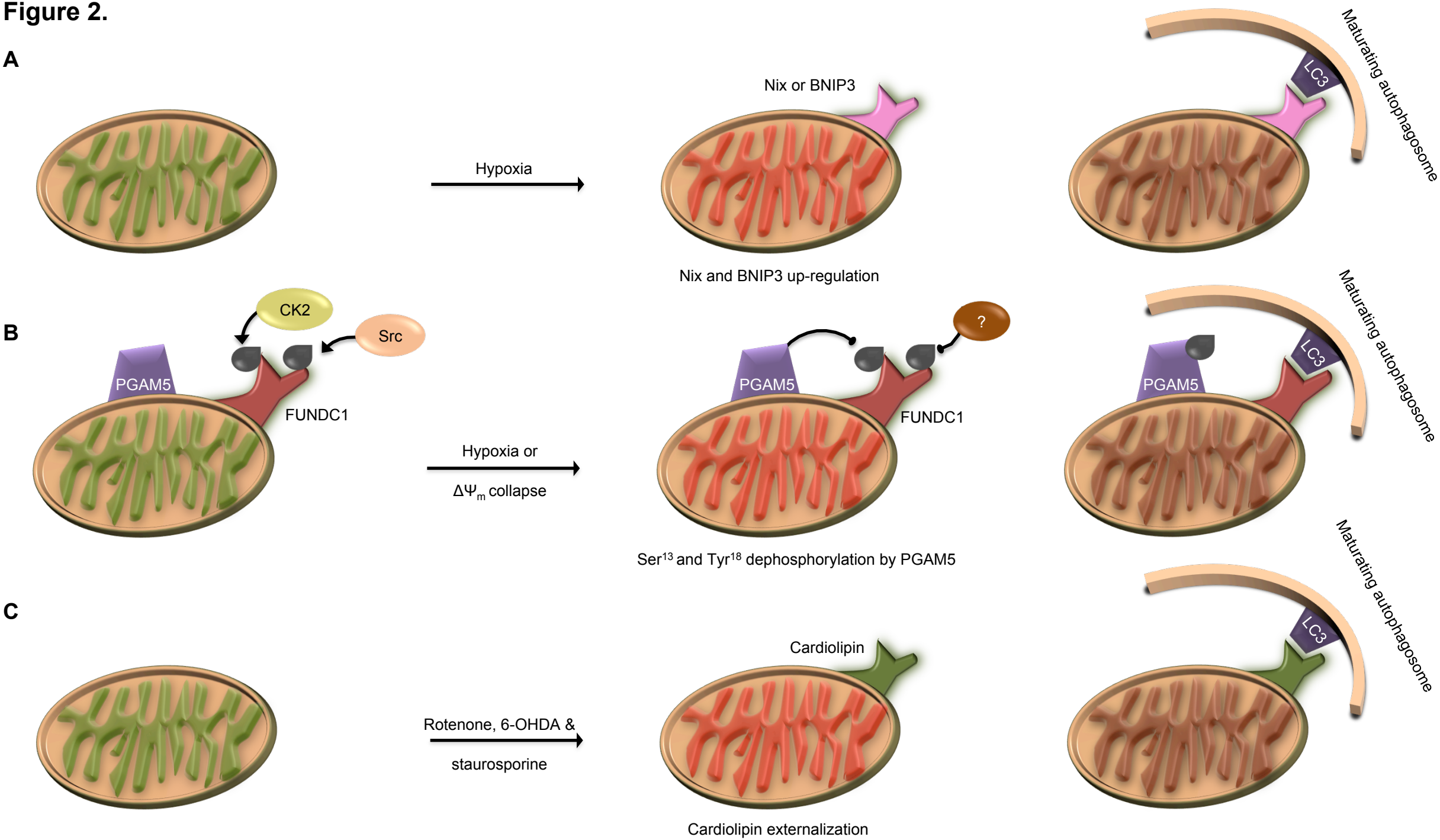

A
Hypoxia
Nix or BNIP3
Nix and BNIP3 up-regulation
LC3
Maturating autophagosome
B
CK2
Src
PGAM5
FUNDC1
Hypoxia or
ΔΨm collapse
?
PGAM5
FUNDC1
Ser13 and Tyr18 dephosphorylation by PGAM5
LC3
Maturating autophagosome
C
Rotenone, 6-OHDA &
staurosporine
Cardiolipin
Cardiolipin externalization
LC3
Maturating autophagosome

Figure 3.

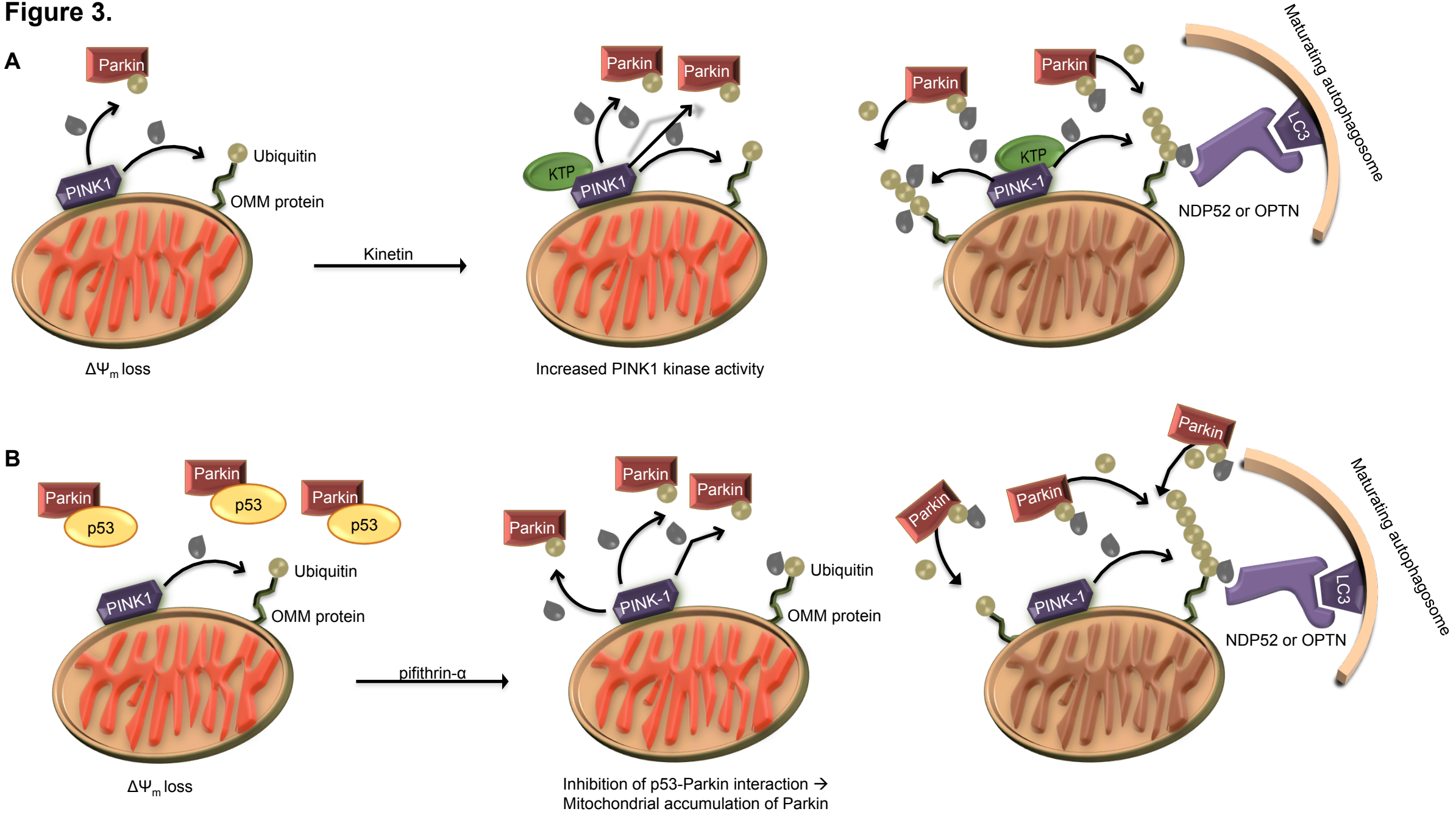

A
Parkin
Ubiquitin
PINK1
OMM protein
ΔΨm loss
Kinetin
Parkin
Parkin
KTP
PINK1
Increased PINK1 kinase activity
Parkin
Parkin
KTP
PINK-1
LC3
Maturating autophagosome
NDP52 or OPTN
B
Parkin
p53
Parkin
p53
Parkin
p53
Ubiquitin
PINK1
OMM protein
ΔΨm loss
pifithrin-α
Parkin
Parkin
Parkin
Ubiquitin
PINK-1
OMM protein
Inhibition of p53-Parkin interaction →
Mitochondrial accumulation of Parkin
Parkin
Parkin
Parkin
PINK-1
LC3
Maturating autophagosome
NDP52 or OPTN

Figure 4.

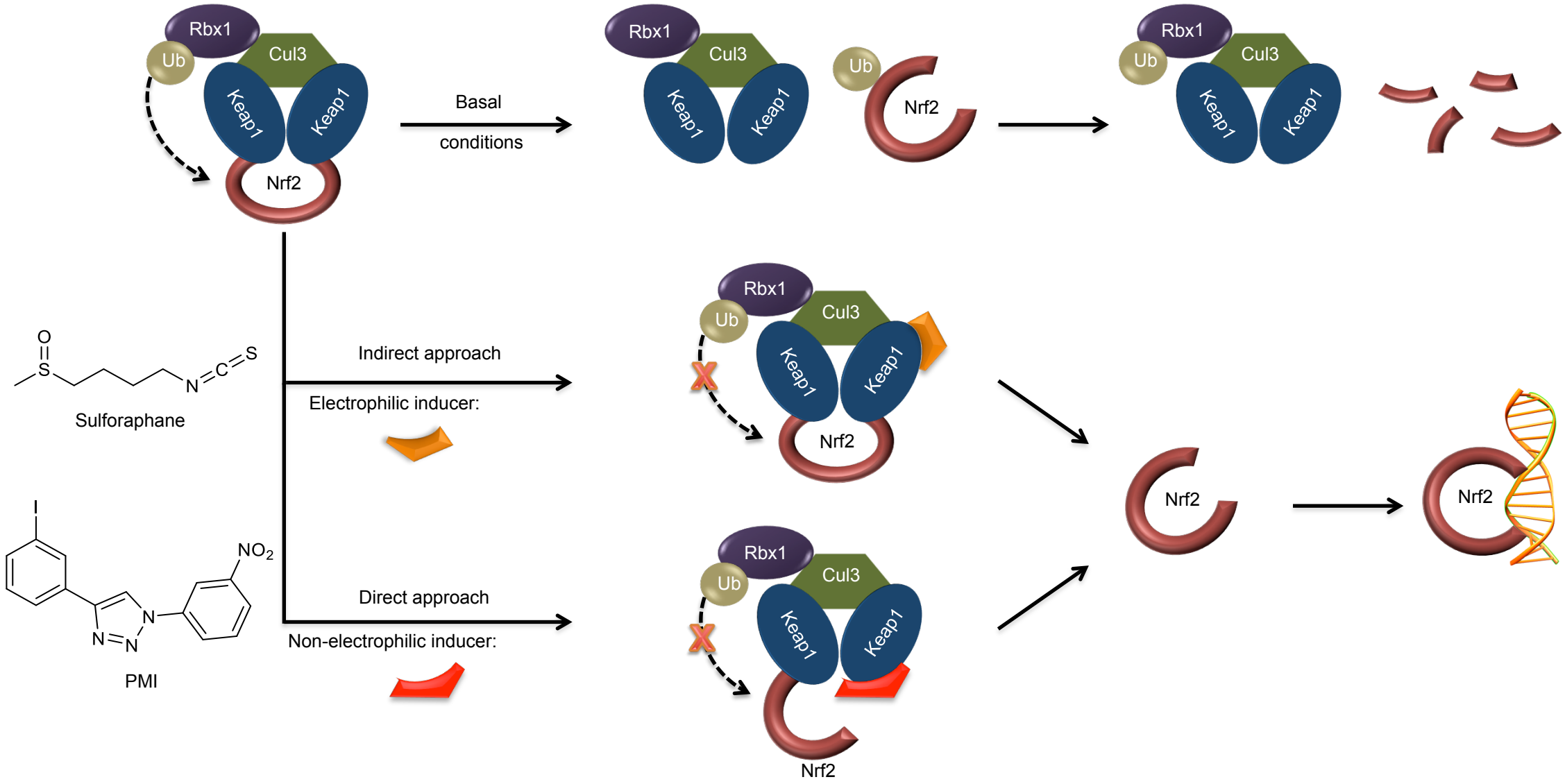
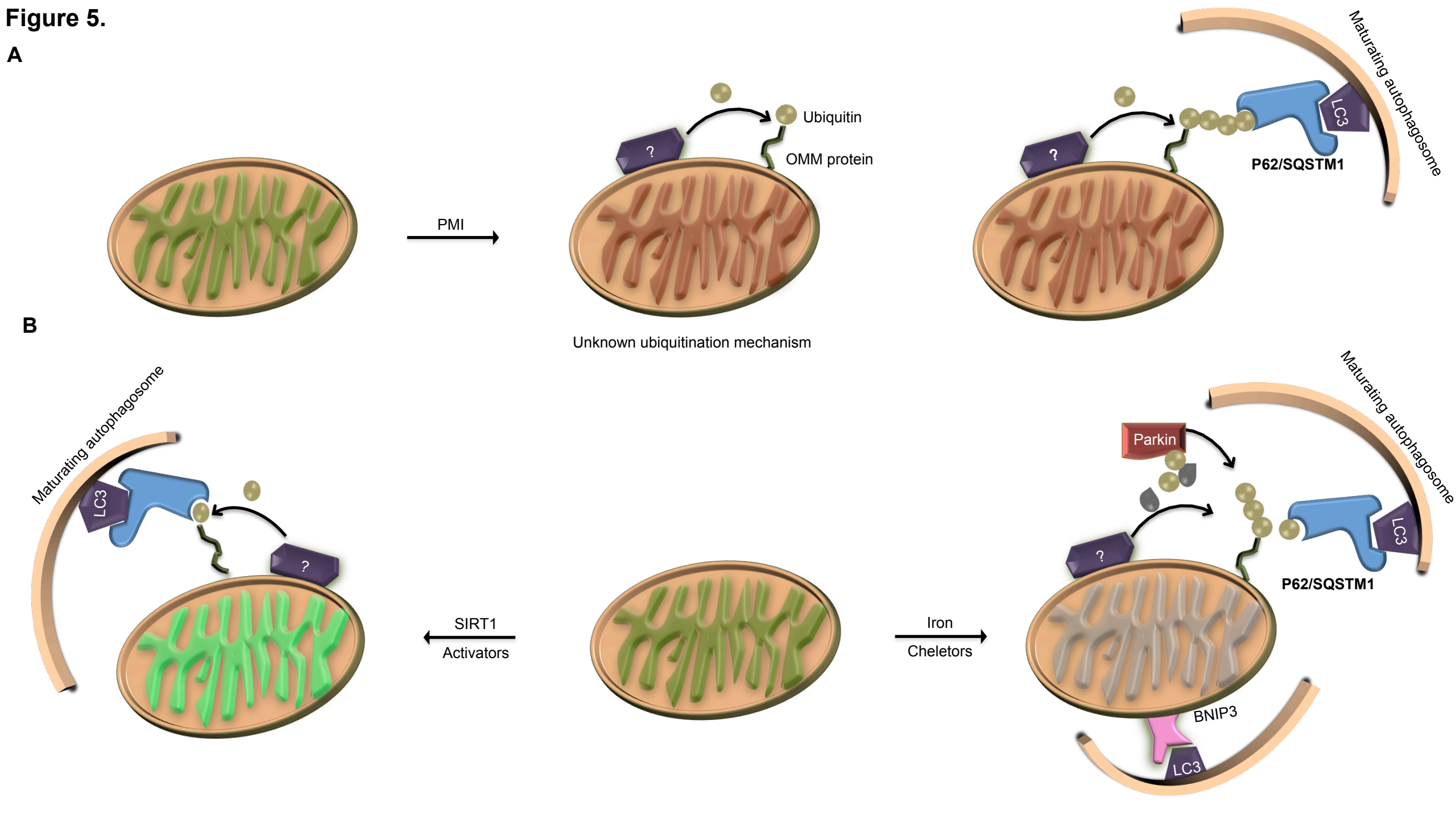

Rbx1
Ub
Cul3
Keap1
Keap1
Nrf2
Basal
conditions
Rbx1
Cul3
Keap1
Keap1
Ub
Nrf2
Rbx1
Ub
Cul3
Keap1
Keap1
Sulforaphane
Indirect approach
Electrophilic inducer:
Rbx1
Ub
Cul3
Keap1
Keap1
Nrf2
PMI
Direct approach
Non-electrophilic inducer:
Rbx1
Ub
Cul3
Keap1
Keap1
Nrf2
Nrf2
Nrf2

Figure 5.


A
PMI
Ubiquitin
OMM protein
?
Unknown ubiquitination mechanism
Maturating autophagosome
LC3
P62/SQSTM1
B
Maturating autophagosome
LC3
?
SIRT1
Activators
Iron
Cheletors
Parkin
P62/SQSTM1
BNIP3
LC3