Organelles in focus **MITOCHONDRIA**

Mitophagy and the therapeutic clearance of damaged mitochondria for neuroprotection

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Organelle Facts

- Mitochondria are the primary producers of cellular energy
- Mitochondria are also a major source of ROS
- Mitochondrial macroautophagy (mitophagy) is crucial for mitochondrial quality control
- There are several different mechanisms of mitophagy
- Dysregulation of mitophagy contributes to various neurological pathologies
- Compounds which can modulate mitophagy have great potential to enhance the understanding of and therapies for neurodegeneration

Abstract

Mitochondria are the foremost producers of the cellular energy currency ATP. They are also a significant source of reactive oxygen species and an important buffer of intracellular calcium. Mitochondrial retrograde signals regulate energy homeostasis and pro-survival elements whereas anterograde stimuli can trigger programmed cell death. Maintenance of a healthy, functional mitochondria network is therefore essential, and several mechanisms of mitochondrial quality control have been described. Mitochondrial dysfunction is linked to several neurodegenerative conditions including Parkinson, and Huntingdon diseases as well as Amyotrophic lateral sclerosis. Understanding mechanisms governing mitochondrial quality control may reveal novel strategies for pharmacological intervention and disease therapy.

Key words: mitochondria, mitophagy, neurodegeneration, therapy

Introduction

The mitochondrial network has evolved as a highly specialised system for coordinating ATP production, via the TCA cycle and oxidative phosphorylation, to meet cellular energy demands for maintenance and replication (Wallace, 2012). Mitochondria are also central to several other processes in cellular homeostasis. The ability to buffer cytosolic calcium (Ca^{2+}), is fundamental in shaping cellular Ca^{2+} signals (East and Campanella, 2013), and reactive oxygen species (ROS) generated as a by-product of respiration act as both signalling molecules and a major source of oxidative stress (Duchen and Szabadkai, 2010). Disruption of these systems (Energy, Ca^{2+}, ROS) elicit retrograde signals from mitochondria to the nucleus that can regulate mitochondrial biogenesis and quality control, antioxidant responses, as well as Ca^{2+} and glucose metabolism (Quirós et al., 2016) (**Figure 1**). In addition to proliferative and pro-survival signals, mitochondria are also intrinsically linked to apoptosis(Martinou and Youle, 2011). If the cellular damage sustained is irreversible, release of cytochrome C (CytC) from mitochondria and subsequent activation of caspase enzymes, results in efficient processing of the dying cell for removal by phagocytosis (Li and Dewson, 2015). Mitochondrial homeostasis is a delicate balancing-act: too few mitochondria will result in low ATP levels and bioenergetics failure, whereas too many can generate detrimental levels of ROS and leak CytC. Consequently, maintenance of a healthy mitochondrial population is essential for cellular function and survival, and mitochondrial dysfunction has been implicated in a variety of neurological pathologies (Kamat et al., 2014). This review will focus on the physiological aspects of mitochondrial quality control and allude to pharmacological strategies that not only could advance the current understanding of the processes involved, but also be exploited for therapy.

Molecular physiology of mitochondrial quality control

Targeted autophagy of mitochondria – mitophagy (Kim et al., 2007) - is the process by which dysfunctional or damaged mitochondria are selectively targeted by autophagosomes and delivered to lysosomes to be recycled by the cell. The most extensively characterised mechanism regulating the recruitment of autophagosomes to mitochondria (**Figure 2A**) is that driven by PINK1 and Parkin (Narendra and Youle, 2011). Under normal conditions PTEN-induced putative kinase 1 (PINK1) is imported into mitochondria and subsequently cleaved by PARL (Jin et al., 2010). However, in response to mitochondrial membrane depolarisation, PINK1 (Valente et al., 2004) is not degraded and accumulates on the outer membrane of dysfunctional mitochondria where it triggers the recruitment of the E3 ubiquitin ligase Parkin (Narendra et al., 2010). Additionally, molecular regulators of mitochondrial bio-energy during stress conditions have been recently reported to play part in this process. For examplethe overexpesssion and activation of ATPIF1, the endogenous ATPase inhibitor, is essential to induce mitochondrial accumulation of of PINK1 (Lefebvre et al., 2013; Matic et al., 2016).

Once present at the mitochondria, Parkin ubiquitinates several OMM proteins including the voltage dependent anion channel VDAC1 (Sarraf et al., 2013) which are consequently targeted by P62 (Geisler et al., 2010). P62 recognizes ubiquitinated substrates and acts as an adaptor molecule through direct interaction with autophagosome-associated LC3-II, driving the recruitment of autophagic membranes to the mitochondria (Pankiv et al., 2007). Parkin also recruits AMBRA1, which enhances mitochondrial clearance by binding LC3 via a LC3-interacting regions (LIR) (Strappazzon et al., 2015; Van Humbeeck et al., 2011). Targeting of mitochondria to autophagosomes by Parkin is also achieved in part via the regulation of mitochondrial trafficking along microtubules. This is accomplished via Parkin-mediated recruitment of ubiquitin-binding protein deacetylase, HDAC6 which promotes

mitochondrial transport (Lee et al., 2010), and by the ubiquitination, and subsequent degradation of MIRO, a GTPase involved in the tethering of mitochondria to kinesins (Wang et al., 2011). Finally, Parkin has non mitochondrial targets such as transcriptional repressor PARIS which inhibits PGC1α expression and therefore mitochondrial biogenesis (Shin et al., 2011). The importance of ubiquitination systems in mitochondrial quality control is reinforced by several redundant ubiquitination mechanisms identified in more recent studies. For example, the ubiquitin ligase Mul1 also induces mitophagy by ubiquitinating mitofusin 2, required for mitochondrial fusion, which is consequently degraded increasing the rate of mitochondrial fission and mitophagy (Lokireddy et al., 2012). Conversely, negative regulation is achieved via deubiquitinases such as USP30 (Bingol et al., 2014) and the 18 kDa TSPO, which is upregulated during cellular stressaccumulates on mitochondria preventing autophagic clearance by limiting ubiquitination of OMM proteins (Gatliff et al., 2014).

An alternative mechanism (**Figure 2B**), most well studied during hypoxia-induced mitophagy involves two molecular regulators, BNIP3 and NIX (Zhang and Ney, 2009), which are targets of the hypoxia inducible factors (HIFS) (Guo et al., 2001) as well as other transcription factors including NFkB and P53 (Fei et al., 2004; Feng et al., 2011; Shaw et al., 2008) Inhibition or disruption of BNIP3/NIX function can lead to non-apoptotic cell death suggesting their roles in quality control are critical (Chourasia et al., 2015).

BNIP3 and NIX integrate into the outer mitochondrial membrane (OMM), each with amino termini protruding into the cytoplasm. The direct interaction between BNIP3/NIX and LC3 target mitochondria to the autophagosome for delivery to the lysosome (Hanna et al., 2012; Schwarten et al., 2009). This interaction is regulated by phosphorylation of serine residues close to the LIR of BNIP3/NIX however the responsible kinases have yet to be identified (Zhu et al., 2013) Furthermore BNIP3 and NIX can both bind to Bcl2/Bcl-XL, and it has been suggested that these interactions can also regulate binding to LC3 (Zhu et al., 2013).

In addition to LC3 binding, BNIP3 has been shown to disrupt the action of Opa1, which promotes mitochondrial fusion cristae remodeling, whilst inducing the activity of Drp1, a protein involved in mitochondrial fission. In this way BNIP3 may facilitate the excision of damaged mitochondrial from network and prevent re-fusion (Landes et al., 2010; Lee et al., 2011). Finally, under conditions stimulating high levels of oxidative phosphorylation, Rheb, a small GTPase, is recruited to mitochondria and interacts directly with NIX and LC3 (Melser et al., 2013). Overexpression of Rheb increases LC3 processing and induces mitophagy in a NIX-dependent manner, suggesting NIX has a fundamental role in Rheb recruitment under conditions of high oxidative stress where maintaining a health population of mitochondria is vital (Melser et al., 2013). Another novel mechanism involving direct interaction with LC3 involves the mitochondrial membrane protein FUNDC1, which interacts with membrane associated LC3 via a LIR domain in the amino terminus (Liu et al., 2012). Regulation is achieved via phosphorylation of a serine residue in the LIR site by ULK-1 which promotes binding to LC3II whereas SRC1 inhibits this interaction by phosphorylating an adjacent tyrosine (Wu et al., 2014).

Mitochondrial dysfunction in neurodegeneration

Due to the high ATP levels required for neuronal function, neurons typically contain an increased mitochondrial population (Rugarli and Langer, 2012). Dysfunctional mitochondria produce less ATP and exhibit diminished Ca^{2+} handling capability, leading to increased ROS levels, which can induce neuronal stress and death (Lionaki et al., 2015). However, post-mitotic neuronal cells must endure for the lifetime of the organism, therefore neuronal survival and function is governed by mitochondrial quality control. The unique neuronal architecture poses an additional challenge in that mitochondria, the majority of which are located at distal neuronal processes must be transported back to the cell body where acidic lysosomes are mainly located, making regulation of mitochondrial trafficking especially important in neurons (Sheng, 2014).

Parkinson disease (PD) is characterised by the loss of neurons in the substantia nigra and the formation of intra-neuronal α-synuclein aggregates (Kamat et al., 2014). Mutations in the PINK1 and Parkin genes are well known causes of autosomal recessive forms of PD, and numerous studies (Pickrell and Youle, 2015) link this to the role of these gene products in mitochondrial quality control (**Figure 2**). Aberrant activity of several other proteins induce mitochondria dysfunction in PD, exacerbating a situation in which mitophagy is already impaired. For example, DJ-1 is a multifunctional protein with chaperone, protease, reductase and transcriptional regulatory activities. Its localisation on mitochondria is neuroprotective, however PD-associated mutations in DJ-1 inhibit this mitochondrial association (Tanti and Goswami, 2014). Additionally, aggregates of α-synuclein are reported to inhibit the activity of mitochondrial respiratory complexes (Subramaniam et al., 2014), increase mitochondrial fragmentation and disrupt ER-mitochondria contacts sites leading to deregulation of Ca^{2+} buffering (Guardia-Laguarta et al., 2014). Finally, LRRK2, another multifunctional protein with essential kinase functions, interacts with several key regulators of mitochondrial dynamics (Stafa et al., 2014). PD-associated mutations in LRRK2 cause increased fragmentation of the mitochondrial network, a reduction in ATP levels with a corresponding increase in ROS generation and increased susceptibility towards environmental insults (Ryan et al., 2015)

Huntington disease (HD) is a neurodegenerative condition that affects muscle coordination and leads to cognitive deterioration, behavioural changes and dementia. It is caused by a mutation in the Huntingtin gene (HTT), yielding huntingtin proteins containing polyglutamine repeats that become misfolded and resist degradation (Michalik and Van Broeckhoven, 2003). Although the function of the huntingtin protein is not yet fully understood it is thought to have a role in endosome and lysosome dynamics (Caviston et al., 2011). Mutant huntingtin has also been observed to associate with mitochondria resulting in alterations to mitochondrial ultrastructure. This is accompanied by a reduction in mitochondrial membrane potential ($\Delta\Psi_m$), impaired energy metabolism and deregulated Ca^{2+} handling (Orr et al.,

2008; Panov et al., 2002). Furthermore it has been proposed that HTT elicits toxicity through disruption of mitochondrial trafficking such that dysfunctional mitochondria are not adequately cleared form the cell resulting in oxidative stress and neuronal death (Martinez-Vicente et al., 2010; Orr et al., 2008). Finally, the toxicity associated with accumulation of dysfunction mitochondria may be exacerbated by inhibition mitochondrial biogenesis, as mutant HTT inhibits transcription of PGC1α, the master regulator of mitochondrial biogenesis (Cui et al., 2006).

Indeed, overexpression of PGC1α in HD model mice, restored biogenesis and eliminated protein aggregates by activation of TFEB, a regulator of the autophagy-lysosome pathway (Tsunemi et al., 2012)

Amyotrophic lateral sclerosis (ALS) is characterised by a progressive loss of motor neurons in the brain and spinal cord leading to fatal muscle paralysis (Kawamata and Manfredi, 2010). The specific causes of cell death are still under active investigation however several studies have implicated mutant superoxide dismutase SOD1, a key antioxidant enzyme, and TDP-43, a DNA binding protein, to mitochondrial dysfunction in ALS. SOD1 mutant mice develop bioenergetic abnormalities in motor neurons characterised by a reduced ATP production consequence of impaired ETC activity (Mattiazzi et al., 2002). Abnormal mitochondrial ultrastructure is also observed in SOD1 mutant mice, which may cause defects in mitochondrial trafficking (Magrané et al., 2009) Additionally, aberrant interaction of mutant SOD1 with OMM proteins such as VDAC1 can cause mitochondrial damage, and mutant SOD1 aggregates accumulate inside mitochondria causing oxidative stress (Palomo and Manfredi, 2015). Mutant TDP-43 conversely, forms aggregates which reduce contacts sites between mitochondria and the endoplasmic reticulum, possibly through and interaction with Mfn-2 (Stoica et al., 2014; Wang et al., 2013). Perturbations to these contacts sites lead to deregulation of mitochondrial Ca^{2+} homeostasis.

Pharmocological interventions

When developing new therapies for neurodegererative disorders the most logical approach might be to replace mutant genes that lead to mitochondrial dysfunction and neuronal death. Although it is an attractive prospect, gene therapy still in its infancy and significant hurdles of safety and regulation mean it may be many years before it is a viable solution. In the interim then, the best candidates for therapy remain small molecule drugs. Since mitochondrial damage is a hallmark of several neurological pathologies, drugs that are able to enhance the clearance of dysfunctional mitochondrial are likely to have significant therapeutic benefit.

The compounds routinely used to trigger mitophagy *in vitro* such as FCCP or antimycin/oligomycin combinations, are generally unsuitable as therapeutic agents they are toxic, non-specific and commonly trigger unphysiological levels of mitophagy (Padman et al., 2013; Vives-Bauza et al., 2010) . The goal for neuroprotective drugs should be to restore or boost endogenous mechanisms of mitochondrial quality control, however to date, few have been described. One promising candidate, P62-mediated mitophagy inducer (PMI) is a KEAP1 inhibitor, based on naturally occurring sulphoraphane (East et al., 2014). PMI-mediated KEAP1 inhibition activates NRF2 (**Figure 1**) resulting in expression of antioxidant response genes including P62 (Jain et al., 2010). The authors report a greater expression and localisation of P62 on mitochondria and subsequently increased mitophagy in cells treated with PMI suggesting that availability of P62 may be a rate-limiting step in mitochondrial clearance. PMI or compounds that act in a similar manner are of particular interest for translation to clinical applications as, unlike FCCP or antimycin/oligomycin, PMI triggers mitophagy without the toxicity associated with disruption of $\Delta\Psi m$ or inhibition of respiratory chain activity (East et al., 2014).

In conditions such as ALS and Huntington disease where the PINK1/Parkin machinery remains intact novel deubiquitinases inhibitors may be beneficial, tipping the balance and promoting mitochondrial quality control (Bingol et al., 2014). Similarly in PD in which the PINK1/parkin pathway is impaired, molecules that enhance the expression or activity of BNIP3/Nix and FUNDC1 have the potential to promote therapeutic levels of mitophagy. In

the future a deeper understanding of the complex mechanisms involved in mitochondrial network dynamics coupled with the emergence of novel drug molecules will be key to developing innovate therapies for neurodegenerative disorders.

Furthermore, compounds such as PMI, which enhance endogenous levels of mitophagy, are likely to be the most favourable candidates.

Financial Interest

We declare that none of the authors has a financial interest related to this work.

Acknowledgements

The research activities led by M.C. are supported by the following funders gratefully acknowledged: Biotechnology and Biological Sciences Research Council [grant number BB/M010384/1]; 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.

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Figure 1 – Mitochondrial retrograde signalling

Damage or distribution of the electron transport chain (ETC) can result in increased levels of ROS generation and a decrease in ATP synthesis. Both these conditions can activate AMKP which induces mitochondrial biogenesis and quality control via PGC1α. ROS also inhibits KEAP1, a negative regulator of the NRF2 transcription factor, promoting NRF2-mediated activation of antioxidant response elements. Insults which trigger loss of ΔΨm, including disruptions to ETC function, cause a release of mitochondrial Ca^{2+} stores. Several Ca^{2+} sensitive kinases then active transcription factors leading to an increase in expression of genes involved with Ca^{2+} homeostasis and glycolysis. Ca^{2+} can likewise active NFκB (which is also ROS sensitive) which drives expression of pro-survival factors.

Figure 2 – Mechanisms of mitochondrial quality control

(**A**) PINK1 recruits Parkin to damaged mitochondria. Parkin catalyses the transfer of ubiquitin (Ub) to several OMM proteins including VDAC1 and Mfn2. Ubiquitinated substrates are recognised by P62 which recruits autophagic membranes, associated with LC3, to the mitochondria. AMBRA1 is also recruited enhancing mitophagy via an interaction with LC3. Parkin also ubiquitinates MIRO, preventing its interaction with microtubules and PARIS, preventing inhibition of PGC1α and therefore stimulating mitochondrial biogenesis. In the absence of Parkin, redundant ligases such as Mul1 are able to ubiquitinate mitochondrial targets and deubiquitinases such as USP15 and USP30 oppose the effect of parkin and other ubiquitin ligases on mitochondria. Similarly, in stress conditions TSPO prevents ubiquitination of OMM proteins (**B**) BNIP3 and NIX are recruited to mitochondria during hypoxia and interactions with Bcl-2/Bcl-XL modulate their direct affinity for LC3 and the autophagosome. BNIP3 inhibits OPA1 mediated mitochondrial fusion, and stimulates fission via Drp1 and can also bind to Rheb, inhibiting activation of MTOR and cellular proliferation. NIX similarly bind Rheb enhancing the recruitment and processing of LC3. FUNDC1 is likewise recruited to dysfunctional mitochondria and its direct interaction with the autophagosome is regulated by kinases including ULK1 and SRC.

Fig 1

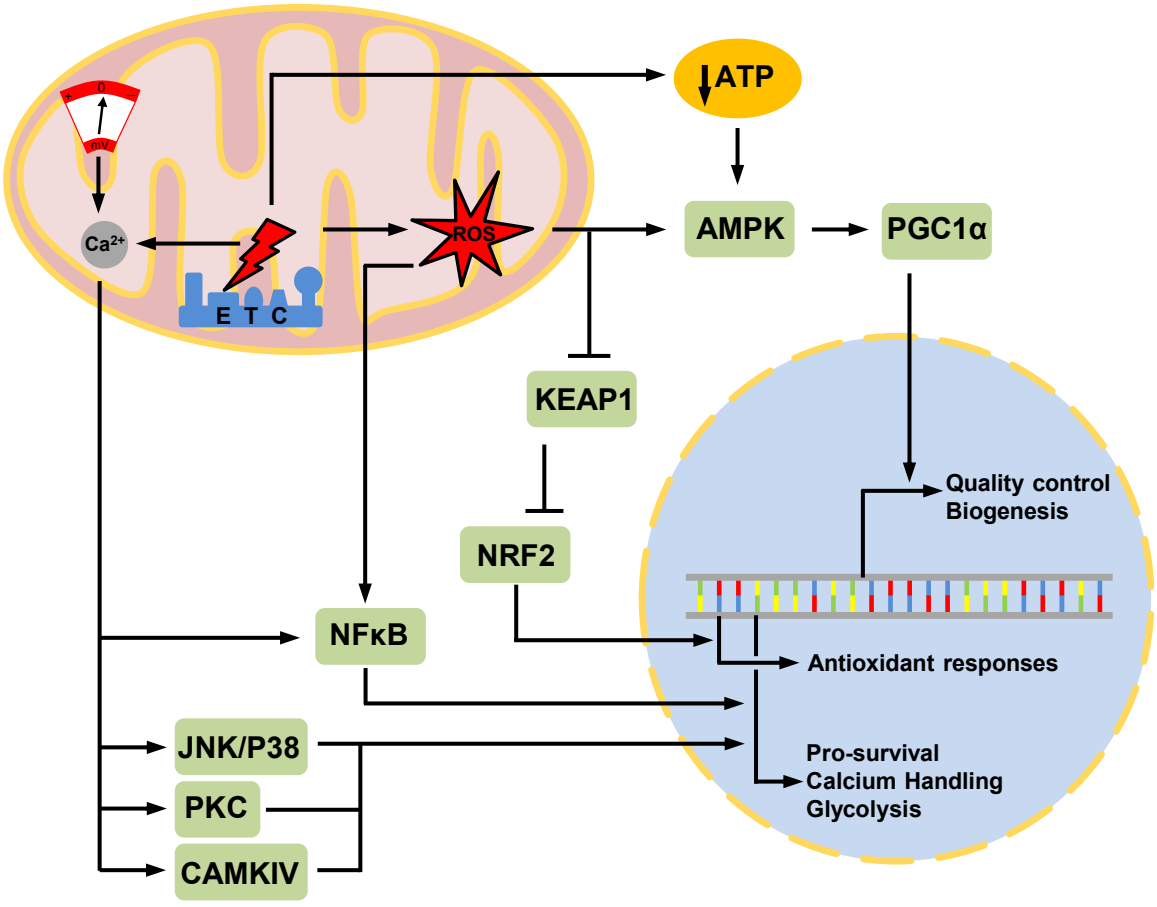
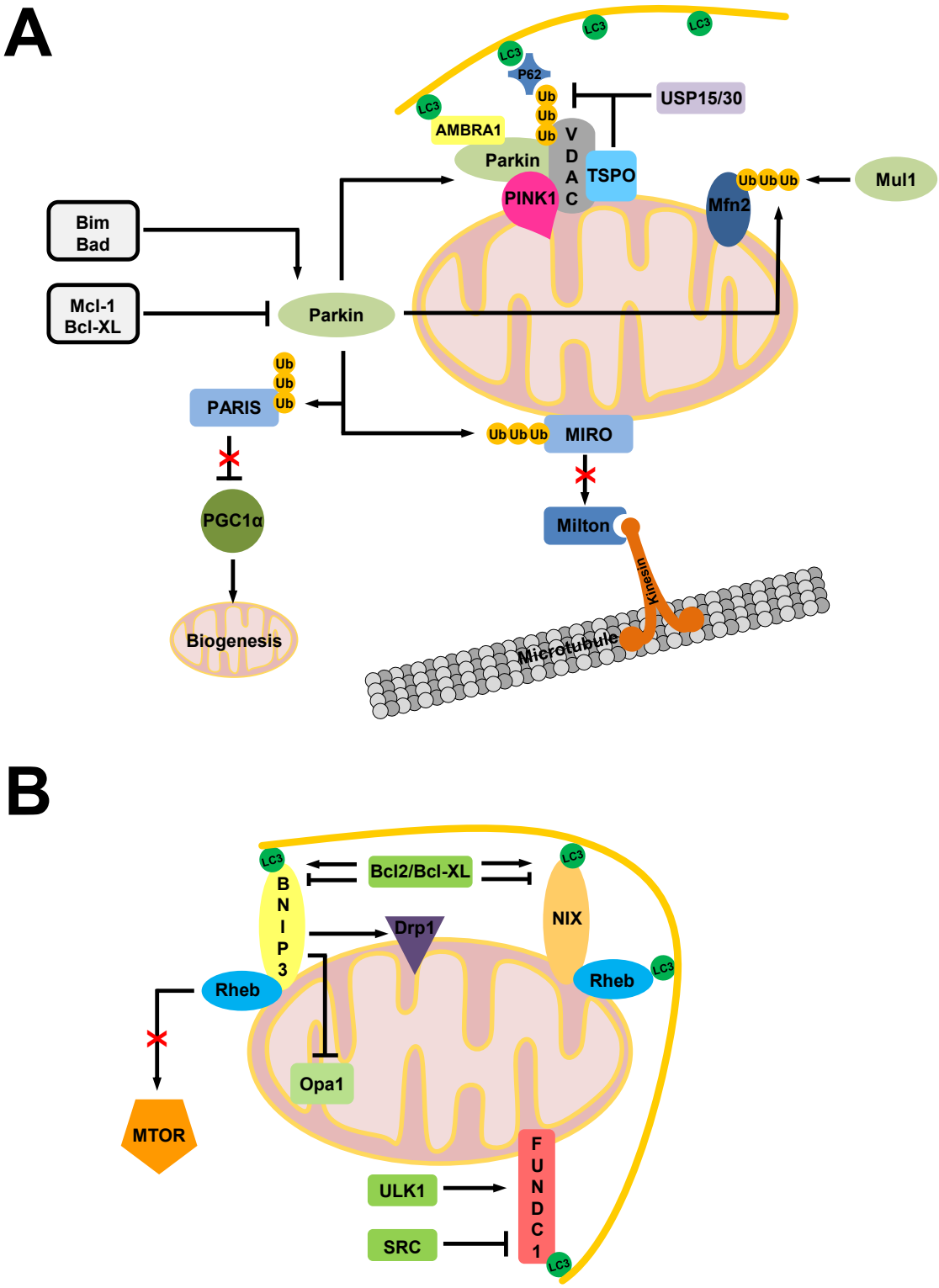

ATP
AMPK
PGC1α
ROS
Ca2+
E T C
KEAP1
NRF2
NFκB
JNK/P38
PKC
CAMKIV
Quality control
Biogenesis
Antioxidant responses
Pro-survival
Calcium Handling
Glycolysis

Fig 2


A
LC3
P62
Ub
USP15/30
AMBRA1
Parkin
V
D
A
C
TSPO
PINK1
Mfn2
Mul1
Bim
Bad
Mcl-1
Bcl-XL
Parkin
PARIS
MIRO
PGC1α
Milton
Kinesin
Biogenesis
Microtubule
B
Bcl2/Bcl-XL
B
N
I
P
3
Drp1
NIX
Rheb
Rheb
MTOR
Opa1
ULK1
SRC
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