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TITLE

Mitophagy during development and stress in *C. elegans*

KEYWORDS

mitochondria, mitochondria homeostasis, mitophagy, *Caenorhabditis elegans*, paternal mitochondria elimination, aging, stress, heteroplasmy

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ABSTRACT

Mitochondria is a key cellular organelle, which is tightly supervised by multiple oversight cellular mechanisms regulating mitochondrial biogenesis and mitochondria maintenance and/or elimination. Selective autophagy of mitochondria, *id est* mitophagy, is one of the cellular mechanisms controlling mitochondria homeostasis. The nematode *Caenorhabditis elegans* has recently emerged as a powerful model organism to study the roles and functions of mitophagy. We present here the current knowledge on cellular and molecular mechanisms underlying the selective elimination of mitochondria by autophagy in *C. elegans* in the context of developmental processes, aging and adaptive responses to various stresses

1) Mitochondria homeostasis is regulated through multiple supervision systems.

1.1 Mitochondrial functions

Mitochondria are double-membrane organelles originating from an endosymbiotic event whereby an alpha protobacteria has been domesticated by eukaryotic cell (Sagan, 1967). This organelle possesses its own genome (mtDNA) (Nass and Nass, 1963) and integrates multiple catabolic pathways through oxidative phosphorylation to produce adenosine triphosphate (ATP) (Hall et al., 2012). This organelle shelters essential metabolic pathways such as the Krebs cycle and fatty acid β -oxidation, as well as the biosynthesis of heme and lipids and part of the urea cycle. Mitochondria is the major site for reactive oxygen species (ROS) production, which could act either as signaling molecule when produced at low doses or be detrimental for the cells when produced at higher dose (Shadel and Horvath, 2015). Mitochondria are involved in the early step of the signaling pathway of apoptosis and participate to cell calcium homeostasis. Mitochondria are dynamic organelles that consistently undergo fission and fusion events and move over the cell cytoskeleton tracks (Summerhayes et al., 1983). Such a dynamic is essential for the quantitative and qualitative adaptation of mitochondria to the cell physiology variations, and the participation to calcium regulation and spatial segregation of mtDNA and matrix components. This dynamic allows the specific localization of mitochondria to subcellular places where energy is required (Sun et al., 2013), and eventually modulate the way mitochondria establish contact sites with other organelles. Large mechanochemical GTPases from the Dynamin family are responsible for the fluidity of the mitochondria network reticulum by controlling fusion and fission of mitochondria (Praefcke and McMahon, 2004; van der Blik et al., 2013). Because mitochondria are essential to the cell, its dysfunction could jeopardize cell homeostasis and trigger apoptosis, inflammation or senescence (Bock and Tait, 2019). Indeed, numerous human diseases are associated with mitochondria dysfunction (Alston et al., 2017).

1.2 Mitochondrial maintenance

Mitochondrial homeostasis is the result of a constant equilibrium between biogenesis, reparation and degradation processes.

On the one hand, a series of mechanisms allow the building of new mitochondrion, increasing the mitochondrial mass. These processes include the replication of mtDNA, the expression of mitochondrial genes, the import of nuclear-encoded proteins and lipids, and mitochondrial dynamic, which encompasses transport, fusion and fission. Overall, these

mechanisms constitute the mitochondrial biogenesis, which requires a tight crosstalk between two different genomes that is called the nucleo-mitochondrial interaction (Grivell, 1995; Melber and Haynes, 2018).

On the other hand, there is a set of mechanisms involved in maintenance, reparation and eventually the removal of dysfunctional and damaged mitochondria. A first level of action is the regulation of protein degradation via mitochondrial proteases and the ubiquitin- and proteasome-dependent OMMAD (outer mitochondrial membrane associated degradation) (Xu et al., 2016). A second mechanism is the mitochondrial unfolded protein response (UPR^{mt}), an evolutionary conserved adaptive transcriptional stress response, which promotes normal mitochondrial function recovery. UPR^{mt} modifies nuclear genes transcription to foster protein folding and to prevent aggregate formation, to induce proteases and to limit ROS accumulation. Noticeably, UPR^{mt} can also promotes mitochondrial biogenesis by stimulating the mtDNA replication and the mitochondrial import of lipids and proteins (Naresh and Haynes, 2019). An additional maintenance mechanism relies on the formation of mitochondrial-derived vesicles that allows the selective elimination of mitochondrial damaged proteins and lipids in response to an oxidative stress (Sugiura et al., 2014). It depends on the formation of buds, containing the jeopardized mitochondrial material, which are addressed to late endosomes for further degradation within the lysosomes (Soubannier et al., 2012). Finally, the massive elimination of dysfunctional mitochondria could occur through jettisoned exosphere containing mitochondria (Melentijevic et al., 2017), endosomal microautophagy (Hammerling, 2016) or mitochondrial-selective autophagy. Overall, these multiple mechanisms involved in quality control of mitochondria are modulated by intrinsic and environmental contexts.

This review focusses on the functions and the molecular mechanisms of selective degradation of mitochondria by autophagy, *id est* mitophagy, in the model organism *Caenorhabditis elegans*.

2) Overview of the autophagy mechanisms

Autophagy allows the addressing of intracellular components to lysosome for degradation and recycling. It was first termed by de Duve (De Duve, 1963) to describe the cell degradation process taking place in the double-membrane organelle containing parts of cytosol and pieces of organelle structures. Indeed, three different types of autophagy co-exist within the cell depending on the mechanisms of delivery of cytoplasmic components to the lysosome: microautophagy, chaperone-mediated autophagy and macroautophagy (Boya et al.,

2013). Microautophagy (MI) is defined as the engulfment of cytosolic cargoes directly via invagination of the lysosomal/vacuolar membrane. It can degrade soluble proteins or even bigger cargoes such as portion of the nucleus, lipid droplets, mitochondria and endosomes (Kissová et al., 2007; Tuttle et al., 1993; Tuttle and Dunn, 1995; Veenhuis et al., 1983; Yokota et al., 1993). MI depends on the activity of the Endosomal Sorting Complexes Required for Transport (ESCRT) machinery (Oku et al., 2017). Interestingly a microautophagy related mechanism, named endosomal microautophagy (eMI), shares with MI many features but involves endosome (Mukherjee et al., 2016; Sahu et al., 2011; Uytterhoeven et al., 2015). For example, in mouse embryonic fibroblasts, organelle such as mitochondria could be guided to the Rab5 positive early endosome and are engulfed in a ESCRT-0, -I and -II-dependent mechanism (Hammerling et al., 2017).

Chaperon-mediated autophagy (CMA) is another type of autophagy that directly imports substrates to lysosome for degradation (Majeski and Dice, 2004; Massey et al., 2004). In a similar way to eMI, CMA degrades protein, which interact with HSPA8 through a KFERQ like motif, and nearly 30% of cytosolic components could be degraded via CMA (Dice, 1990). Neither microautophagy nor CMA has been yet scrutinized in *C. elegans*, but while the former is evolutionary well conserved the latter is presumably not conserved in *C. elegans*, which is devoid of LAMP2A (Eskelinen et al., 2005; Kaushik and Cuervo, 2018).

Macroautophagy (hereafter referred to as autophagy) is present in all eukaryotes and characterized by the formation of a double-membrane vesicle, the autophagosome, which is dedicated to cytoplasmic materials sequestration and delivery to the endo-lysosomal system. Initially, autophagy has been studied in starvation condition and was considered a non-selective degradation process, during which autophagosomes engulfed indistinctly bulk part of the cytoplasm (Figure 1). However, a growing number of experimental results demonstrated that selective autophagy could target specific cargoes using a series of dedicated receptors. Autophagy receptors can specifically recognize the cargoes to be degraded and bridge them to autophagosomes by interacting with Atg8/LC3 (Komatsu and Ichimura, 2010). Based on the engulfed cargo, selective autophagy was further classified into mitophagy (mitochondria), pexophagy (peroxisome), nucleophagy (nucleus), ER-phagy (ER), lipophagy (lipid droplets), aggrephagy (protein aggregates), xenophagy (pathogens) and so forth (Galluzzi et al., 2017).

Molecular machinery is well conserved between eukaryotes and Atg genes have been initially characterized in yeast (Tsukada and Ohsumi, 1993). However, Atg genes have been duplicated during evolution resulting in a more complex situation in mammals.

In the last 15 years, *C. elegans* has emerged as a powerful model to study autophagy (Chen et al., 2017) and genetic screens identified new autophagy genes some of which are absent in yeast (Tian et al., 2010).

3) Mitophagy is a selective degradative process that contributes to mitochondria quality control

3.1 Generalities

Mitophagy is the process that selectively engulfs pieces or whole mitochondrion into autophagosomes, to form mitoautophagosome that subsequently fused with lysosomes for degradation. The term mitophagy was first coined by Lemaster (Lemasters, 2005) based on the work performed in yeast by Camougrand and colleagues (Kissová et al., 2004). Since this initial molecular description of mitophagy in yeast, multiple *in vivo* evidences for the physiological importance of mitophagy have been put forward in other organisms. While in yeast, mitophagy is mediated by the receptor ATG32, *C. elegans* and mammals present several receptors and signalling pathways regulating mitophagy (Table1). Mitophagy is involved in multiple physiological processes, including programmed red blood cells maturation, apoptotic cell death, stress-responses (hypoxia, heat shock, etc...), and neurodegenerative diseases that challenge mitochondria functioning. The discovery of mitophagy and the detailed description of its various molecular mechanisms and underlying functions have been recently reviewed (Montava-Garriga and Ganley, 2019; Palikaras et al., 2018; Pickles et al., 2018). Therefore, we briefly state a general overview of the principles that lead to selective elimination of mitochondria via autophagy.

Mitophagy events covers three main categories: the basal, the stress-induced and the developmentally programmed mitophagy. The mitophagy flux can be divided into two major steps, the tagging of mitochondria to be eliminated and the subsequent formation of the mitoautophagosome. Usually, mitophagy is triggered by modifications of the cell environment or stress conditions or developmental signalling that lead to mitochondria activity/parameters alteration and specific mitochondria tagging. It means that environmental or cellular stresses or other mitophagy stimuli are translated into a specific molecular modification of the outer mitochondrial membrane that primes mitochondria for degradation. Thereafter, primed mitochondria recruits the autophagy machinery leading to the formation of an isolation membrane (IM), also called phagophore, nearby mitochondria. Finally, IM enwraps the targeted mitochondria and closes to form a mitoautophagosome, which subsequently fuses with the lysosome for degradation.

3.2 Tagging unwanted mitochondria

Overall, the selective elimination of mitochondria via autophagy requires the specific localization to the mitochondrial surface of a set of molecules recognized as cargoes for autophagosome (Table 1). These evolutionary conserved pathways include ubiquitin and autophagy receptors, both potentially harbouring post-translational modification such as polyubiquitination and/or phosphorylation. The best understood mechanism involves PTEN-induced putative kinase protein 1 (PINK1) and the E3 ubiquitin ligase Parkin (Figure 2). However, alternative E3-ubiquitin ligases could be involved such as Mul1 (Rojansky et al., 2016). When mitochondria are damaged and loss their membrane potential, PINK1 is stabilized on the OMM and thus recruits Parkin, which subsequently ubiquitylates proteins at the OMM. Then, these multiple ubiquitin motifs are recognized by a set of autophagy receptor such as P62, NBR1, NDP52, Optineurin, TAXBP1 and ALLO-1 (Table 1). These receptors harbour both an ubiquitin binding site that interacts with ubiquitylated OMM proteins and a LC3 interacting region (LIR) motif that connects onto the IM, thus enabling the tethering between both organelles (Figure 2). Alternatively, a series of ubiquitin-independent mitophagy employ specific autophagy receptors directly localized to the mitochondrial membranes, such as Atg32, BCL2L13, DCT-1/NIX/BNIP3L, BNIP3, FUNDC1, FKBP8, PHB2 and AMBRA1 (Figure 2 and Table 1). In this case, the mitophagy stimulus triggers the increase of autophagy receptor density at the OMM by both transcriptional and post-translational regulations to promote and strengthen direct interaction with Atg8/LC3 autophagy proteins. Additionally, alternative mechanisms allow the exposure of inner membrane of the mitochondria (IMM) autophagy receptor, PHB-2 and cardiolipin. This set of mitophagy receptors is differently employed depending on the organism, cellular context, developmental stage and environment. For example, when yeast, are submitted to nitrogen starvation the OMM protein Atg32, is upregulated and acts as an autophagy receptor, through its Atg8 interacting motif, leading to the specific recognition of mitochondria by forming autophagosome. Similarly, during the maturation of mammalian red blood cell, mitochondria are removed by a mechanism involving mitophagy via increased expression of the OMM protein NIX, which recruits isolation membranes to mitochondria through its LIR (Table 1).

3.3 Formation and maturation of the mitoautophagosome

The mitophagy receptors, mediate the interactions with the autophagy machinery to trigger autophagosome formation around mitochondria. Damaged mitochondria represent a

platform for the multiple formation of mitophagosomes (Dalle Pezze et al., 2020; Zachari et al., 2019). Different receptors interact with the autophagosome nucleation UNC-51-Like Kinase 1 (ULK1) complex or the ATG8/LC3/GABARAP autophagosomal proteins. For example, ULK1 can interact directly with FUNDC1 and BCL2L13 whereas NDP52 contact FIP200 which is a ULK1 protein complex member to trigger mitochondria degradation (Vargas et al., 2019). Atg8/LC3/GABARAP are ubiquitin-like proteins, which localize to autophagosomal membrane through conjugation to phosphatidylethanolamine and mediate interactions with a number of mitophagy receptors possessing a LIR motif. Moreover, it has been shown that ATG8 homologues use a LIR motif to interact with ULK1 and could regulate autophagy (Alemu et al., 2012; Kraft et al., 2012). However, Atg protein including ULK1, WIPI-1, Atg14 and Atg16L1 can directly associate with depolarized mitochondria in the absence of LC3 (Itakura et al., 2012). In particular conditions, LC3/GABARAP homologs are dispensable for autophagosomes formation during PINK1/Parkin-dependent mitophagy but essential for autophagosomes-lysosome fusion (Nguyen et al., 2016). Additionally, studies in erythrocytes, suggest that the ubiquitin like conjugating system made of Atg5 and Atg7 is dispensable for mitophagy (Nishida et al., 2009). Overall, these studies question the importance of interactions between mitophagy receptors and Atg8/LC3 protein.

Importantly, recent publications showed that the extension of the autophagosomal membrane around the mitochondria is regulated by the Rab family of small GTPases and their canonical regulators Rab-GEF (GTP exchange factor) and Rab-GAP (GTPase activating protein)(Tan and Tang, 2019). During mitophagy, Rab7, whose activity is regulated by two Rab GAPs TBC1D15 and TBC1D17, is also recruited to the OMM where it mobilizes vesicles to build up autophagosomes. Both proteins are also recruited to the outer mitochondrial membrane via the OMM protein FIS and could interact via their LIR motifs with the nascent autophagosomal membrane containing LC3/GABARAP proteins. Inactivation of these GAPs lead to the accumulation around mitochondria of autophagosomal like membranes. It seems that these GAPs help to guide the extension of the autophagosomal membrane around mitochondria and spatially control the Rab7 activity which itself controls vesicle recruitment to the autophagosomal membrane (Yamano et al., 2014). Finally, once mitoautophagosome is formed it ultimately fuses with the lysosome for degradation. Before this ultimate step of mitophagy, mitoautophagosomes acquire different protein complexes to gain any capacity to fuse. These proteins complexes are tethering factors allowing the establishment of contact zones with lysosome and SNARE proteins to trigger membrane fusion between tethered organelles.

239

240 **4) Tools to study mitophagy in *C. elegans***

241 In recent years, the model organism *C. elegans* has been a leading animal to study the
242 physiological role of mitophagy (Figure 3). This organism allows addressing key biological
243 questions regarding mitophagy functions during the ageing process and in the selective
244 clearance of paternal mitochondria. *C. elegans* has multiple experimental advantages amongst
245 them its genetic toolkit and its ease for cellular biology approaches. Specifically, strategies
246 used to observe and analyse mitophagy in *C. elegans* are mainly based on imaging techniques
247 employing combinations of photonic and electron microscopies. Immunostaining approach on
248 fixed tissue to visualize both autophagosome and mitochondria has been developed.
249 Antibodies against LGG-1 or LGG-2, the two homologues of the ubiquitin-like protein ATG8
250 that label the autophagosome membrane could be combined with marker of mitochondria. A
251 colocalization analysis is indicative of possible mitophagy events or close contacts between
252 both organelles. Hence, in *C. elegans*, live imaging of mitophagy events is often monitored by
253 colocalization between fluorescent tagging of mitochondrial and autophagosomal markers
254 (Figure 4). Multiple ways are available to visualize mitochondria, which have been recently
255 reviewed (Rolland, 2014). Briefly, multiple transgenic lines expressing different
256 mitochondrial protein (either addressed to the OMM, IMM or the matrix) tagged with
257 fluorophore are available. These strains include known *C. elegans* mitochondria autophagy
258 receptors such as DCT-1/NIX or ALLO-1 (Palikaras et al., 2015; Sato et al., 2018).

259 Alternatively, mitochondria could be labelled with fluorescent dyes (red and deep red
260 Mitotracker, tetramethylrhodamine ethyl ester (TMRE) and rhodamine 6G) (Rolland, 2014).

261 *C. elegans* autophagosome are generally monitored in transgenic lines expressing either GFP
262 or mCherry tagged LGG-1 and LGG-2 proteins. Interestingly, transgenic lines expressing
263 autophagy proteins, LGG-1 and SQST-1, tagged with a novel fluorophore, CemOrange2 that
264 is separable from GFP and far-red fluorescent proteins should facilitate multiplex imaging in
265 the future (Thomas et al., 2019). Several combination of organelle tags have been developed
266 by N. Tavernarakis and his colleagues. They created strains with a dual fluorescent reporter
267 system that combines a mitochondria marker, the mitochondrial-targeted GFP, expressed
268 along with an ubiquitously expressed LGG-1 tagged with DsRed. Additionally, they adapted
269 the Rosella tool (Rosado et al., 2008) by constructing a *C. elegans* strain expressing the
270 mitoRosella synthetic gene, to study mitophagy in *C. elegans* (Palikaras et al., 2015). The
271 mitoRosella is a mitochondria-targeted pH sensitive GFP fused to the pH insensitive DsRed.
272 In the acidic autolysosome, the GFP part of the biosensor is quenched while the DsRed

fluorophore is resistant. Thus, healthy/normal mitochondria could be observed thanks to both green and red signals they emitted. Subsequently, once mitophagy events occur, autophagosome engulfs mitochondria and then fuses with lysosome, which results in the quenching of the GFP signal and the reduction of the GFP/DsRED ratio indicative of the mitophagy flux. This tool has been originally adapted to study mitophagy in the large body wall muscle cells of *C. elegans* but also adapted to the nervous system (Fang et al., 2019). In addition, the overall mitochondrial homeostasis could be analysed through its mass modification. These observations could be assessed in *C. elegans* by quantifying the overall fluorescence signal from mitochondrial targeted GFP specifically expressed in intestinal cells under the control of the *ges-1* promoter (Palikaras et al., 2015). Additionally, addressing the Timer protein to the mitochondria (MitoTimer), allows to monitor mitochondrial biogenesis and mitophagy (Laker et al., 2014). The dual emission fluorescent “Timer” probe initially fluoresces in green then shifts irreversibly to red fluorescence when it mature following a form of oxidation (dehydrogenation) of the Tyrosine 67 residue (Terskikh et al., 2000). The MitoTimer Red/Green fluorescence ratio does not depend on the overall expression of MitoTimer nor in changes in mitochondria pH. On one hand, the green fluorescence level indicates newly synthesized MitoTimer protein addressed to the mitochondrial matrix and reflects the increase of MitoTimer production or import and mitochondrial biogenesis. On the other hand, the level of red fluorescence, which indicates older mitochondria, mainly depends on degradative pathways such as autophagy, mitochondrial proteases or the ubiquitin proteasome system. Overall, measurements of red fluorescence over the green signal emitted by MitoTimer allows collection of quantitative data that mirrors multiple parameters controlling the mitochondrial homeostasis and therefore should be interpreted cautiously for monitoring mitophagy. In *C. elegans*, MitoTimer has been so far only expressed in mechanosensory neuron but it is a promising complementary tool to monitor mitochondria biogenesis in other cells and tissues. Alternatively, a dual Fluorescent Protein tag fused to the mitochondrial protein TOMM-7 has been proposed to monitor the mitophagy flux by western blot (Chapin et al., 2015). Finally, direct observation of mitoautophagosome could be performed in *C. elegans* with electron microscopy (EM) (Al Rawi et al., 2011; Meléndez et al., 2003). This technic allows visualization of autophagosome, possibly at different maturation steps (Manil-Ségalen et al., 2014). Additionally, EM analysis of autophagosomes from wild type and mutant strains could provide insights into the mechanism defects that are associated with genome alteration (Wang et al., 2016). It helps to monitor and distinguish normal mitochondria from modified ones, targeted by autophagosome, with altered structure

such as matrix aggregates or cristae disappearance (Wang et al., 2016; Zhou et al., 2016). Interestingly, EM and direct observation of fluorescent reporter construct labelling either distinct autophagosomal or mitochondrial proteins could be combined in *C. elegans* by applying the Correlative Light and Electron Microscopy (CLEM) technique. This technique has been used to analyse the mechanism underlying apoptotic corpse elimination through consecutive recruitment of LGG-1 and LGG-2 during *C. elegans* development (Largeau and Legouis, 2019) and could be adapted to further study mitophagy in *C. elegans*.

5) Physiological and developmental mitophagy in *C.elegans*

The model organism *C. elegans*, due to its intrinsic multiple experimental advantages and specific autophagy tools developed by the research community, has lead the way to the discovery of key developmental and physiological roles for mitophagy. Hereafter, we will present and discuss mitophagy mechanisms involved in *C. elegans* development and stress response.

5.1 Elimination of paternal mitochondria

In humans, as in most animals, the mtDNA is maternally inherited (Birky, 2001), with exceptional pathological cases in which paternal mtDNA could be transmitted to the offspring (Luo et al., 2018, 2013). Multiple experimental evidences using *C. elegans* and other model organisms have demonstrated that paternal mitochondria are not simply diluted upon fertilization but actively eliminated (see Box 1). Albeit some variations between species, the process of paternal mitochondria elimination (PME) is essential and relies on a selective mitophagy mechanism named allophagy. Allophagy designs the allogeneic (non-self) organelle autophagy in which paternal mitochondria, and membranous organelles (MO) that enter the oocyte are eliminated by autophagy (Al Rawi et al., 2011; Sato and Sato, 2011; Zhou et al., 2011) (Figure 5). MO are sperm specific organelles that derived from the Golgi apparatus and probably play a role similar to the vertebrate acrosome (Nishimura and L'Hernault, 2017). Electron microscopy analyses of the fertilized oocyte revealed that paternal mitochondria are small and rounded whereas maternal ones are thin and tubular. Paternal mitochondria lose their membrane potential, have no cristae but present thicker membranes containing holes and dark speckles within the matrix. In the meantime, ubiquitination events take place on paternal mitochondria and MO (Sato et al., 2018) and the depletion of the E1 ubiquitin activating E1 enzyme UBA-1 or the E2 ubiquitin conjugating enzymes UBC-16 and UBC-18 alters the PME (Molina et al., 2019). This ubiquitination is at

least partially Parkin-independent since *pink-1* and *pdr-1* mutants display no defect in PME (Lim et al., 2019). Probably, two parallel but independent mechanisms contribute to PME. One is dependent on ubiquitination of the OMM proteins and involves both proteasome and mitophagy, the other is ubiquitination-independent and involved FNDC-1, the orthologue of FUNDC1 (FUN14 domain containing 1)(Lim et al., 2019). The paternal mitochondria ubiquitination triggers the recruitment of the mitophagy receptor ALLO-1, which upon phosphorylation by the IKKE-1 kinase interacts with LGG-1, via a LIR domain, and participates to the formation of autophagosome around the paternal mitochondria (Sato et al., 2018). An additional mitophagy receptor, the prohibitin PHB-2, is localized to the IMM and becomes accessible for allophagy following alteration of the OMM (Wei et al., 2017) (Figure 5). The initial alteration of the OMM could be due to the process OMMAD because depletion of the two proteasomal subunits RPN-10 and RAD-23 affects PME (Molina et al., 2019; Zhou et al., 2011).

Several studies reported differential functions for LGG-1 and LGG-2, the homologues of the mammalian GABARAP and LC3, respectively, during allophagy. After fertilization, they both localize in clusters containing paternal mitochondria and MOs, which degradation is completed at the 8-cell stage (Al Rawi et al., 2011; Sato and Sato, 2011; Zhou et al., 2011). Genetic studies revealed that the allophagic clusters are completely abolished in *lgg-1* null mutant but persist for longer time in *lgg-2* mutants, due to a delay in the formation of autolysosome. These data illustrate that *lgg-1* is essential for autophagosome formation, whereas *lgg-2* has a role in autophagosome fusion with lysosome. Moreover, LGG-2 interacts with VPS-39, an Homotypic fusion and vacuole Protein Sorting (HOPS) complex subunit, to mediate the tethering and acidification of autophagosomes with lysosomes downstream of Rab5-Rab7 (Djeddi et al., 2015; Manil-Ségalen et al., 2014).

5.2 Mitophagy and iron metabolism

Several proteins of the mitochondrial electron transport chain need iron as a key element to accomplish their functions, and iron deregulation is often detrimental for mitochondria functions. Recently, a study on *C. elegans* identifies that the induction of mitophagy is part of an adaptive iron starvation response induced as a protective mechanism against mitochondrial stress (Schiavi et al., 2015). Mitochondrial respiration is dependent of iron-sulfur (Fe-S) clusters, which biosynthesis is stimulated by the mitochondrial protein Frataxin (FXN) (Gervason et al., 2019). Mutations of *fxn* result in dysfunctions of the respiratory chain and lead to multiple symptoms observed in Friedreich's ataxia. In *C.*

C. elegans, the moderate depletion using RNAi, of FXN homologue FRH-1, induces lifespan extension (Ventura and Rea, 2007). Interestingly, mitophagosome formation and mitophagy are induced in *frh-1* RNAi worms, and lifespan extension depends on PDR-1, PINK-1, DCT-1, as well as on the autophagy receptor p62/SQST-1. EM analysis of *frh-1* worms revealed the accumulation of electron-dense iron deposits within mitochondrial matrix, which is consistent with data in mouse (Puccio et al., 2001). *Frh-1* (RNAi) mimics a hypoxia state through cytosolic iron deprivation, which activates the hypoxia-inducible factors HIF-1 and AHA-1, to modulate longevity (Zhang et al., 2009). Moreover, *frh-1* RNAi can also upregulate the expression of *dct-1* and a series of globin genes, while it downregulates the expression of *sqst-1/p62*. Globin genes encode iron-containing proteins that regulate the cellular redox status in response to hypoxia (Hoogewijs et al., 2007). Upon iron deprivation, using the iron chelator 2,2'-dipyridyl, the worms present a mitophagy-dependent increased longevity, which overlaps the FRH-1 depletion.

Interestingly, *C. elegans* infection by *Pseudomonas aeruginosa* also induces an iron related mitophagy. Amongst the virulence factors produced by *P. aeruginosa*, siderophores, such as pyoverdine, retrieve the iron from its host. Consequently, *C. elegans* infected by *P. aeruginosa* experiments an acute iron sequestration similar to the slight decrease of *frh-1* mRNA level. This iron sequestration induced a mitochondrial turnover through a protective mitophagy process. For example, *pink-1* and *pdr-1* depletion sensitize animals to *P. aeruginosa* (Kirienko et al., 2015) indicating that mitophagy is necessary for *C. elegans* resistance to the infection, which probably impairs mitochondrial function through iron chelation and damages mitochondria. Therefore, iron chelation-induced mitophagy eliminates damaged mitochondria to preserve energy homeostasis during this pathogen infection.

5.3 Dual effect of mitophagy on heteroplasmy maintenance.

In most animals, mtDNA is maternally inherited and typically, individuals harbour a unique mtDNA genotype defining the homoplasmic state. However, germline and somatic mutations can lead to the coexistence of a mixture of mutant and normal mtDNA within the same individual, qualified as heteroplasmy and responsible of numerous human mitochondrial diseases (Wallace and Chalkia, 2013). Several *C. elegans* heteroplasmic strains have been isolated (Konrad et al., 2017; Tsang and Lemire, 2002). These strains combined with powerful novel technical possibilities such as purification of mitochondria from specific tissues (muscle, neuron, intestine, epidermis, gonad) even from a single worm make *C. elegans* a powerful model for studying mechanisms regulating heteroplasmy state

maintenance (Ahier et al., 2018). The *C. elegans* heteroplasmic strain, harbouring a 3.1-kilobase deletion (*uaDf5*) has twice mtDNA molecules compared to the wild type. Dissection of worms shows that *uaDf5* content varies within tissues of individual worms (Liau et al., 2007). Interestingly, PDR-1/Parkin is required for maintaining low levels of *uaDF5* mtDNA in *C. elegans* suggesting that this key protein involved in triggering mitophagy could have a beneficial role (Valenci et al., 2015). However, the mechanism underlying heteroplasmic status maintenance seems much more complicated. Two recent reports have shown that the heteroplasmic strain constitutively induces UPR^{mt}, which promotes proliferation of the *uaDF5*. Interestingly, the loss of the UPR^{mt} transcription factor ATFS-1 facilitates mitophagy activity and induces a decrease of mtDNA copy-number (Gitschlag et al., 2016; Lin et al., 2016). The expression of an error prone mtDNA polymerase in *C. elegans* generates multiple mtDNA deletions with a variable heteroplasmic state, and displays multiple phenotype defects such as neuromuscular dysfunction. Surprisingly, a genetic screen to identify modulators of this mutator phenotype reveals the paradox that a reduced mitophagy may be beneficial for this worm (Haroon et al., 2018). Altogether, these reports point out a very complex implication of mitophagy in the regulation of the heteroplasmic state in *C. elegans*.

6) Mitophagy during aging and neurodegeneration in *C. elegans*

6.1 Mitophagy in aging and stress conditions

A recent work revealed a novel physiological role for mitophagy, which allows mitochondria clearance all along the aging process in *C. elegans* (Figure 6). The progressive accumulation of the mitochondrial mass, observed during aging of the worm, could induced some persistent cell malfunctions and be responsible of the decline of health associated with aging (Palikaras et al., 2015). Interestingly, this phenotype is further enhanced when key autophagy gene or the mitophagy Pink/parkin pathway are depleted, demonstrating that the mitochondria homeostasis is partly modulated via mitophagy during aging. The mitophagy receptor DCT-1 (DAF-16/FOXO Controlled, germline Tumour affecting-1), homologue of NIX/BNIP3L and BNIP3, is crucial during this process and its depletion results in the accumulation of abnormal mitochondria, similarly to *pdr-1* and *pink-1* animals. DCT-1 is localized at the OMM, and contains a LIR motif, which mediates its interaction with LGG-1 (Palikaras et al., 2015). The overexpression of DCT-1 allows worm to better adapt to various stressful condition such as heat shock, UV exposure and pharmacological treatment challenging mitochondria (FCCP, paraquat). The ubiquitination of the lysine 26 of DCT-1 increases upon mitophagy induction, in a PDR-1 and PINK-1-dependent manner and the three

mutants are more sensitive to mitochondrial stress. DCT-1 participates with PINK-1 and PDR-1 to the extension of lifespan dependent on both the insulin pathway (*daf-2* mutant) and mitochondrial proteins (*clk-1* and *isp-1* mutants).

This study also showed that *dct-1* gene expression and mitophagy are both regulated by the transcription factor SKN-1, the homologue of the mammalian NRF2/NFE2L2. SKN-1 is activated under oxidative stress conditions (Ghose et al., 2013; Ploumi et al., 2017) and promotes mitochondrial biogenesis by up-regulating several mitochondria genes. SKN-1 depletion leads to the modification of mitochondrial morphology, an increase of cytoplasmic Ca^{2+} and a mitochondrial membrane depolarization and blocks DCT-1 mediated mitophagy. By coupling mitophagy and mitochondrial biogenesis, SKN-1 is a key player of a loop which regulation is important during the aging of animals (Figure 6). Interestingly, an additional mechanism of control of mitophagy during aging in *C. elegans* relies on the specific interaction of two mRNAs, from *puf-8* and *mff-1* genes. MFF-1 is an OMM protein that recruits the key mitochondrial fission factor DRP-1 to mitochondria. In old adult worms, *puf-8* mRNA inhibits the translation of *mff-1* mRNA, decreasing the mitochondrial fission and also the mitophagy, which, ultimately results in the accumulation of altered mitochondria (D'Amico et al., 2019).

Finally, a recent report studied the involvement of mitophagy in *mev-1* mutants that are highly sensitive to environmental stresses. This study also demonstrates that the mitophagy genes *dct-1* and *pdr-1* but *pink-1* are required for *mev-1* to survive to heat shock (Civelek et al., 2019). This conclusion is further supported by results indicating that mitophagy deficient animals are more sensitive to the ultraviolet C radiation (Hartman et al., 2019).

Overall, these results indicate that mitophagy is a key cellular process that helps *C. elegans* to cope with stressful environmental context.

6.2 Pharmacological modulation of mitophagy protects from age-induced decline

Three molecules that specifically modulate mitophagy in *C. elegans* have been identified: Urolithin A (UA), tomatidine and nicotinamide adenine dinucleotide (NAD⁺). UA, a part of the organic compounds known as benzo-coumarins, is the first natural compound shown to induces mitophagy in worm as well as rat and mammalian cells (Ryu et al., 2016). The UA precursor, ellagitannin, is found in multiple fruits and is processed in UA by the microbiota. UA can extend lifespan and improve worm fitness by inducing mitophagy. In young adult, UA treatment induces mitochondria fragmentation and leads to the reduction of

mitochondrial content. The latter phenotype depends on the mitophagy genes *pink-1*, *dct-1*, *skn-1* or the autophagy genes *bec-1*, *sqst-1*, *vps-34*, which depletion inhibits the lifespan extension following UA exposure. Indeed, UA treatment induces autophagy, which is detected by the increased LGG-1 positive autophagosomes. In older worms, UA exposure leads to mitochondrial content increase and to a higher transcription rate of respiratory chain subunit genes, compared to control animals. During the UA long-term treatment, the mitochondrial biogenesis is facilitated by the initial mitophagy induction observed in young adult. The enhanced mitochondrial biogenesis improves mitochondrial function through stabilizing ATP content and increasing the respiration capacity, and therefore leads to a prolonged locomotion and pharyngeal pumping activities of treated worms. Interestingly, this result suggest that UA is capable to uncouple mitophagy and mitochondrial biogenesis during aging; stimulating first mitophagy then mitochondrial biogenesis. Interestingly, most of the effects of UA on *C. elegans* are also observed in mammalian muscle and intestinal cells and, mice and rat aging models treated with UA show improvement of their muscle activity. This molecule shows a high potential to be used as a nutritional supplement to improve mitochondria function and muscle quality in human elderly people and clinical trials aiming to test the effect of UA on human health have been undertaken (Andreux et al., 2019).

Tomatidine is a steroid like compound enriched in unripe tomatoes. This molecule have beneficial effects in mouse cells for anti-inflammatory (Chiu and Lin, 2008) and anti-tumorigenic effects (Fujiwara et al., 2012). In mouse skeletal muscle cells, tomatidine promotes muscle fitness partially through increasing mitochondrial mass (Dyle et al., 2014). Interestingly, tomatidine treatment extends worm lifespan, as well as health span (Fang et al., 2017), as manifested by the improvement of swimming and pharyngeal pumping in aged animals. Interestingly, tomatidine induces moderate ROS production, which in turn activates SKN-1/Nrf-2 pathway. SKN-1, in turn, promotes PINK-1/DCT-1-dependent mitophagy and mitochondrial biogenesis to keep mitochondria healthy. Additionally, tomatidine action seems to be dependent on the key UPR^{mt} gene *atfs-1* and its beneficial effect has been further extended to primary rat cortical neurons and human HEK-203 cells (Fang et al., 2017). This study further provides experimental evidences indicating that somehow pharmacological treatment, activating mitophagy through SKN-1 pathway, may provide new approaches to improve healthy aging. It is highly possible that UA induced mitophagy and lifespan extension shares the same pathway triggered by tomatidine treatment.

NAD⁺ is an important coenzyme linked to the cell energy homeostasis. It is part of the redox couple NAD⁺/NADH, which provides the mitochondria respiratory chain with electrons

and protons. NAD⁺ is also a coenzyme for the nuclear deacetylase sirtuin 1 that modulates chromatin structure and regulates multiple cellular pathways including aging. In *C. elegans*, NAD⁺ depletion inhibits mitophagy while its replenishment stimulates mitophagy by upregulating DCT-1 through the sirtuin homolog SIR-2.1 and DAF-16 dependent mechanism (Fang et al., 2017). Interestingly, it has been also shown that NAD⁺ depletion is a major driver of the metabolic defects observed in the premature aging autosomal recessive disease Werner syndrome (WS). Both in WS model worm, *wrn-1* mutant, and in patient cells, a depletion of NAD⁺ is associated with mitochondria dysfunction and defective mitophagy. The NAD⁺ replenishment improves the mitochondrial function by restoring mitophagy in a DCT-1- and UNC-51 dependent mechanism (Fang et al., 2019), which opens future therapeutic strategy for several diseases (Connell et al., 2019).

Overall, *C. elegans* has been a leading model organism to put forward drug that selectively modulate mitophagy and contribute to set up numerous pre-clinical and clinical trials aiming to analyse putative translation of these mitophagy inducers drug beneficial effect to human.

6.3 Mitophagy and neurodegenerative processes

Alzheimer's disease (AD), Parkinson's disease (PD) and amyotrophic lateral sclerosis associated with frontotemporal dementia (ALS/FTD) are the major diseases that affect the functioning of the brain in the human population. These pathologies have currently no effective treatment and result in a progressive destruction of the nervous system leading to a loss of: memory, cognitive functions, sensory abilities, speech and locomotion.

AD, PD and ALS/FTD share similar cellular dysfunctions regarding calcium homeostasis, lipid metabolism, energy production and recycling of cellular waste products, related to mitochondria and autophagy, and which are mostly linked to mitochondrial associated membranes function (Area-Gomez and Schon, 2017; Paillusson et al., 2016).

Several *C. elegans* models of neurodegenerative diseases have been used to challenge the possible causal link between these disease-associated gene mutations and nervous system dysfunctions (Alexander et al., 2014). The exploration of the link between mitophagy and neurodegeneration, in animal models of AD, indicates that conserved mitochondrial stress response signature emerges comprising UPR^{mt} and mitophagy pathways (Sorrentino et al., 2017). Mitophagy induction by NAD⁺ boosting compound attenuates A β toxicity in *C. elegans*. The stimulation of the UPR^{mt} and mitophagy can decrease protein aggregation, increase worm fitness and delays progression of symptoms. By adapting for neural expression

the genetic tools for studying mitophagy (see section 4), a recent report provided additional evidences for mitophagy involvement in neurodegenerative diseases in *C. elegans* (Fang et al., 2019). The treatment of worms with UA (see section 6) triggers a *dct-1*, *pink-1* and *pdr-1* dependant mitophagy flux in nerve cells without inducing bulk autophagy. The authors also tested the beneficial effect of mitophagy in two *C. elegans* models of AD, the pan-neuronal expression of the human A β 1-42 protein (Vogliss and Tavernarakis, 2008) and the neuronal expression of the pro-aggregant F3 Δ K280 tau fragment (Fatouros et al., 2012). The pharmacological stimulation of mitophagy is beneficial for both models and restore the cognitive defects observed on learning and memory. A similar effect has been reported in mice model of AD, suggesting a conserved role of mitophagy in neurodegenerative diseases (Fang et al., 2019).

Multiple *C. elegans* models of PD, including genetic and pharmacological induced models have been reported in the literature (Cooper and Van Raamsdonk, 2018). Mutations in parkin and Pink1 cause autosomal recessive form of PD and the depletion of both genes in *C. elegans* lead to accumulation of defective mitochondria. *pink-1* null mutant presents mitochondrial cristae defect both in muscle and neuron, as well as increased sensitivity to oxidative stresses and neuron dysfunction (Sämann et al., 2009). Surprisingly, the defects in *pink-1* mutant could be suppressed by depleting *lrk-1*, the homologue of LRRK2 which is associated with PD in human (Biskup et al., 2006). LRK-1 functions in axonal-dendritic polarity of synaptic vesicles in neurons of *C. elegans* (Sakaguchi-Nakashima et al., 2007). LRK-1 and PINK-1 act antagonistically in neurite outgrowth, suggesting that the mitophagy defect in *pink-1* mutant could affect neuron outgrowth defect. Finally, patients with ALS/FTD accumulate TDP-43 (Tar DNA-binding protein 43) in degenerating neuron (Neumann et al., 2006). Interestingly, knockdown of TDP-43 enhances autophagosome and lysosome biogenesis indicating somehow a link between ALS/FTD and autophagy. (Xia et al., 2016). When overexpressed in the *C. elegans* nervous system, the human TDP-43 protein accumulates in the nucleus and results in an uncoordinated locomotion associated with abnormal synapse structure (Ash et al., 2010). The full exploitation of these worm models regarding any defect in mitophagy and the implication on ALS/FTD is still pending.

7) CONCLUSION

Recent researches performed with the nematode *C. elegans* have clearly contributed to the better understanding of the molecular mechanisms of mitophagy and its physiological roles at the scale of a whole animal. The most representative inputs from these studies

concerns the fields of developmental biology, aging, adaptation to stress and neurodegenerative pathologies.

Studies in *C. elegans* revealed that the maternal restricted transmission of mtDNA requires the selective elimination of paternal mitochondria by mitophagy and led to the identification of an original mechanism that is independent of Pink/parkin and relies on the mitophagy receptor ALLO-1. These data, supported by studies in the model *Drosophila melanogaster* paved the way for understanding this essential question in mammals (see Box 1). Additionally, the worm *C. elegans* provided the first clue on a regulatory mechanism that coordinates mitochondrial biogenesis and mitophagy during aging and involved the master regulator SKN-1. It also contributed to demonstrate that an inner membrane protein, Prohibitin 2, could act as a mitophagy receptor (Wei et al., 2017), and to identify the links between iron deficiency and mitophagy (Schiavi et al., 2015). Finally, *C. elegans* studies have been important to better understand the relationships between neurodegenerative diseases and mitophagy and allowed to test and validate novel molecules that could be further developed as therapeutics to enhance mitophagy and improve healthy aging.

Several technological improvements for studying mitophagy and mitochondrial biogenesis have been possible using *C. elegans*, taking advantage of its transparency to developed the fluorescent biomarkers Rosella (Palikaras et al., 2015) and MitoTimer (Laker et al., 2014; Terskikh et al., 2000). The power of genetic approaches and engineering of transgenic nematodes using molecular biology approaches strongly suggests that additional live-imaging tools will appear soon in *C. elegans*. Nevertheless, the mitochondrial degradation *per se* often lacks clear experimental demonstration in *C. elegans*, and would strongly benefit of the development of biochemical tools for the quantification of the delivery/degradation of mitochondrial cargos into the lysosome (Chapin et al., 2015).

One can expect that in the near future, studies in *C. elegans* will bring new insights on molecular mechanism controlling mitophagy, and identify the roles for mitophagy in many biological processes. The important question of the basal mitophagy is particularly difficult to address, which explains why most of the studies that identified the mitophagy mechanisms are generally done in the context of a massive and non-physiological stress of the mitochondria. *C. elegans* could be useful to identify whether the signalling events, the mitophagy receptors and the regulation of the basal mitophagy are different from the developmental or pathological processes. By the simplicity of its anatomy and the presence of multiple tissues, *C. elegans* is also a powerful system to decipher the variations of autophagy/mitophagy functions between organs and during the whole life cycle. Neurology and aging pathologies

are one obvious target but the differentiation and physiology of other cell types could be studied as well as trans-differentiation processes that have been described in the nematode. *C. elegans* could also open the study of autophagy/mitophagy in very complex biological processes such as circadian rhythm, the sleep-like state lethargus or very long starvation survival period.

Finally, another key point in the field of mitophagy is to understand the mechanisms of cooperation and coordination between the various mitochondria quality control pathways. How UPR^{mt}, mitophagy, proteasome and other processes like the exophers jettison (Melentijevic et al., 2017) are globally regulated to cope with a dysfunctional mitochondria network remains unclear and *C. elegans* could be useful to address such a question.

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Box 1. Convergence of struggles to eliminate paternal mitochondria

Multiple mechanisms, depending on the animal, act before or just after fertilization during the early embryogenesis, to eliminate paternal mitochondria. Early work from Sutovsky and colleagues showed that paternal mitochondria are ubiquitinated for an efficient degradation mediated by proteasome in fertilized cow and monkey eggs (Sutovsky et al., 2000). Nishimura and colleagues then showed, in the fish medaka (*Oryzias latipes*), that the paternal mtDNA number is progressively reduced at the end of the spermatogenesis and then is specifically and progressively digested just after fertilization (Nishimura et al., 2006). In *Drosophila melanogaster*, sperm mtDNA is eliminated during spermatogenesis, by two mechanisms. The first one occurs at the elongation stage of postmeiotic sperm development, during which, mitochondrial nuclease, Endonuclease G (EndoG), eliminates only mtDNA rather than the complete mitochondria. Later on during spermatogenesis, the residual mtDNA is eliminated during spermatid individualization stage, through a cellular remodelling process that modify spermatid tail (DeLuca and O’Farrell, 2012). This mechanism is similar to the

one observed in *C. elegans* whereby the endonuclease CPS-6 originally localized to the intermembrane space has access to the mtDNA, after rupture of the IMM by an unknown mechanism. This step seems to be important for the subsequent PME steps (Zhou et al., 2016). Moreover, an additional active mechanism exists also in drosophila eggs to get rid of paternal mitochondria (Politi et al., 2014). This mechanism uses non conventional autophagy and endocytic pathways to eliminate sperm mitochondria within the eggs even if these sperm mitochondria is devoid of DNA. Paternal mitochondria destruction (PMD) initiates with vesicles resembling MVB that put mitochondria into small fragments that are subsequently sequestered in autophagosomes that finally fuse with lysosomes. P62 and ubiquitination are involved but this mechanism is not Parkin dependent (Politi et al., 2014). In mice, the situation is debated. One report indicates that most motile sperm eliminate their mtDNA and leave only vacuolar mitochondria before fertilization. Then the sperm mitochondria persist until morula stage, even they are observed to colocalize with autophagy proteins p62 and LC3 just after fertilization. Nonetheless, their clearance is independent of autophagy pathway (Luo et al., 2013). However, this conclusion is challenged by a functional analysis showing that paternal mitochondria are maintained in 60% of the embryos depleted for Parkin and Mul1. Therefore, the authors concluded that in mice, PMD required a Parkin and Mul1 dependent autophagy (Rojansky et al., 2016). Sutovsky and colleagues addressed the PME question in another vertebrate, the porcine zygote, and provided evidences for another type of active paternal mitochondria destruction mechanism. This mechanism is based on a P62/SQSTM1-dependent autophagy, VCP-mediated dislocation of ubiquitinated mitochondria protein and presentation to the 26S proteasome (Song et al., 2016). Overall, multiple different systems have been selected during the evolution to specifically eliminate paternal mitochondria in most animals suggesting that PME step is critical for the subsequent animal development.

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Legends of figures

Figure 1. The three types of autophagy in most eukaryotic cells

(A) Macroautophagy, which is commonly named autophagy, is characterized by the sequestration of cargos within a double membrane structure, called the autophagosome. In mammals, the autophagy starts by the formation of a flat membranous structure, the phagophore, which elongates and close to generate a double membrane vesicle. Finally, the external membrane of the sealed autophagosome fused with the lysosome to generate an acidic and degradative compartment named the autolysosome, allowing recycling of autophagic cargoes. The core autophagy machinery forms various complexes that acts in four major steps. the ULK1/2 (unc-51 like autophagy activating kinase 1/2) complex is involved in the initiation step of the process. The class III phosphatidylinositol 3-kinase (PI3K) complex induces locally PI3P (phosphatidylinositol 3 phosphate) synthesis and allows the nucleation of the phagophore by recruiting the PI3P binding complex. The Atg12 and the Atg8 conjugation systems are controlling the elongation of the phagophore to form the autophagosome through lipidation of Atg8s to phosphatidylethanolamine (PE).

(B) Membrane invagination of either the endosome or the lysosome allows the direct sequestration of cargoes within degradative compartments by endosomal microautophagy or microautophagy, respectively.

(C) Chaperone-mediated autophagy (CMA) targets proteins containing the KFERQ motif that is recognized by several chaperones, including Hsp70 (Heat shock protein 70kDa). During CMA, LAMP2 (Lysosomal-Associated Membrane Protein) mediates the transfer of Hsp70-protein aggregates within the lysosome.

Figure 2. Ubiquitin-dependent or independent mitophagy pathways involve multiple receptors.

Two main mitophagy pathways allow the selective degradation of mitochondria by autophagy in physiological and stress induced conditions. **(A)** On one hand, mitochondrial receptors mediate mitophagy through the direct interaction with Atg8/LC3 proteins. Such mitochondrial receptors are outer mitochondrial membrane proteins NIX BNIP3 (NIX- NIP-3-Like Protein X / BNIP3L- BNIP3 like), FUNDC1 (FUN14 Domain Containing 1), BCL2L13 (Bcl2-like protein 13), and inner membrane protein prohibitin or the phospholipid cardiolipin. **(B)** On the other hand, the Pink1 (PTEN-induced putative kinase 1)-Parkin dependent mitophagy pathway requires cytosolic protein adapters. Upon mitochondrial damage, PINK1, that is usually cleaved and processed, is stabilized at the outer mitochondrial membrane where it phosphorylates both ubiquitin residues on mitochondrial protein and the E3 ubiquitin ligase Parkin. Activation of Parkin results in the high density of phospho-ubiquitin chains linked to substrates, which triggers the mitochondrial recruitment of the receptors OPTN (Optineurin), NDP52(Nuclear dot protein 52 kDa), SQSTM1(sequestosome 1), NBR1 (Neighbor of BRAC1) and TAX1BP1 (Tax1-binding protein) and their interaction with the autophagy machinery. In both pathways, Atg8/LC3s can interact with ULK1 (unc-51 like autophagy activating kinase 1) to induce autophagosome formation.

Figure 3. Schematic representation of *C. elegans* life cycle indicating autophagy and mitophagy characterized processes.

The development cycle of *C. elegans* is rapid and stereotyped (3.5 days at 20 °C), and consists of an embryonic stage and four larval stages before producing an adult animal that can live several weeks. In response to environmental stress an alternative developmental pathway results in the formation of a diapause stage called dauer larva. The figure only shows hermaphrodite animals, which are fertile for several days in absence of cross-fertilization by males. The various types of autophagy and the associated cargo are indicated in orange.

Figure 4. Detection of mitophagy by microscopy in *C. elegans*

(A) Live imaging confocal microscopy of mitophagy events (yellow arrows) monitored by the colocalization between mitochondria stained with the fluorescent Mitotracker (red) and autophagosomes labelled with GFP::LGG-1 (green), the *C. elegans* homolog of GABARAP. **(B)** Transmission electron microscopy image of muscle cells showing regular mitochondria

(m) and a stressed-mitochondria containing dark inclusions, engulfed by an autophagosome (yellow arrow).

Figure 5. Degradation of spermatozoid-inherited mitochondria by autophagy in *C. elegans*

Three processes are involved in the degradation of paternal mitochondria during fertilization (see also Box 1). **(Left)** The internal membrane (IMM) breakdown occurs shortly after fertilization and causes the relocation of the endonuclease CPS-6 from the intermembrane space to the matrix of paternal mitochondria. CPS-6 catalytic activity triggers the degradation of paternal mitochondrial DNA (mtDNA) and promotes paternal mitochondrial elimination (PME) through autophagy. **(Right)** As soon as fertilization occurs, paternal mitochondria are further ubiquitinated, allowing the recognition and binding by the autophagy receptor ALLO-1. ALLO-1 interacts with the IKKE-1 kinase, which phosphorylates ALLO-1 and probably other substrates that could be present on mitochondria. Then ALLO-1 interacts with LGG-1 through its LIR domain to allow the formation of autophagosomes around paternal mitochondria. **(Middle)** Paternal mitochondria are also tagged by two specific mitophagy receptors, the outer mitochondrial membrane (OMM) protein FNDC-1 and the inner mitochondrial membrane protein, prohibitin PHB-2. After the depolarization and rupture of the OMM, which is dependent of the proteasome, PHB-2 is exposed to the cytosol and interacts with LGG-1.

Figure 6. SKN-1 coordinates mitochondrial biogenesis and mitophagy during *C. elegans* aging.

The mitochondrial homeostasis depends on the balance between mitochondrial biogenesis and degradation. In physiological condition, a basal mitophagy flux regulates the mitochondrial content. Mitochondrial damages result in the increase of cytosolic Ca^{2+} and ROS causing the releasing of several transcription factors, including SKN-1 (SKiNhead-1). The mitochondrial quality control is first ensured by UPR^{mt} (Unfolded Protein Response in mitochondria), an adaptive transcriptional response that promotes protein refolding and mitochondrial recovery. When dysfunctions are persistent and the UPR^{mt} overwhelmed, elimination of the defective mitochondria occurs through mitophagy. The transcriptional factor SKN-1 promotes the activation of nuclear genes coding for mitochondrial proteins and mitophagy components, such as DCT-1 (Daf-16/ FOXO controlled germline tumor affecting-1) which is localized at the outer mitochondrial membrane and can interact with LGG-1 to mediate mitophagy.

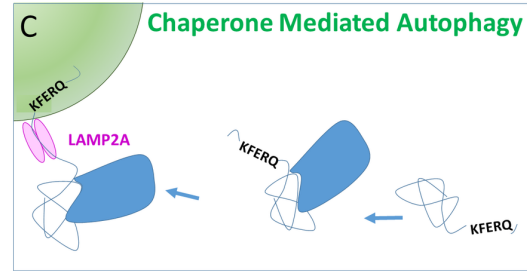
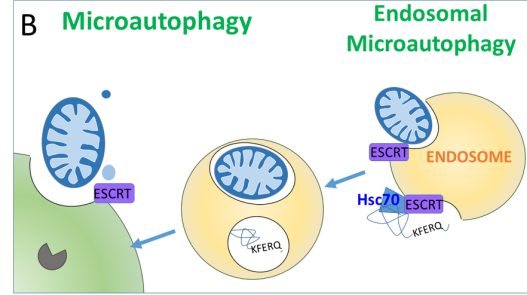
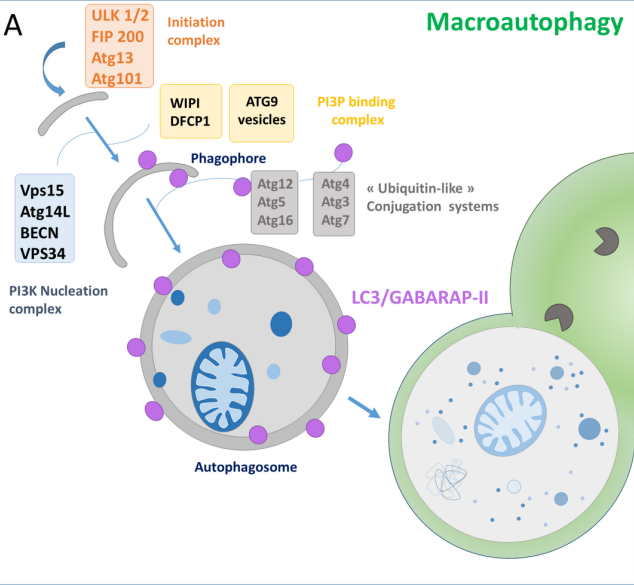
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Table 1 : Key mammalian proteins involved in mitophagy and their homologues in *C. elegans*
Autophagy receptors as well as cargos are engulfed by autophagosomes for degradation
References are key publications linked to the mitophagy functions of the proteins. n.d : not determined.

| Mitophagy actors in mammals | <i>C.elegans</i> homologues | Function | Localisation | References |
|--|---|---|---|--|
| AMBRA1 - Activating molecule in Beclin-1-regulated autophagy | n.d | Mitophagy inducer Parkin and LC3 interactor | cytosolic | (Strappazzon et al., 2015; Strappazzon and Cecconi, 2015; Van Humbeeck et al., 2011) |
| BCL2-L-13 – Bcl2-like protein 13 -Homologue of yeast Atg32 | n.d | Mitophagy receptor | OMM | (Murakawa et al., 2015; Sakakibara et al., 2015) |
| BNIP3 - BCL2/adenovirus E1B 19kDa protein interacting 3 | DCT-1 - Daf-16/ FOXO controlled germline tumor affecting-1 | Mitophagy receptor | OMM | (Hanna et al., 2012; Quinsay et al., 2010; Schiavi et al., 2015) |
| BNIP3L - BNIP3 like / NIX - NIP-3-Like Protein X | DCT-1 - Daf-16/ FOXO controlled germline tumor affecting-1 | Mitophagy receptor | OMM | (Palikaras et al., 2015; Schweers et al., 2007) |
| Cardiolipin is a major membrane phospholipid localized in mitochondria | Cardiolipin | Lipid mitophagy receptor | Translocates from IMM to OMM | (Chu et al., 2013; Sakamoto et al., 2012) |
| FKBP8 - FK506-binding protein (FKBP) family | n.d | ATG8 interacting protein- Adaptor | OMM | (Bhujabal et al., 2017) |
| FUNDC1 – FUN14 Domain Containing 1 | FNDC-1 | Mitophagy receptor | OMM | (Lim et al., 2019; Liu et al., 2012) |
| LC3 - light chain 3 / GABARAP - GABA(A) receptor-associated protein -Homologue of yeast Atg8 | LGG-2 / LGG-1 | Initiation, elongation and closure of autophagosome/ recognize specific cargos to degrade | Cytosolic protein conjugated to autophagosome | (Alemu et al., 2012; Manil-Ségalen et al., 2014) |

| | | | | |
|--|---|------------------------------------|-----------------------|--|
| NDP52 - Nuclear dot protein 52 kDa | n.d | Ubiquitin-binding receptor protein | Cytosolic protein | (Vargas et al., 2019) |
| NBR1 – Neighbor of BRAC1 gene | n.d | Ubiquitin-binding receptor protein | Cytosolic protein | (Hollville et al., 2014) |
| NRF2/NFE2L2 - Nuclear factor (erythroid-derived 2)-like 2 | SKN-1 -SKiNhead 1 | Transcription factor | Cytosolic and nucleus | (Fang et al., 2017; Ghose et al., 2013) |
| OPTN - Optineurin | n.d | Ubiquitin-binding receptor protein | Cytosolic protein | (Padman et al., 2019) |
| PHB2 – Prohibitin 2 | PHB-2 | Mitophagy receptor | IMM | (Wei et al., 2017a) |
| PINK1 -PTEN-induced putative kinase 1 | PINK-1 | Serine/threonine kinase | OMM | (Kawajiri et al., 2010; Narendra et al., 2010) |
| PRKN – Parkin | PDR-1 Parkinson's Disease Related 1 | E3 ubiquitin ligase | Cytosolic protein | (Narendra et al., 2008; Schiavi et al., 2015) |
| SQSTM1/p62 –sequestosome 1 | SQST-1 | Ubiquitin-binding receptor protein | Cytosolic protein | (Geisler et al., 2010; Zhang et al., 2017) |
| TAX1BP1 – Tax 1 binding protein 1 | n.d | Ubiquitin-binding receptor protein | Cytosolic protein | (Whang et al., 2017) |

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● Atg8 family

■ Chaperones

ESCRT Endosomal Sorting Complexes Required for Transport

Figure 1 Leboutet et al.

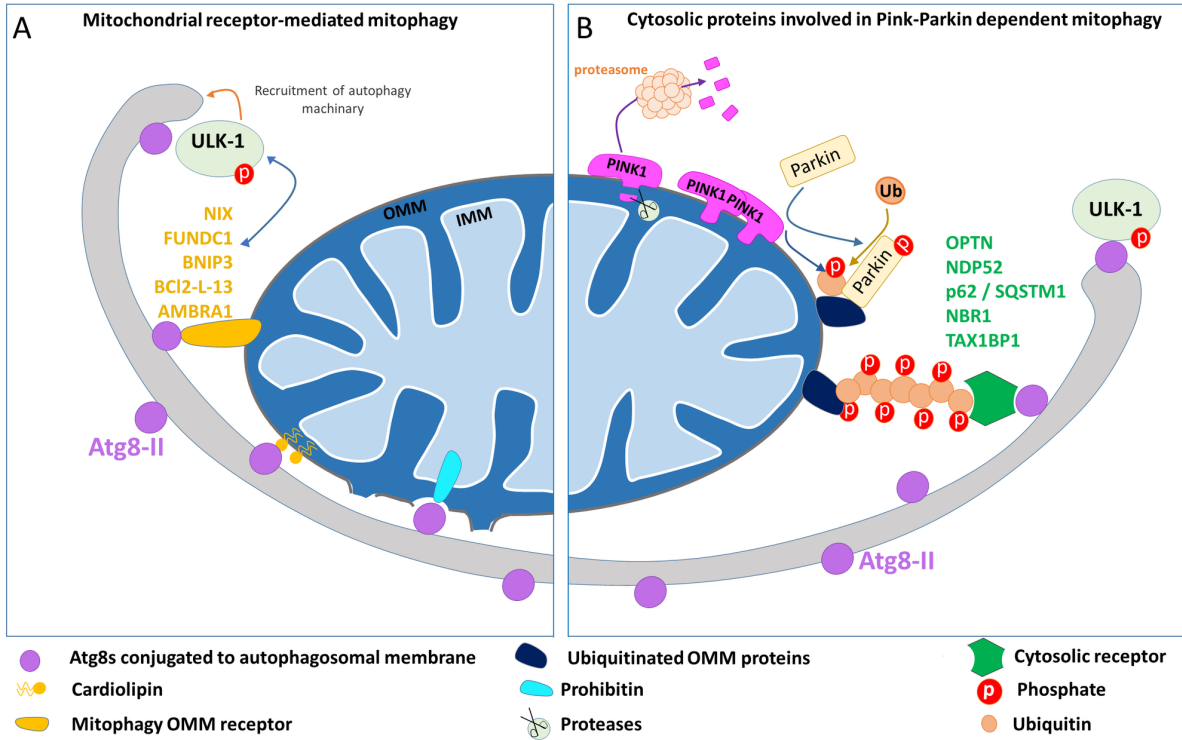


Figure 2 Leboutet et al.

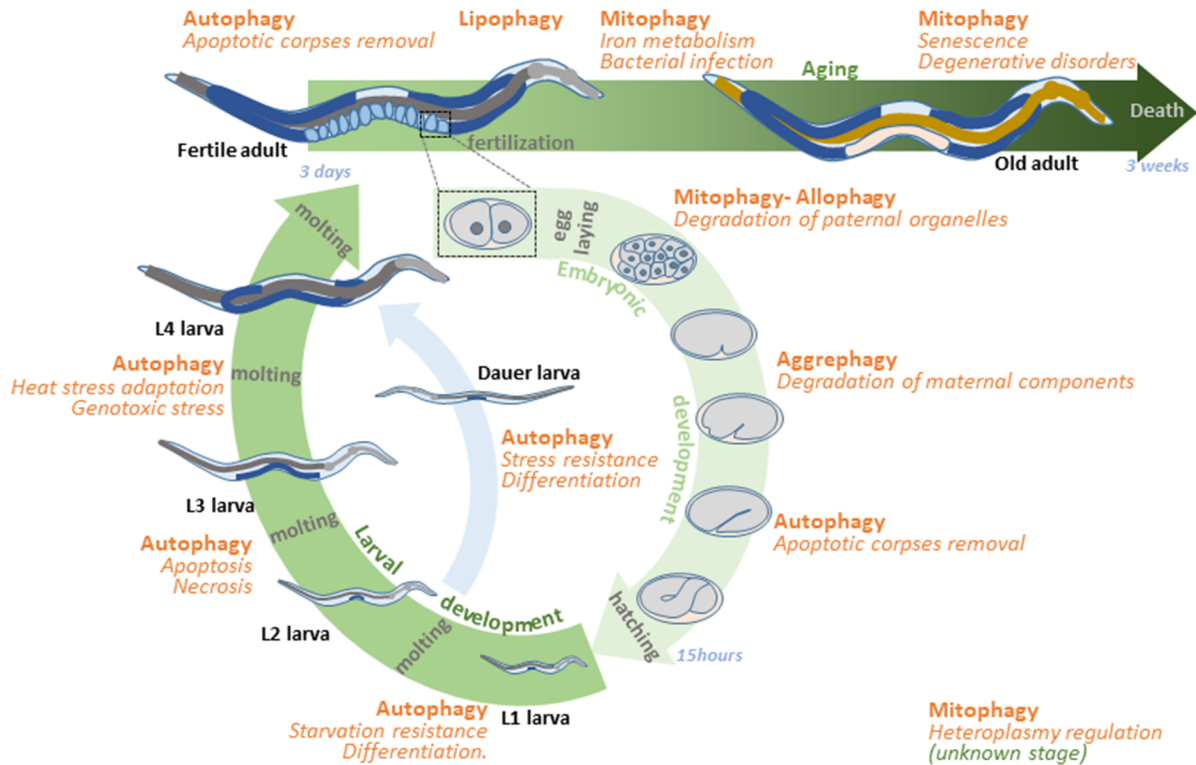


Figure 3 Leboutet et al.

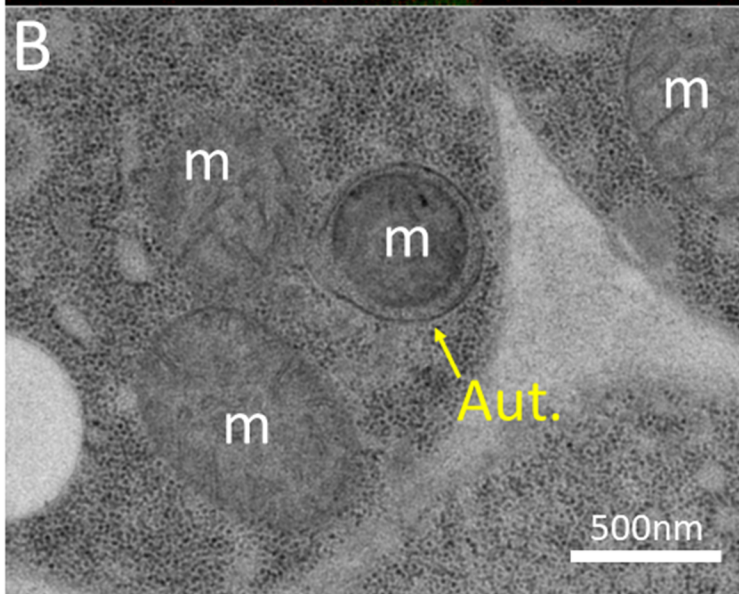
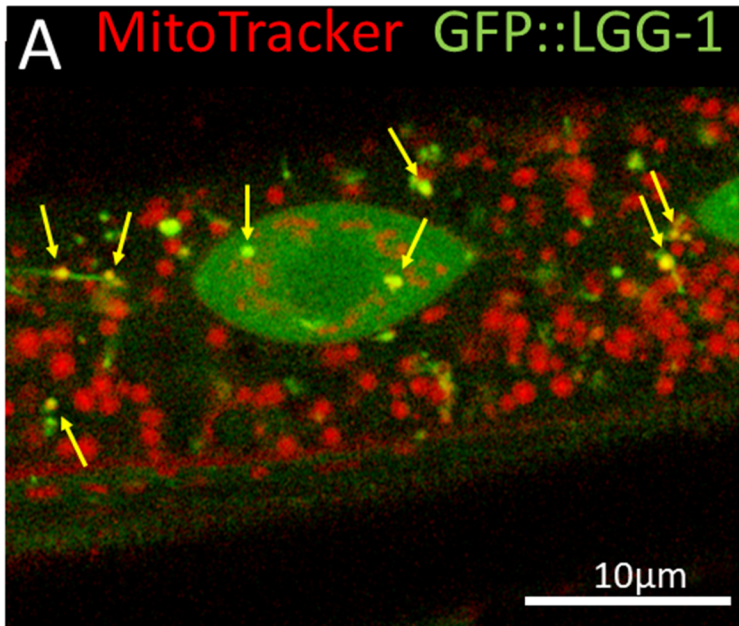


Figure 4 Leboutet et al.

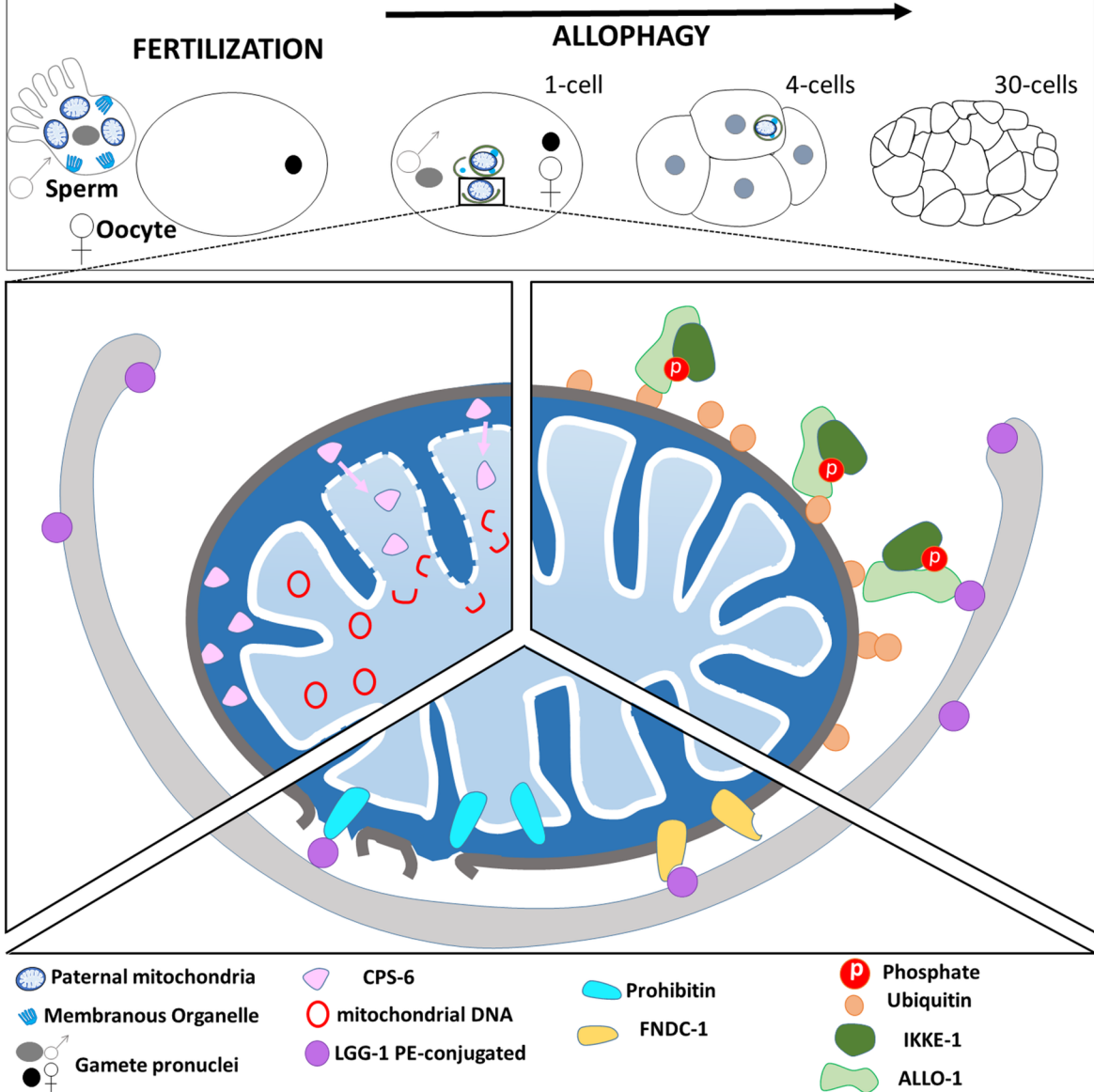


Figure 5 Leboutet et al.

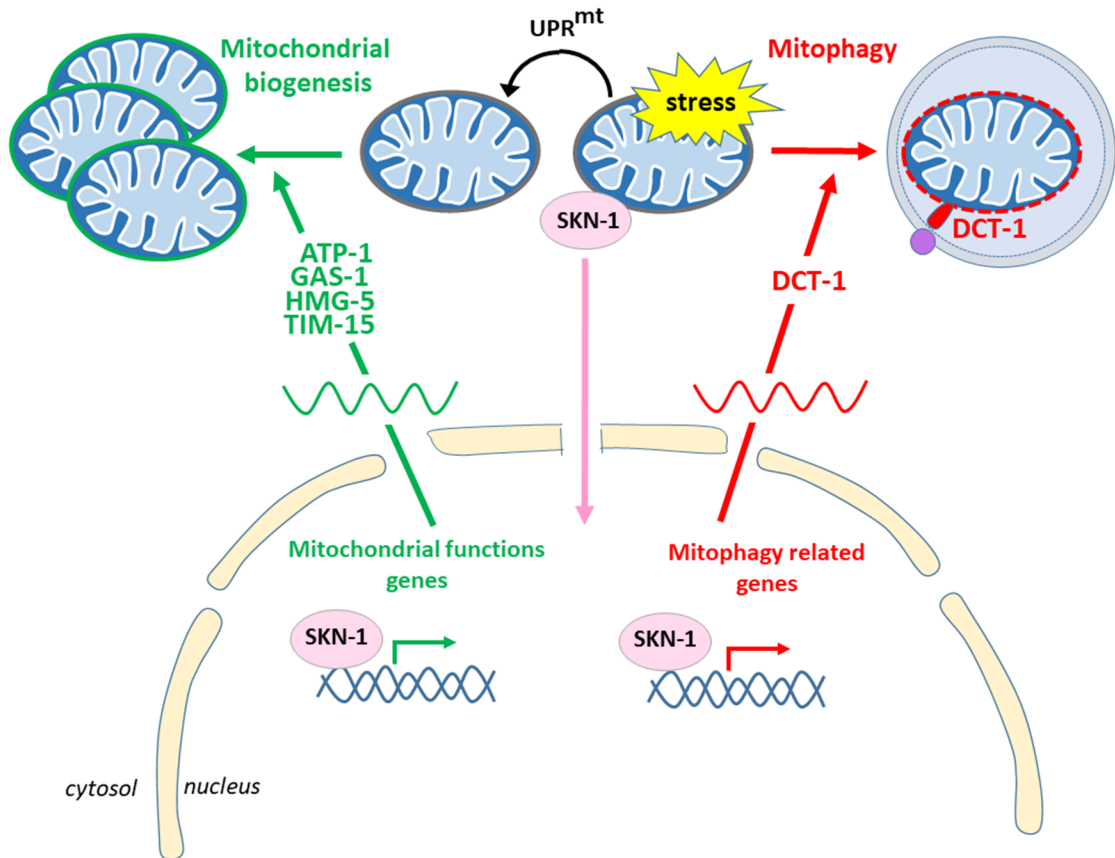


Figure 6 Leboutet et al.