AUTOPHAGY AND MITOPHAGY INTERPLAY IN MELANOMA PROGRESSION

Hannelore Maes and Patrizia Agostinis[§]

Laboratory of Cell Death and Therapy, Department Cellular and Molecular Medicine, KU Leuven, Leuven, B-3000, Belgium

[§] Corresponding author: Patrizia Agostinis, Ph.D.
Laboratory of Cell Death Research & Therapy
University of Leuven, Campus Gasthuisberg O&N1
Herestraat 49, B-3000, Leuven, Belgium
tel: 32-16-330650; fax: 32-16-345995.
Email: patrizia.agostinis@med.kuleuven.be

Abstract

Autophagy, or self-eating, is the most extensively studied lysosomal degradation pathway for the recycling of obsolete or damaged cytoplasmic materials, including proteins and organelles. Although this pathway was initially thought to function as trafficking system for 'in bulk' degradation by the lysosomes of cytoplasmic material, it is now widely appreciated that cargo selection by the autophagic machinery is a major process underlying the cytoprotective or –possibly- pro-death functions ascribed to this catabolic process. Indeed increasing evidence suggests that in mammalian cells the removal of dysfunctional or aged mitochondria occurs through a selective degradation pathway known as 'mitophagy'. Due to the crucial role of mitochondria in energy metabolism, redox control and cell survival/death decision, deregulated mitophagy can potentially impact a variety of crucial cell autonomous and non-autonomous processes. Accumulating evidence indicates that during malignant transformation aggressive cancers hijack autophagy to preserve energy fitness and to acquire the plasticity required to adapt to the hostile microenvironment. However, whether and how mitophagy contributes to carcinogenesis, which pathways regulates this process in the cancer cells and how cancer cell-mitophagy impacts and modifies the tumor microenvironment and therapeutic responses, remain largely unanswered issues. In this review, we discuss novel paradigms and pathways regulating mitophagy in mammalian cells and the impact this process might have on one of the most dreadful human malignancies, melanoma.

Keywords: Autophagy, mitophagy, reactive oxygen species, signaling, cancer, melanoma, anticancer therapy.

Abbreviations: **AMPK**: AMP–activated protein kinase; **Atg**: autophagy-related genes; **BNIP3**: BCL2 and adenovirus E1B 19 kDa-interacting protein 3; **BNIP3L**: BNIP3 like protein; **CQ**: chloroquine; **Drp1**: Dynamin-1-like protein**; ER**: endoplasmic reticulum; **Fis1**: Mitochondrial Fission Protein: **FIP200**: focal adhesion kinase family interacting protein of 200 kDa; **HIF1**: hypoxia-inducible factor 1; **HKI**: hexokinase I; **IMM**: inner mitochondrial membrane; **MAMs:** mitochondria-associated membranes; **Mff**: Mitochondrial fission factor; **MPP**: mitochondrial processing proteases; **mTORC1**: mechanistic target of rapamycin complex 1; **NIX**: NIP3-like protein X; **LC3**: microtubules-associated light chain-3; **LIR**: LC3 interacting region; **Mfn**: mitochondrial

outer membrane guanosine triphosphatase mitofusin; **OMM**: outer mitochondrial membrane; **PARL**: mitochondrial inner membrane rhomboid protease presenilin-associated rhomboid-like protein; **PE**: phosphatidylethanolamine; **PGC-1α**: transcription factor PPARγ co-activator 1α; **PI3KC3**: class III PI3K; **PARKIN:** *parkin* gene product; **PINK1**: Phosphatase and tensin homolog (PTEN)-induced putative protein kinase 1; **ROS**; reactive oxygen species; **TOM**: the protein translocase of the outer mitochondrial membrane complex; **TIM23**: translocase of inner mitochondrial membrane 23 homolog; **ULK1/2**: Unc51-like kinase 1/2; **VDAC:** the voltage-dependent anion channel**.**

1. Autophagy; basic molecular machinery

Macroautophagy (hereafter referred to as autophagy) is a regulated process of self-eating by which part of the cytoplasm, including long-lived or unfolded proteins and superfluous or damaged organelles, is trafficked through the aid of double-membraned vesicles, known as autophagosomes, to the lysosomes where cargo is degraded. Besides functioning as a key quality control mechanism crucial to control organelle number and fitness during homeostasis in all eukaryotic cells, recycling of intracellular components via this catabolic process supplies an alternative energy resource to survive in conditions of famine or reduced oxygen supply [1]. Although autophagy is constitutively active at basal levels sufficient to preserve homeostasis and cytoplasmic quality control, it can be stimulated 'on demand' whenever cells sense nutrient or oxidative stress, organellar damage or loss of proteostasis [1]. Stimulation of autophagy under these stress conditions is a pro-survival mechanism, although under certain circumstances autophagy may favor cell death [2].

Autophagy initiates by the formation of an isolation membrane (called phagophore), usually formed at the contact sites between the endoplasmic reticulum (ER) and mitochondria. Additionally other organelles, like the Golgi apparatus, post-Golgi compartments, as well as the plasma membrane may provide alternative sources [3], although the exact molecular players in phagophore-building and membrane trafficking in mammalian cells are only beginning to emerge [4, 5]. The isolation membrane is further elongated to form double membrane vesicles, which deliver their cargo to the lysosomes for degradation and recycling (schematically presented in Fig. 1).

At the molecular level the different stages of autophagy are spatially and hierarchically controlled by a set of autophagy-related genes (Atg), conserved between yeasts and mammals [6, 7]. A key regulator of autophagy-initiation in mammalian cells is the Unc51-like kinase 1/2 (ULK1/2) complex, containing Atg13, Atg101 and FIP200 (focal adhesion kinase family interacting protein of 200 kDa). This complex is negatively controlled by the mechanistic target of rapamycin complex 1 (mTORC1) kinase. mTORC1 activation under nutrient/growth factor-rich conditions leads to phosphorylation and inactivation of ULK1/2 and Atg13. Starvation-induced activation of AMP-activated protein kinase (AMPK), reactive oxygen species (ROS), or rapamycin treatment cause the inactivation of mTORC1, which results in the

induction of autophagy (Fig. 1). Upon activation, the ULK-complex is involved in correctly localizing the class III PI3K (PI3KC3)/Vps34 complex, containing p150, Atg14L and Beclin1, which regulates nucleation and assembly of the phagophore membrane. The activity of this complex is tightly controlled by positive regulators like UVRAG, AMBRA1, Rab5 and BIF1 and negative regulators like Rubicon (Fig.1). Beclin 1 in this complex can bind –through agency of its BH3 domain- anti-apoptotic BCL2 family members, which can by this mechanism exert a negative control on autophagy induction. Upon activation ULK1 phosphorylates AMBRA1, hereby releasing the AMBRA1 containing PI3KC3-complex from the microtubules, resulting in its relocalization to the ER [6, 7]. Important signaling molecules for the regulation of the elongation, shaping, and sealing of the autophagosomal membrane are two-ubiquitin-like conjugation systems, regulating the formation of the Atg5–Atg12–Atg16L complex and the phosphatidylethanolamine (PE)-conjugation of the microtubules-associated light chain-3 (LC3), which becomes associated to the autophagosomes through the entire process serving as a marker for their identification (see Fig. 1 for a more extensive description).

Mature autophagosomes can merge with endocytic vesicles (early or late endosomes) to become amphisomes or directly fuse with lysosomes, where the cargo is degraded by lysosomal hydrolases. Fusion of the autophagosomes with the lysosomes is mediated by Rab7, which promotes microtubule transport and Syntaxin17, a SNARE protein that is localized on mature autophagosomes [8]. After autophagolysosome formation building blocks are freed into the cytosol and LC3 located at the cytosolic surface of the autophagosolysosome undergoes Atg4-mediated decoupling from PE to be recycled.

Because autophagy is usually activated by cellular stress that may concurrently initiate apoptotic cell death, accumulation of autophagosomes is often detected in cells committed to die. This has led to the suggestion that autophagy may be causative for cell death. However, the occurrence of the so called autophagic cell death, i.e. cell death caused by the autophagy genetic machinery, has been documented so far during developmental stages/tissues in lower organisms, whereas in mammalian cells appears to be limited to selective pathological and/or drug-induced conditions (for an extensive review see [2]).

2. Mitochondria quality control through mitophagy

Originally autophagy was thought to be a non-selective, bulk degradation pathway to recycle obsolete cytoplasmic materials. However during the last decade the selective removal of organelles, lipid droplets, proteins and pathogens has been extensively documented [9]. The autophagic process governing the specific removal of damaged or superfluous mitochondria is known as mitophagy [10].

Because of the crucial role of mitochondria in metabolism, Ca^{2+} signaling and redox control and as a signaling hub of a variety of pathways impinging on the cell death/survival balance, it is not surprising that the selective removal of damaged or excessive mitochondria under homeostatic and stress conditions, has evolved as a tightly regulated and selective autophagic process. Besides its developmental role in erythrocytes maturation, mitophagy serves as a crucial quality control mechanism. In line with this, defective mitochondria clearance is exacerbated in aged cells and accumulation of unhealthy mitochondria associates -or is possibly causative- to crucial pathological conditions, like neurodegeneration and cancer.

Just like for autophagy, the molecular players of this selective degradation process were first described in yeast. Through a genetic screen for yeast mutants defective in mitophagy, Atg32, a protein not involved in other autophagy pathways, was identified as prime and selective mitochondrial receptor [11]. Upon induction of mitophagy Atg32 is phosphorylated by casein kinase 2 [12] and binds the scaffold protein Atg11, which regulates cargo selection during autophagy. The Atg32-Atg11 complex formation then enables the recruitment of mitochondria to the pahgophore assembly site. Although in mammalian cells (as described further), some crucial mitochondrial receptors have been recently characterized, the functional homologue of the yeast Atg32 has not been identified yet.

Also the mechanisms allowing the selection and isolation of individual mitochondria from the dynamic mitochondrial network and regulating the encapsulation of this rather large cargo within the autophagosomes, are not completely understood. In both yeast and mammalian cells, mitochondria fission, a process regulated by the conserved family of dynamin-related proteins and instigated by dissipation of the membrane potential, has been shown to precede mitophagy (for an extensive review on mitochondria dynamic and mitophagy, see [13]).

Interestingly, fragmentation of mitochondria, which likely facilitates their engulfment by the autophagosomes but *per se* is not absolutely required for mitophagy, appears to occur at specific subdomains located between the endoplasmic reticulum (ER) and the mitochondria. An elegant study in yeast by Böckler and Westermann revealed that yeast mutants lacking essential components of the endoplasmic reticulum mitochondria encounter structure (ERMES), the ER-mitochondrial tethering complex, displayed a reduced mitophagy rate while a synthetic mitochondria-ER tether rescued these mitophagy defects [14]. Based on the major findings of that study, it was suggested that the ER-mitochondria contact sites may provide sufficient supply of ER-derived lipids required for the isolation and expansion of autophagosomal membrane for the engulfment of an isolated mitochondrion, and/or recruit key components of the fission machinery [14, 15]. The relevance of ER-mitochondria contact sites in mitophagy is also supported by recent studies in mammalian cells. The mitochondria-associated membranes (MAMs), i.e. the ER subdomains that physically tether the ER to mitochondria, have been shown to define the position of mitochondrial fragmentation that precedes the recruitment components of the fission (Drp1) and autophagy machinery, like Atg14 and Atg5 [16, 17]. All together these evidence strongly indicate that the ER-mitochondria contact sites have evolved as a privileged subdomains to coordinate mitochondrial morphological changes and fission mechanisms regulating mitophagy in eukaryotic cells [18].

Finally, emerging evidence indicate that mammalian cells dispose of complex and redundant signaling mechanisms that prime the mitochondria for selective recognition by the autophagic machinery.

In this review we describe the major pathways governing mitochondria priming and pruning in mammalian cells, along with the identified molecular players involved. A more detailed overview of these pro-mitophagic molecules is presented in Table 1.

3. Signaling mechanisms in mitochondria priming

a) The PINK1-PARKIN signaling pathway

The best characterized signaling mechanism for the priming of mitochondria for autophagic removal in mammalian cells is the poly-ubiquitylation of mitochondrial proteins mediated by PARKIN. PARKIN (also known as PARK2) is a RING-HECT

hybrid E3 ubiquitin ligase, which is recruited to the outer mitochondria membrane (OMM) upon mitochondrial membrane depolarization or accumulation of unfolded proteins in the mitochondrial matrix, through a mechanism requiring PINK1 (Phosphatase and tensin homolog (PTEN)-induced putative protein kinase 1).

PINK1 (also known as PARK6) is a kinase with an N-terminal mitochondrial import sequence, which under basal conditions results in its import into the mitochondria via the TOM (the protein translocase of the outer mitochondrial membrane complex) and TIM23 (translocase of inner mitochondrial membrane 23 homolog) complexes. Following import in the mitochondria PINK1 is cleaved by mitochondrial processing proteases (MPP) in the matrix and by the mitochondrial intramembrane protease PARL (mitochondrial inner membrane rhomboid protease presenilin-associated rhomboid-like protein) localized in the inner mitochondrial membrane (IMM), hereby generating an N-terminal destabilizing amino acid. The cleavage product is subsequently translocated from the mitochondria into the cytosol, where it is degraded by the proteasome, thus guaranteeing constant low levels of mitochondrial PINK1 under basal conditions [19, 20] (Fig. 2). When mitochondrial membrane potential is lost or misfolded proteins accumulate in the mitochondrial matrix, PINK1 is no longer imported into the IMM and mitochondrial matrix and its proteolysis by MPPs and PARL is prevented [19, 21] (Fig. 2). This allows PINK1 stabilization, autophoshorylation and its dimerization in the OMM [22]. A RNAi screen by Hasson and co-workers identified TOMM7 as an essential component of the TOM complex to shunt and retain PINK1 on the OMM upon mitochondrial damage [23]. Upon stabilization on the OMM, PINK1 recruits PARKIN from the cytosol to the damaged mitochondria by phosphorylation of its linker region on T175 and T217 in humans [24]. Other proteins that have been reported to mediate the relocation of PARKIN to the damaged mitochondria are PINK1-phosphorylated Mfn2 (mitochondrial outer membrane guanosine triphosphatase mitofusin 2) [25], BNIP3 (BCL2 and adenovirus E1B 19 kDa-interacting protein 3) [26] and BNIP3L/NIX (BNIP3 like protein/ NIP3-like protein X) [27] (see below), however the underlying molecular mechanisms are not known. Upon recruitment to the mitochondria PARKIN poly-ubiquitylates several mitochondrial proteins, including VDAC1 (the voltage-dependent anion channel1) [28], mitochondrial hexokinase I (HKI) [29], Miro1 [30], Mfn1 and Mfn2 [31]. The ubiquitylated mitochondrial proteins are subsequently degraded by the proteasome or recruit ubiquitin-binding LIR-containing adaptors, like

p62/SQSTM1 and NBR1, resulting in the clustering of the damaged mitochondria around the nucleus and their incorporation into the LC3-containing autophagosomes [13]. Indeed, the recruitment of p62/SQSTM1 to poly-ubiquitin-positive clusters of mitochondria has been shown to require PARKIN, and to be necessary for the complete clearance of unfunctional mitochondria [28]. Although PARKIN-mediated ubiquitylation is an important signal tagging mitochondria for degradation, little is known about the molecular mechanisms that link autophagic isolation of mitochondria, autophagosomal membrane expansion and recognition/encapsulation of this rather large cargo. Recently members of the TBC family of RAB GTPases deactivators, TBC1D15 and TBC1D17, have been identified as important molecules for the proper autophagic encapsulation of mitochondria. During PINK1-PARKIN-mediated mitophagy TBC1D15 and TBC1D17 act in concert with Fis1 – an OMM protein part of the mitochondrial fission machinery along with Mitochondrial fission factor(Mff) and Drp1- and LC3 homologue proteins to control autophagosomes morphology by coordinating the activity of the small GTPase Rab7, a protein that governs autophagosomes biogenesis [32, 33]. Interestingly, Fis1 has been shown to interact with ER proteins and to enable the recruitment of the fission machinery at the contact sites established between these organelles, where also Drp1 has been shown to localize [18, 34]. These studies reinforce the role of the ER-mitochondria contact sites as privileged interface orchestrating the morphological (i.e. mitochondrial fission/division), structural (i.e. membrane source for encapsulation) and functional (recruitment of both fission and autophagy machineries).

Although PINK1-PARKIN-mediated mitophagy has been studied and characterized in various neuronal and non-neuronal cell lines, including cancer cell lines, its relevance has been mostly explored as a mechanism implicated in the pathogenesis of Parkinson's disease. PINK1 and PARKIN are frequently found to be mutated in familial forms of Parkinson's disease, thus material derived from those patients offers a unique potential to validate the role of PINK1 and PARKIN in *ex vivo/in vivo* situations [35]. *Ex vivo* evidence for the importance of the PINK1-PARKIN system in mitophagy was obtained in fibroblasts derived from patients with PINK1 mutations, where the recruitment of overexpressed PARKIN to the mitochondria after treatment with mitochondrial uncouplers was found to be disturbed [36]. Defects in mitochondrial metabolism and morphology have been described in tissue derived from patients with PARKIN mutations, although the effect on mitophagy was not

thoroughly investigated in these studies [37, 38]. However direct evidence for the role of the PINK1-PARKIN system in mitophagy under physiological conditions is limited, and only one study in the drosophila model system has reported the importance of this pathway for mitophagy *in vivo,* by showing an increased half-life of mitochondrial proteins, especially of the respiratory chain components, in *parkin* mutant flies [39]. However, also PARKIN/PINK1 -independent mechanisms of mitophagy have been described [40], highlighting a certain degree of functional redundancy in the signaling mechanisms implicated in this selective degradation process in higher organisms.

b) FUNDC1; a mitochondrial receptor

Most of the molecules known to function as receptors for selective autophagy interact with LC3 and LC3-like proteins, via a LIR. Recently FUNDC1, an OMM protein containing a LIR motif has been shown to act as a mitochondria receptor under hypoxia by binding LC3 and enabling engulfment of mitochondria by the autophagosomes [41]. FUNDC1 is an integral OMM protein, which becomes post-translationally modified under stress conditions stimulating mitophagy, thus enabling FUNDC1 to function as mitophagic receptor. For example upon mitophagy induction by hypoxia or mitochondrial uncouplers, ULK1 is upregulated and localizes to fragmented mitochondria, where it phosphorylates FUNDC1 at serine 17, hereby increasing its binding to autophagosome-associated LC3 [42].

The role of FUNDC1 in mitophagy has still has to be confirmed *in vivo,* since its function has only been addressed in cellular models. In this context, it would be interesting to investigate the role of FUNDC1 during development and under pathological conditions in which removal of ROS-producing mitochondria has been shown to play a major role, like ischemia-reperfusion, neurodegenerative diseases and cancer.

c) BNIP3 and NIX regulated mitochondrial clearance

BNIP3 and NIX, are two BCL2 family members that contain an atypical BH3-only domain and a C-terminal transmembrane domain, essential for their homodimerization and mitochondrial localization [43]. BNIP3 and BNIP3L/NIX (thereafter named NIX) were first recognized for their role in the regulation of mitochondrial apoptosis [44-46]. However, recent studies suggest that BNIP3 and

NIX rather than directly promoting cell death, regulate mitochondrial fragmentation and mitophagy by establishing interactions with different mitochondrial proteins. The expression of BNIP3 and NIX is strongly upregulated by the transcriptional activation of the hypoxia-inducible factor (HIF-1) [43] [47], induced by hypoxia, malignant transformation [48] or even chemotherapeutics e.g. doxorubicin [49].

Recently, key molecular mechanisms through which BNIP3 and NIX regulate mitochondrial clearance have been elucidated. Interestingly, some of these mechanisms are common while others are unique for one of the two proteins, suggesting that upstream regulators of these pro-autophagic proteins may utilize BNIP3 or NIX in a context and possibly stress specific manner.

A well described mechanism by which BNIP3 mediates mitophagy induction entails the binding of its BH3-domain with BCL2, hereby scavenging BCL2 away from Beclin1, resulting in the release of the pro-autophagic activity of Beclin1 [50] (Fig. 1 and Fig. 2). Additionally, BNIP3 can activate the autophagic machinery via direct binding and inhibition of Rheb, a Ras related small GTPase and key upstream activator of mTORC1 [51]. A more direct mechanism enabling mitophagy induction, involves a role for BNIP3 as mitochondrial receptor. BNIP3 can associate with the OMM and interact with LC3/ LC3 like proteins through its LIR domain [52, 53] (Fig. 2). The interaction of BNIP3 with the LC3 family members, LC3B or GATE-16, has been shown to be enhanced by phosphorylation of the serine residues 17 and 24 flanking the BNIP3 LIR domain [53]. Interestingly, induction of mitophagy in cardiac myocytes by overexpression of BNIP3 was independent of mitochondrial Ca^{2+} uptake, ROS generation and opening of the permeability transition pore, mitochondria-regulated processes that can instigate their fragmentation [54].

Interestingly, mitochondria-localized BNIP3 has been shown to impair mitochondrial respiration by inducing the degradation of components of the electron transfer chain through mitochondrial proteases, a process stimulating the removal of mitochondria through mitophagy [55].

In adult cardiac myocytes, BNIP3 favors the translocation of PARKIN at the mitochondria in a Drp1-dependent manner, a cytoprotective process that promotes Drp1-mediated mitochondrial fission and PARKIN-regulated degradation of unhealthy mitochondria [26] (Fig. 2).

A functional connection between BNIP3 and mitochondrial clearance has been suggested under different physiopathological conditions and observed in several tissues. For example, using rat or mouse models BNIP3 has been linked to the induction of mitophagy after acute kidney injury in renal tubular cells [56], in aged kidneys [57], after acute spinal cord injury [58], during skeletal muscle atrophy [59], in the diaphragm after chronic hypoxia [60], in the liver especially under fasting conditions [61] as well as in tumor-associated fibroblasts [62]. This suggests a rather widespread and important role for BNIP3 in the regulation of mitochondria degradation induced by a variety of endogenous and exogenous cellular insults.

Also for NIX several mechanisms for mitophagy induction have been described. In analogy to BNIP3, NIX can induce the autophagic machinery either by releasing Beclin1 [50], or by increasing mitochondrial ROS production after CCCP-treatment, leading to the inhibition of mTORC1 signaling. NIX also functions as a LIR-containing mitochondrial receptor [63-65] and can contribute to mitochondrial priming by controlling the mitochondrial localization of PARKIN [27]. Scattered evidence exists delineating the connections between PINK1-PARKIN pathway and the BNIP3/NIX regulation of mitophagy [26, 27], but the exact mechanism underlying their interaction is still obscure and requires further study. Another level of mitophagy regulation was recently found by Li and coworkers, who showed that both FUNDC1 and NIX levels are down-regulated by the hypoxia-responsive microRNA-137 (miR-137), which when overexpressed inhibits hypoxia-induced mitochondria clearance without impairing global autophagy [66]. Thus reduction of miR-137 under hypoxia, allowing the increase of NIX and FUNCD1 bound to LC3, may represent a mechanism regulating mitophagy, while leaving general autophagy unrestrained.

Although in most studies mitochondrial degradation has been linked to the removal of superfluous or damaged mitochondria, a recent report links energetic status of mitochondria with NIX-mediated mitophagy. Conditions stimulating increased oxidative phosphorylation in HeLa cells, induced by switching glucose to glutamine as primary carbon source for ATP production, were found to be associated with heightened mitophagy. Mitophagy mediated by stimulated oxidative phosphorylation, was independent on mTORC1, but required the recruitment of Rheb to the mitochondria through a NIX-dependent mechanism and the formation of a Rheb-NIX-LC3 complex [67]. Although the signaling mechanism by which NIX mediates the recruitment of Rheb to mitochondria is still elusive, the local induction of hypoxia

generated by the increased rate of mitochondrial oxygen consumption was suggested as a potential triggering signal. Irrespective of the exact molecular mechanism, NIX-mediated mitophagy was found to be vital for the maintenance of maximal respiration and ATP production. This suggests that this pathway sensing the energetic status of the mitochondria, is activated to preserve cellular bioenergetic efficiency, by ensuring the renewal of a healthy mitochondrial pool and preventing the accumulation of damaged mitochondria [67]. Why this process is selective for NIX is not yet clear, as BNIP3 is also hypoxia-responsive and shares with NIX the ability to directly interact with Rheb.

In vivo NIX has been shown to be essential for the recruitment of LC3/GABARAP pro-autophagic proteins to the mitochondria during mitophagy in maturing murine erythrocytes [64] and it has been linked to the induction of mitophagy after acute spinal cord injury in rats [58].

All together these studies indicate that BNIP3 and possibly NIX, regulate different signaling mechanisms that either precede mitochondrial clearance (i.e. Drp1-mediated fission of the mitochondrial network) or act directly at the core of the mitophagy machinery, by modulating degradative processes (i.e. activation of mitochondrial proteases), acting as a mitophagy receptor (via LIR domain) or inducing the translocation of pro-mitophagic mitochondrial complexes (i.e. PARKIN).

4. Autophagy, mitophagy and cancer

Metabolic stress present within the tumor microenvironment principally nutrient deprivation and hypoxia, have been reported to induce autophagy in cancer cells and the surrounding stroma [68]. In agreement with this, autophagy in tumors is found particularly heightened in hypoxia areas, where energy and nutrient demand are higher and stress adaptation is a pre-requisite for cancer cells to survive.

However, while the pro-survival function of autophagy may enable cancer cells to adapt to a hostile environment and resist cancer therapy, emerging evidence supports a highly dynamic and context dependent role for autophagy in cancer [69, 70]. In early stages of tumorigenesis, damage mitigation by autophagy serves as a tumor suppressor mechanism, limiting ROS-driven genomic instability and preventing necrosis-associated inflammation [71-73].

Moreover, by removing damaged mitochondria in conditions of stress, mitophagy prevents the accumulation of toxic ROS, which may propagate damage to other macromolecules and the nucleus [74-76]. Additionally given that mitochondrial DNA mutations are also incited by mitochondria ROS production, mitophagy may be a mechanism to decrease the rate mtDNA mutations, which may be further propagated to daughter cells and could contribute to cancer progression [77]. In more advanced tumors, autophagy provides energy for accelerated tumor cell growth and helps tumor cells to withstand metabolic stress [71, 72, 78].

Moreover, the dynamic and contextual role of autophagy in cancer appears to be closely linked to the genetic landscape of the developing tumors, as recently shown in a mouse model of pancreatic ductal adenocarcinoma, where the pro-tumorigenic role of autophagy was dependent on the presence of tumor suppressor p53 [79].

Recent studies indeed disclose that aggressive tumors driven by activating Ras mutations [80, 81]), such as pancreatic and lung cancer, are particular reliant on autophagy to support energy metabolism, cell growth and viability in nutrient-deprived conditions [82-85]. Using genetically engineered mouse models of *KRas*^{G12D}-driven non–small cell lung carcinoma, the genetic ablation of autophagy was shown to convert aggressive adenomas and adenocarcinomas into benign tumors (oncocytomas). These tumors displayed an accumulation of dysfunctional mitochondria suggesting that mitophagy contributed to cancer progression in this Ras-driven lung cancer model [79]. Similar findings were recently confirmed in lung tumors harboring activating mutations of BRAF [86], a downstream effector of Ras and activator of the ERK-pathway whose oncogenic BRAFV600E variant is expressed in a variety of cancers. Hence, this ‘autophagy addiction’ may be a characteristic of aggressive human cancers. Moreover in this BRAFV600E-driven lung cancer model the tumor-suppressive function of autophagy are largely explained by the preservation of redox homeostasis early in tumorigenesis, whereas later during cancer progression maintenance of mitochondrial clearance is essential to supply essential metabolic substrates, like glutamine, to feed mitochondrial function and to confer cancer cell’s fitness and growth [86].

Which components of the mitophagy pathway(s) and signaling mechanisms are implicated in the pro-tumorigenic function of autophagy remain to be characterized, although it can be hypothesized that hypoxic-responsive NIX/BNIP3 and Ras-regulated BNIP3 [87] may be critical molecular candidates. This notion seems to be

particular relevant for melanoma, the most recurrent BRAFV600E-driven malignancy, for which the relevance of autophagy and BNIP3-mediated mitochondrial quality control mechanisms are emerging.

5. Relevance of autophagy and mitophagy in melanoma

Cutaneous malignant melanoma (hereafter referred to as melanoma) is a cancer originating from the melanocytes, the pigment producing cells that reside in the epidermis of the skin. Despite numerous health campaigns for early diagnosis and protection, melanoma is the most rapidly increasing cancer in the white populations [88]. If melanoma is diagnosed in the early stages (radial growth phase) it can be cured by surgical resection. However, metastatic melanoma is largely refractory to existing therapies and has a very poor prognosis, with a median survival rate of 6 months and 5-year survival rate of less than 5% [89]. Like many other cancers, melanoma is the result of the interplay between environmental and genetic components, (readers are referred to a recent review on the genetic landscape of melanoma [90]).

a) Deregulation of autophagy pathways during melanomagenesis

The first clues indicating a deregulation of autophagy during melanomagenesis were already found in the early 1980s, when pathologists described the presence of giant autophagic melanosomes complexes in malignant melanoma [91]. Despite the early recognition of the involvement of autophagy in melanoma, only a limited amount of studies have investigated the alterations in the autophagic status of the cells during melanomagenesis.

In benign nevi autophagy has been reported to be increased to support oncogene-induced senescence (e.g. driven by BRAFV600E expression) [92, 93]. However during the early stages of malignant transformation autophagy is decreased as compared to benign nevi [92, 94] and melanocytes [95]. In metastatic melanoma along with the acquisition of other mutations altering the oncogenic landscape, the autophagy capacity is re-established to support cancer cell's high metabolic demands and survival in the face of the stressful tumor microenvironment [95-98]. Thus these studies indicate that melanoma progression is coupled to alterations in

the amount of autophagosomes as well as pro-autophagic proteins, supporting a dynamic role for autophagy in melanoma progression (see Fig. 3). Importantly, a high level of autophagy in melanoma patient's tumors is associated with a lower therapeutic response and a worse outcome, further supporting the relevance of autophagy as possible prognostic marker and suggesting autophagy as a therapeutic target in melanoma [85, 96].

Despite its increasing relevance in cancer, mitophagy is a virtually unexplored field in melanoma [99]. However the existing data, combined with the importance for mitochondria in redox control, metabolism and cell death hint towards an important role for mitophagy in melanomagenesis and melanoma progression. Indeed exposure to sunlight, one of the main risk factors for melanoma, which has been reported to induce mitochondrial DNA mutations/deletions, has been recently shown to induce changes in mitochondrial morphology and mitophagy in melanocytes [100]. This suggests that functional mitophagy in the melanocytes is required to maintain mitochondrial quality and as a mechanism preventing melanocyte transformation. These studies suggest that expression levels of pro-mitophagic proteins during melanomagenesis could allow the identification of novel biomarkers of melanoma progression [101], a premise that deserves to be validated in future studies.

b) Metastatic melanoma are addicted to autophagy and mitochondrial quality control

Recent studies delineate a crucial role for autophagy in metastatic melanoma. Marino *et al* showed that under mildly acid cell culture conditions, the number of autophagosomes is rapidly increased and inhibition of autophagy via knockdown of Atg5 reduces melanoma cell survival [102]. In the same line Sheen and co-workers, reported that a leucine-free diet combined with an autophagy inhibitor, synergistically reduced the growth of xenografted human melanoma tumors and induced widespread apoptosis of the cancer cells [103]. However, even under nutrient-rich growth conditions metastatic melanoma cells display an addiction to autophagy. This is supported by data from several groups who reported that pharmacologic inhibition of autophagy or knockdown of key autophagy genes (e.g. Beclin1 and Atg7) in

melanoma induces spontaneous melanoma cell death [104] [105]. Recent data from our group show that inhibition of mitophagy through BNIP3 knockdown or blockage of autophagic flux though chloroquine (CQ) or Atg5 silencing, increased similarly spontaneous cell death and reduced the clonogenic expansion of metastatic melanoma cells *in vitro* [106]. Intriguingly, loss of melanoma clonogenic growth under autophagy/mitophagy inhibition was coupled to an increased ROS production and a metabolic shift from oxidative phosphorylation towards glycolysis (*Maes et al,* unpublished data). These observations highlight that metastatic melanoma rely on mitophagy to maintain the functional pool of mitochondria required to support their high metabolic demands ([106] and Maes et al., *unpublished data*). Impairing this BNIP3-regulated process ultimately compromised their ability to survive, by instigating a redox and bioenergetic failure, a conjecture that needs to be further validated *in vivo* by deleting *BNIP3* in melanoma cells. Whether the NIX-Rheb pathway described before [67] also contributes to the modulation of mitochondria bioenergetics and growth in melanoma requires further studies. The observation that inhibitors of farnesylation, a post-translational modification that is required for the mitochondria anchorage of Rheb to ensue mitophagy [107], reduces melanoma cell growth *in vitro*, supports further studies aiming at revealing this connection.

Moreover, given the additional, mitophagy-independent role of BNIP3 in preserving the architecture of actin-cytoskeleton and CD47 levels in metastatic melanoma [106], BNIP3 deficiency has the potential to impact both melanoma cell intrinsic (metabolism, redox signaling, migration, adhesion) and extrinsic (cell-cell interactions, phagocytosis, immunosurvelliance) features. Interestingly increased transcript-expression levels BNIP3 tend to correlate with reduced overall survival in a melanoma patient cohort [106], thus suggesting that BNIP3 may become an interesting therapeutic target in melanoma.

Besides harboring heightened basal levels of autophagy, various anti-melanoma therapeutic regimens have been shown to stimulate autophagy with a predominant cytoprotective function [68], thus suggesting that interfering with autophagy may ameliorate their therapeutic effects. For example, Lotze *et al* found that interleukin-2 therapy induces massive autophagy activation, which when inhibited with the lysosomotropic drug CQ ameliorated therapeutic outcome. in line with this treatment of different melanoma cell lines with the mTORC1 inhibitor

temsirolimus, induced pro-survival autophagy and the combination of temsirolimus with CQ treatment significantly increased therapy response [104]. This finding is interesting as in a phase I clinical trial assessing the potential temsirolimus in combination with the CQ derivative hydroxychloroquine (HCQ) 73% of patients with metastatic melanoma showed stabilized tumor growth, as compared to temsirolimus treatment alone, which failed to stabilize the disease [108]. Interestingly Haq *et al* reported that melanoma cells become addicted to oxidative phosphorylation after treatment with inhibitors of BRAFV600E, targeting the most common genetic alteration in melanoma. The increased oxidative phosphorylation after BRAFV600E inhibition is driven by the activation of MITF, that augments the expression of transcription factor PPARγ co-activator 1α (PGC-1α), resulting in increased expression of genes involved in citric acid cycle and oxidative phosphorylation [109]. Importantly up to 30% of melanoma harbor genomic amplifications of *MITF*, which drives PGC1α overexpression, leading to increased mitochondrial phosphorylation and capacity for reactive oxygen species detoxification, hereby increasing survival under oxidative stress conditions [110]. Interestingly PGC1α has been reported to co-activate the expression of TFEB, a promoter of the expression of genes in the autophagy pathway as well as genes required for lysosome biogenesis and function [111], suggesting a role for autophagy in the prosurvival activities of the MITF-PGC-1α axis. Indeed Ma *et al* reported that after BRAFV600E inhibition, autophagic flux was increased and therapeutic response was ameliorated when the mice where co-treated with the autophagy inhibitor Lys-05 [112]. Taken together these results support a model whereby melanoma cells displaying high MITF activity depend on oxidative phosphorylation for ATP production and concomitantly become addicted to mitophagy to maintain a functional pool of mitochondria. Another piece of the puzzle was provided by Armstrong *et al*, who reported that BRAFV600E-melanoma cells become resistant to mTORC1-mediated autophagy induction (e.g. via rapamycin, fenretinide or bortezomib), hereby limiting the therapeutic advantage of autophagy inhibition [113, 114].

Taken all together, these data suggest that the outcome of melanoma patients harboring wild type BRAF following anticancer therapy may be ameliorated by the combination with an autophagy blocker. On the other hand, in case of those patients expressing the BRAFV600E mutant, autophagy blockers will only be effective when

combined with a BRAFV600E inhibitor, indicating a prognostic role for BRAF mutant status for the efficacy of autophagy-blocking therapies.

The encouraging preclinical activities support the use of CQ/HCQ as first-generation autophagy blockers in combination with anti-cancer treatments. Importantly, the ability of CQ to inhibit the autophagic removal of damaged cellular materials may not be the only mechanism by which CQ exerts its anti-cancer action, since by blocking lysosomal degradation, this lysosomotropic agent could have anti-cancer effects that are independent on autophagy blockage. Additionally systemic treatment with autophagy blockers not only affects the cancer cells, but also the tumor stroma cells, important players in tumor progression and therapy response. Indeed recent *in vitro* data [115] and our recent *in vivo* data (Maes et al, *under revision*) indicate that the effects of cannot be completely reproduced by silencing key autophagy genes like Atg5 or Atg7 and that CQ also alters the tumor stromal compartment. Our systematic analysis of the effects of the systemic delivery of chloroquine or targeted inhibition of cancer cell-intrinsic autophagy, via shRNA-mediated knockdown of Atg5 in cancer cells, in mouse models of melanoma revealed that CQ, besides inhibiting melanoma-intrinsic autophagy, also 'normalized' the highly disorganized tumor vasculature. CQ increased tumor vessels tightening and perfusion, hereby ameliorating tumor perfusion and reducing tumor cell intravasation and metastasis (Maes et al, *under revision*). Instead, $Atg5^{-/-}$ tumors displayed no vascular changes and increased necrosis. Moreover, in sharp contrast with the vasculo-normalizing effects of CQ, melanomas grown in mice with endothelial cell-specific ablation of Atg5 displayed an even more abnormal vasculature, thus indicating that CQ ameliorates the tumor vasculature through mechanisms that are autophagy-independent. Intriguingly, we found that CQ caused a stimulation of Notch1 signaling, by interfering with its endocytic trafficking, which was found responsible for the vessel normalizing effects observed in CQ-treated tumors (Maes et al, *under revision*). This study thus delineates novel and important assets of CQ in anti-cancer treatment, as this agent appears to be capable of targeting cancer cell-intrinsic prosurvival autophagy, while reinforcing cancer-cell extrinsic barriers, like a more impenetrable endothelial cell barrier and tumor oxygenation, crucial to fend off tumor dissemination and improve chemo/radiotherapy responses.

6. Concluding remarks

Although the molecular and signaling mechanisms of mitophagy in mammalian cells still need to be completely elucidated, selective mitochondrial degradation is emerging as a key process involved in mitochondrial quality control and regulating crucial mitochondrial functions like cellular redox control and bioenergetics. Moreover, accumulating evidence is unraveling the critical role of this autophagy pathway in supporting energy metabolism and growth of cancer cells. Several outstanding questions remain to be answered including how mitochondria are selected for degradation in physiological and pathological conditions, the impact that their removal has for metabolic reprogramming and stress adaptation and how mitochondrial degradation affect the interface between cancer cells and their environment. Certainly these unanswered questions will provide a great incentive to further study the role of key autophagy pathways as mitophagy in dreadful human pathologies, like melanoma.

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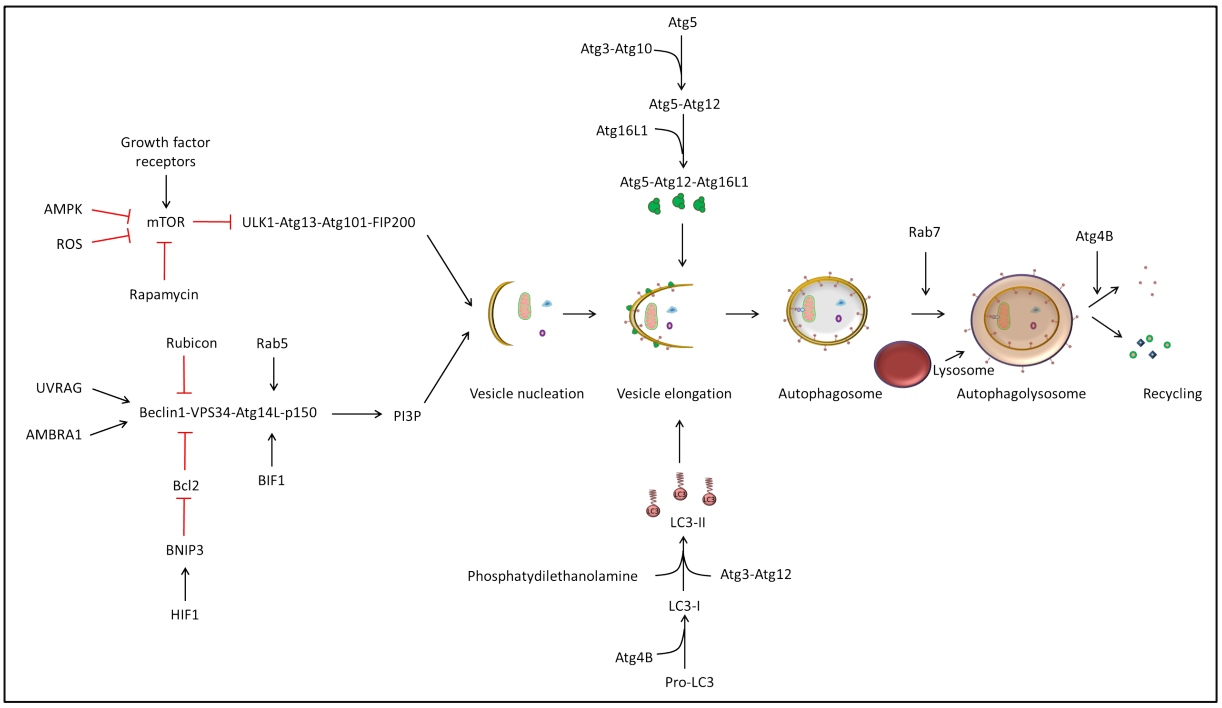


Figure 1: Molecular signaling during autophagy. The figure schematically depicts the different stages of autophagy and their major molecular regulators**. Vesicle nucleation,** is controlled by the ULK1-Atg13-Atg101-FIP200 complex which is negatively regulated by the mTORC1 complex. Upon autophagy activation, mTORC1 dissociates from the ULK complex, leading to ULK1/2 and Atg13 dephosphorylation and translocation of the complex to the autophagosomes formation site. Autophagy activation by reduced cellular energy occurs through the AMP-activated protein kinase (AMPK), which phosphorylates Raptor, hereby inhibiting mTOR. Upon activation, the ULK-complex is involved in the correct localization of the Beclin1-VPS34-Atg14L-P150 complex, whose activity is positively regulated by Bif-1, UVRAG, Rab5 and AMBRA1 or inhibited by Bcl2 and Rubicon. Upon autophagy activation, ULK1 phosphorylates Ambra1, releasing the PI3K3 complex from the cytoskeleton and enabling the relocalization of this complex to the ER. **Vesicle elongation,** is mediated by two-ubiquitin-like conjugation systems. Firstly, Atg5 conjugates to Atg12 assisted by Atg7 and Atg10 (E-1 and E-2 like enzymes, respectively). The Atg5-Atg12 conjugate binds to Atg16L forming the Atg16L complex, which transiently associates to the growing autophagosomes and leaves the autophagosomes after closure. The second ubiquitin-like conjugation system involves LC3 (microtubules-associated light chain-3), which is cleaved by the cysteine protease Atg4 to produce LC3-I. Its glycine residue conjugates to

phosphatidylethanolamine (PE) with the assistance of Atg7, the E2-like enzyme Atg3 and the Atg16L complex to produce the lipidated autophagosomal-localized LC3-II. **Autophagolysosome formation.** Rab7 promotes microtubule transport and fusion of the autophagosomes with the lysosomes. **Recycling.** After autophagolysosome formation LC3-II located at the cytosolic surface of the autophagosolysosome undergoes Atg4-mediated decoupling from PE to be recycled.

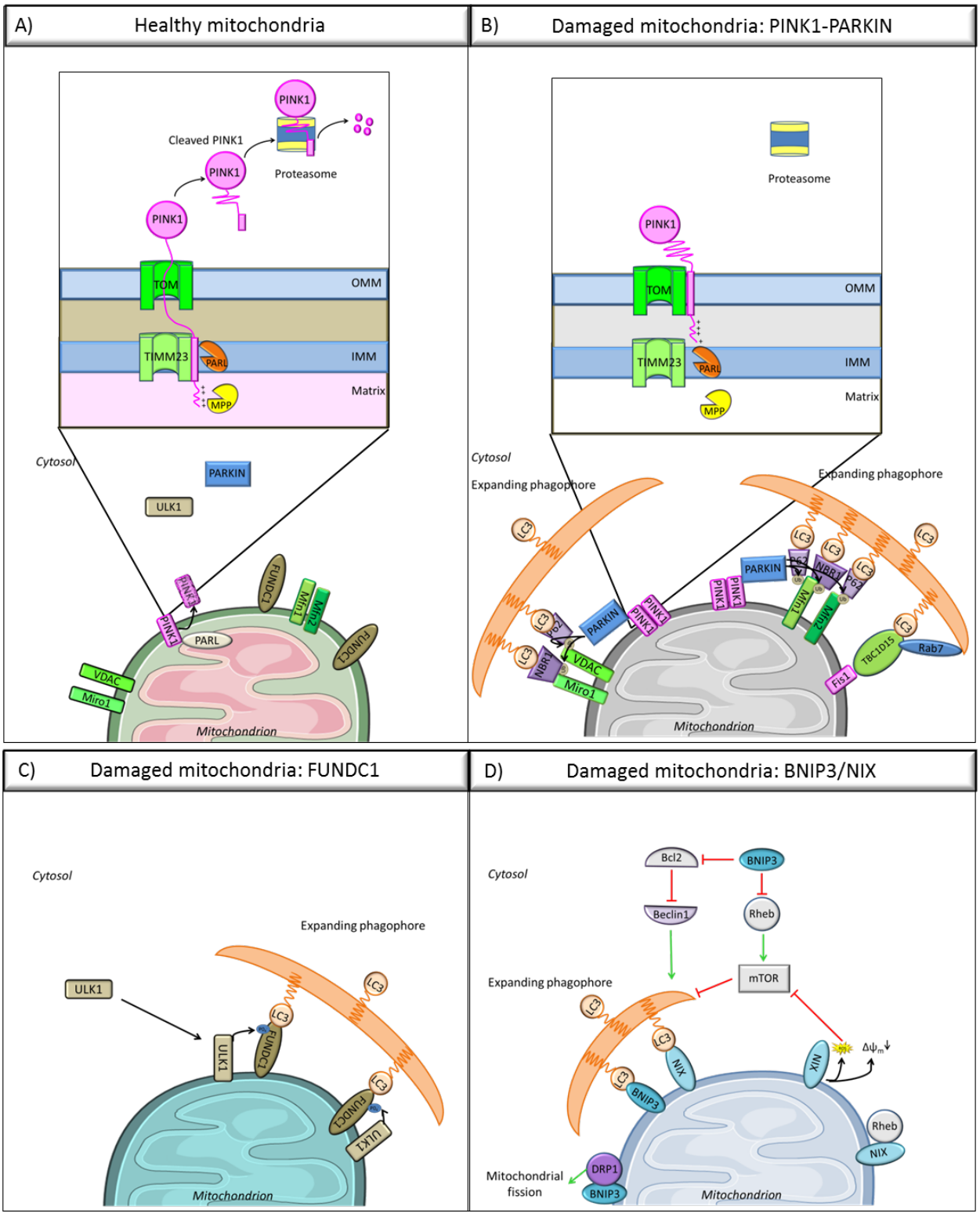
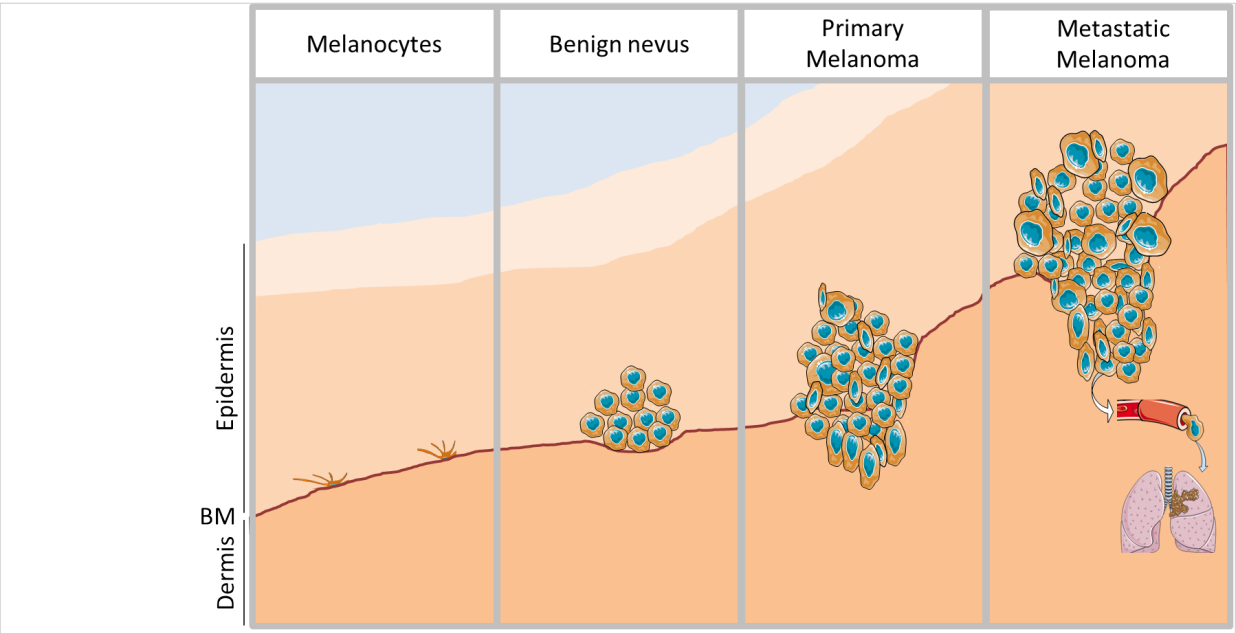


Figure 2. Signaling mechanisms in mitochondria priming.

A) In unstressed, healthy conditions PINK1 is imported into the OMM, IMM and mitochondrial matrix via the TOM and TIM23 complexes. Following import in the mitochondria PINK1 is cleaved by MPPs in the matrix and by the mitochondrial intramembrane protease PARL localized in the IMM, hereby generating an N-terminal

destabilizing amino acid. The cleavage product is subsequently translocated into the cytosol, where it is degraded by the proteasome, thus guaranteeing constant low levels of mitochondrial PINK1 under basal conditions. B) When mitochondrial membrane potential is lost or unfolded proteins accumulate in the mitochondrial matrix PINK1 is no longer imported in the IMM and mitochondrial matrix, but is stabilized on the OMM, resulting in the recruitment of the E3 ubiquitin ligase PARKIN from the cytosol to the damaged mitochondria. Upon recruitment to the mitochondria PARKIN polyubiquitinates several mitochondrial proteins, which recruit ubiquitin-binding LIR-containing adaptors, like p62/SQSTM1 and NBR1, resulting in the incorporation of the damaged mitochondria into the LC3-containing autophagosomes. The size of the autophagosomes is restricted by the RAB GTPases deactivators TBC1D15 and TBC1D17 in concert with Fis1 and LC3 homologue proteins by controlling by coordinating the activity of the small GTPase Rab7. **C)** During hypoxia or mitochondrial depolarization FUNDC1, an OMM protein containing a LIR motif is phosphorylated by ULK1, hereby enabling it as a mitochondrial receptor. **D)** Under hypoxia BNIP3 and NIX are upregulated downstream of HIF-1. BNIP3 induces the autophagic machinery by freeing the pro-autophagic protein Beclin1 from Bcl2 or via direct binding and inhibition of Rheb a key upstream activator of mTOR. BNIP3 also functions mitochondrial receptor as it can associate with the mitochondrial outer membrane and interact with LC3/ LC3 like proteins through its LIR domain. Additionally BNIP3 favors mitophagy by promoting the translocation of DRP1, a fission promoting protein to the mitochondria. Also NIX can induce the autophagic machinery via the disruption of the interaction between Bcl2 and Beclin1 or by increasing mitochondrial ROS production, hereby inhibiting mTOR signaling. In analogy to BNIP3, NIX functions as a LIR-containing mitochondrial receptor. During mitophagy induced by increased oxidative phosphorylation, NIX is essential for the recruitment of Rheb to the mitochondria, hereby preventing the accumulation of damaged mitochondria. For a more detailed description and abbreviations see text.



Autophagy status	basal	↑	↓	↑
Reported alterations in autophagy-modulating proteins		↑ LC3: protein level	↓ Atg5: mRNA and protein level ↓ Beclin 1: mRNA and protein level ↓ LC3: mRNA and protein level	↑ LC3: protein level ↑ Beclin 1: protein level
Oncogenic alterations		BRAF V600E mutation	↑ AKT activation	
Functional consequence of autophagy status	Cellular homeostasis Melanogenesis	Senescence induction	Create a permissive Cellular context for melanomagenesis	Pro-survial mechanism Especially in stressed tumor areas

Figure 3: Autophagy deregulation during melanomagenesis

Abbreviations: BM: basal membrane

For more details and references see text.

Molecule	Mitophagy inducing stimulus	Function	Interaction
PINK	Mitochondrial membrane depolarizers (CCCP) Accumulation of misfolded proteins in the mitochondrial matrix	Recruitment of PARKIN to damaged mitochondria	PARK TOMM PAR
PARKIN	Mitochondrial membrane depolarizers (CCCP, Valinomycin) Accumulation of misfolded proteins in the mitochondrial matrix	Ubiquitination of mitochondrial proteins e.g. VDAC, Mfn1, Mfn2,...	PINK NIX
TBC1D15	Mitochondrial depolarizers (Valinomycin)	Link autophagosomal isolation membrane to mitochondrial cargo size via inhibition of Rab7	LC3/GAB Fis1 TBC1D Rab
TBC1D17	Mitochondrial depolarizers (Valinomycin)	Link autophagosomal isolation membrane to mitochondrial cargo size via inhibition of Rab7	TBC1D
TOMM7	Mitochondrial membrane depolarizers (CCCP)	Stabilization of PINK1 on the outer mitochondrial membrane	PINK
HSPA1L	Mitochondrial membrane depolarizers (CCCP)	Positive regulator of PARKIN translocation to damaged mitochondria	PARK
BAG4	Mitochondrial membrane depolarizers (CCCP)	Negative regulator of PARKIN translocation to damaged mitochondria	PARK
SIAH3	Mitochondrial membrane depolarizers (CCCP)	Mitochondrial protein that inhibits PINK1 accumulation after mitochondrial insult	
PARL	Mitochondrial membrane depolarizers (CCCP)	Mitochondrial protein that cleaves PINK1 under basal conditions	PINK
NIX	Mitochondrial membrane depolarizers (CCCP) Mitochondrial clearance during erythrocyte maturation Hypoxia Differentiation of red blood cells	Increase depolarization and ROS production of mitochondria Induction of autophagic machinery Mitophagic receptor Recruitment of Rheb to mitochondria Recruitment of PARKIN to mitochondria	LC3/GAB
BNIP3	Hypoxia-reoxygenation Mitochondrial membrane depolarizers (CCCP)	Induction of autophagic machinery Recruitment of PARKIN to mitochondria Recruitment of DRP1 to mitochondria Induction of degradation of mitochondrial proteins Mitophagic receptor	LC3/GAB
FUNDC1	Hypoxia Mitochondrial membrane depolarizers (FCCP)	Mitophagic receptor	LC3/GAB ULK
Rab7	Mitochondrial depolarizers (Valinomycin)	Autophagosomal membrane expansion	TBC1D
ULK1	Mitochondrial membrane depolarizers (CCCP) Hypoxia	Phosphorylation of FUNDC1 on the mitochondrial membrane	FUND
KRAB/KAP1-miRNA	Differentiation of red blood cells	Repressing of NIX downregulating hsa-miR-125a-5p	
Smurf1	Mitochondrial membrane depolarizers (CCCP)		
Gp78		E3 ubiquitin ligase	
Atg3	Mitochondrial membrane depolarizers (CCCP)		Atg1
Atg9A	Mitochondrial membrane depolarizers (CCCP)		
MicroRNA-137	Hypoxia	Downregulation of FUNDC1 and NIX under normoxia	