The effect of Autophagy in Regulation of ROS, Mitochondria damage, Aging, Inflammation and Metabolic control and Protects against Genome Instability and Necrosis as a Potential target in Cancer and Autoimmunity Treatment and Prevention

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Abstract

Autophagy is a process of cellular self-degradation in which portions of the cytoplasm are sequestered within cytosolic double-membrane vesicles and delivered to the lysosome/vacuole. autophagy is induced during starvation and hypoxia.

Accumulating data also suggest the involvement of autophagy in aging. For example, the role of various hormones and nutrient sensing pathways in life span extension may involve autophagy.

Autophagy also plays a housekeeping role in removing misfolded or aggregated proteins, clearing damaged organelles, such as mitochondria, endoplasmic reticulum and peroxisomes, as well as eliminating intracellular pathogens.

In addition to elimination of intracellular aggregates and damaged organelles, autophagy promotes cellular senescence and cell surface antigen presentation, protects against genome instability and prevents necrosis, giving it a key role in preventing diseases such as cancer, neuro-degeneration, cardiomyopathy, diabetes, liver disease, autoimmune diseases and infections.

Defective autophagy plays a significant role in human pathologies, including cancer, neuro-degeneration, and infectious diseases.

In this article, I discuss Autophagy, Mitochondria and Autophagic Cell Death, ROS, Mitochondira, Aging, and Autophagy, Metabolic control of mitophagy, Autophagy,

Inflammation and Damaged Mitochondria, Signaling Pathways Regulating Autophagy, Insulin/Growth Factor Pathways and Transcriptional and Epigenetic Regulation of Autophagy

Key Word: Autophagy, Mitochondria, ROS, Aging, Mitophagy, Inflammation, Signaling Pathways Regulating Autophagy, Insulin/Growth Factor Pathways, Cancer, and Autoimmunity

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1. Autophagy

Autophagy, an evolutionarily conserved catabolicpathway in eukaryotic cells, plays an important role instress response and the degradation of damagedcytosolic components(1).There are three generaltypes of autophagy that can deliver intracellular materials into lysosomes for degradation: macroautophagy,microautophagy, and chaperone-mediated autophagy(CMA)(2).CMA is restricted to a subset of proteinsbearing a particular pentapeptide motif(3).Furthermore, CMA involves the translocation ofunfolded substrates across the lysosome membraneand, therefore, is unable to accommodate large proteinaggregates or organelles. Unlike CMA, both micro- andmacroautophagy involve dynamic membrane rearrangements resulting in the ability to enwrap largestructures, such as organelles. Microautophagy refersto the sequestration of cytosolic materials throughdirect invagination of the lysosomal membrane. Incontrast, during macroautophagy, double-membranecytosolic vesicles, termed autophagosomes, sequesterthe cytoplasm and deliver this cargo to the lysosome/vacuole for degradation(2).Although macroautophagy is mainly considered abulk sequestration process, selective autophagy todegrade damaged or excess organelles, such as mitochondria(mitophagy)(4),peroxisomes (micropexophagyand macropexophagy)(5),endoplasmicreticulum (ER; reticulophagy)(4),parts of thenucleus (piecemeal microautophagy of the nucleus)(6), and even ribosomes (ribophagy)(7),also havebeen reported. Most of the autophagy-related pathwaysmentioned above share similar molecular componentsand morphological features. In addition to catabolic degradation, in Saccharomyces cerevisiae, Pichiapastoris, and Candida albicans, a biosyntheticpathway, the cytoplasm to vacuole targeting (Cvt)pathway, also uses the autophagy machinery to deliverthe vacuolar hydrolase aminopeptidase I (2). The autophagy process can be dissected into severalsteps: induction, vesicle nucleation, cargo sequestration,autophagosome expansion and completion,vesicle fusion with the lysosome/vacuole, degradation,and nutrient recycling(8),(9).The identificationof many autophagy-related (ATG) genes providesinsight into the molecular machinery of the autophagyprocess. Among 31 ATG genes that have been identifiedso far, the majority of them function at the step ofautophagosome membrane formation. Depending ontheir function, the Atg proteins can be classified intodifferent groups: The Atg1 protein kinase complex isinvolved in autophagy induction and also acts alongwith Atg2 and Atg18 to regulate the cycling of the integralmembrane protein Atg9; Atg14 is part of a phosphatidylinositol3-kinase complex that is required forvesicle nucleation; the Atg8 and Atg12 ubiquitin-likeconjugation systems are involved in autophagosomeformation, and Atg8 controls the size of the autophagosome; and Atg9, Atg23, and Atg27 are thought to be partof the complex that delivers membrane needed forautophagosome production(8),(10),(11),(12).Owing to the increasing understanding in the biologicalfunctions of autophagy, the significance of thisprocess in diseases, aging, and immunity has beenrecognized(8).Among the different functions ofautophagy that have been studied, growing evidencesupports a role of autophagy in life span extension. For example, mutations that decrease mitogen-responsivepathways, such as in the insulin/insulin-like growthfactor 1 (IGF-1) receptor and target of rapamycin(TOR), as well as a reduction in food intake, are knownto increase life span from yeast to mammals, althoughthe mechanisms of these pathways in aging regulationis not clear(13).Results from various studies indicatethat autophagy may play an important role as a downstreameffector of these life span regulatory pathways.One focus of this review is, therefore, the interactionbetween macroautophagy (referred to as autophagyhereafter) and several distinct longevity regulatorypathways. In addition, mitochondrial respiration andalteration have been linked to aging(14).Mitochondria can regulate autophagy through the generationof ROS. The role of this autophagy induction,however, can serve as an important intervention foreither

mitochondrial turnover to maintain homeostasisor as a signal for autophagy-associated cell death.

2. The basic Autophagy Machinery

As summarized in Figure 1, autophagy begins with an isolation membrane, also known as a phagophore that is likely derived from lipid bilayer contributed by the endoplasmic reticulum (ER) and/or the trans-Golgi and endosomes(15),(16),although the exact origin of the phagophorein mammalian cells is controversial. This phagophore expands to engulf intra-cellular cargo,such as protein aggregates, organelles and ribosomes, thereby sequestering the cargo in adouble-membranedautophagosome(17).The loaded autophagosome matures through fusionwith the lysosome, promoting the degradation of autophagosomal contents by lysosomal acidproteases. Lysosomalpermeases and transporters export amino acids and other by-products ofdegradation back out to the cytoplasm, where they can be re-used for building macromoleculesand for metabolism(17).Thus, autophagy may be thought of as a cellular 'recycling factory'that also promotes energy efficiency through ATP generation and mediates damage control byremoving non-functional proteins and organelles.How is this complex process orchestrated at the molecular level? There are five key stages (a) phagophore formation or nucleation; (b) Atg5–Atg12 conjugation, interactionwith Atg16L and multi-merization at the phagophore; (c) LC3 processing and insertion intothe extending phagophore membrane; (d) capture of random or selective targets fordegradation; and (e) fusion of the autophagosome with the lysosome, followed by proteolyticdegradation by lysosomal proteases of engulfed molecules.

3. Atg5/Atg7-Independent Autophagy

Although Atg5- and Atg7-dependent autophagy has been shown to be critical for survival during the starvation period in the first few days immediately following birth(18),(19),recent evidence has identified an alternative Atg5/Atg7-independent pathway of autophagy(20).This pathway of autophagy was not associated with LC3 processing but appeared to specifically involve autophagosome formation from late endosomes and the *trans*-Golgi(20).Atg7-independent autophagy had been implicated in mitochondrial clearance from reticulocytes (21), and it has consistently been shown that Ulk-1 (a mammalian homologue of Atg1) is required for both reticulocyte clearance of mitochondria(22),and, along with Beclin-1, for Atg5/Atg7-independent autophagy(20).The exact molecular basis of Atg5/Atg7-independent autophagy remains to be elucidated.

4. Autophagic Cell Death

The Greek word autophagy means "self-eating",and indeed this form of cell death is characterized bypathways for the degradation of cytosolic constituentsby the lysosome/vacuole. Autophagy has an essentialrole in differentiation and development, in addition to its role in cellular response to stress. It is activated uponamino acid deprivation and has been associated withneurodegenerative diseases, cancer, pathogen infectionsand myopathies. There are three main autophagicpathways: macroautophagy, microautophagy andchaperone-mediated autophagy (CMA)(23).Macroautophagy involves the sequestration of cytosolic portions, including proteins and organelles,through double-membrane structures,

termed autophagosomes,to the degradation in lysosomes. In microautophagy,cytosolic constituents are engulfed directlyby lysosomes through invaginations of thelysosomal membrane. Both these pathways are conservedfrom yeast to mammals. However, the latterpathway is far less characterized, especially in mammaliansystems. The CMA is found only in mammalian cells and degrades selectively cytosolic proteins thatcontain a specific signal motif, KFERQ. This signal motifis recognized by a specific chaperone, which translocatesthe protein into the lysosome through the interactionwith the Lamp2a receptor(24).Although considered originally nonspecific, 'bulk'degradation pathways, it is now accepted that preferentialautophagy of damaged or excess organelles, suchas peroxisomes, ER and mitochondria, occurs both through micro- and macro-autophagy, under certainconditions. The selective degradation of damaged mitochondriathrough autophagy, termed mitophagy, occursin response to various stimuli, both in yeast and inmammalian cells(25).In mammalian cells, autophagosomes undergo amaturation process by fusing with endocytic compartmentsand lysosomes. Autophagy is active at a basallevel in most cells, and this probably reflects its role inregulating the turnover of long-lived proteins, and gettingrid of damaged structures. In the pathogenesis of various diseases, autophagy has been proposed tohave both beneficial and harmful effects(26).One aspectof this complexity probably reflects the dual role ofautophagy, which is both cell-protective and -destructive. Indeed, controversy exists as to whetherautophagy promotes or prevents cell death. If autophagyremoves damaged mitochondria that would otherwise
activatecaspases and apoptosis, then autophagyshould be protective. In agreement with these item, disruption of autophagic processing and/orlysosomal function promotes caspase-dependent celldeath. In addition, enhanced availability of substratesmay delay cell death upon starvation. However, excessiveand deregulated autophagy may promote celldeath, since enzymes leaking fromlysosomes/autolysosomes, such as cathepsins andother hydrolases, can initiate mitochondrial permeabilization,caspase activation and apoptosis, and in certaininstances deletion of autophagy genes decreasesapoptosis(27).Autophagic cell death is mainly a morphologic definition(i.e. cell death associated with autophagosomes/autolysosomes, autophagic degradationof cytoplasmic structures preceding nuclear collapse),and the specific mechanism is still under debate. Theregulation of autophagy is a very complex process;
together with mTOR (mammalian target of rapamycin),GTPases and a wide range of kinases, Ca2+ is a controllerof autophagy. Indeed, different works(28),(29),suggest that the depletion of Ca2+ pools is responsiblefor the inhibitory effect on autophagy, and that elementsthat modify lysosomal Ca2+ levels, also modifythe total volume of autophagic vacuoles, as well as glycogen degradation. These notions should be revisedin the light of recent work published by Hoyer-Hansenand co-workers(30),that demonstrated that various,related Ca2+ mobilizing stimuli (vitamin D3 compounds,ATP, thapsigargin and ionomycin) inhibit the activity of mTOR and induce massive accumulation of autophagosomesin a Beclin-1 and ATG-dependent manner.The paper concluded that a rise in the free cytosolic[Ca2+] rather than [Ca2+] alterations in other cellularcompartments, is responsible for the induction of autophagy.
This notion is supported by the identification ofcytosolic Ca2+-activated kinase, CaMKK, as an essentialmediator of Ca2+ induced autophagy, and by thefinding that inhibition of autophagy by targeted Bcl-2constructs was related to the effect on agonistinducedCa2+ release from the ER and not to thesteady state of ER [Ca2+] ([Ca2+]ER). The studies inwhich [Ca2+]ER has been measured directly by ERtargetedCa2+-sensitive probes support the view thatBcl-2 mainly acts on [Ca2+]ER by increasing the Ca2+permeability of the ER membrane(31).Bcl-2 might inhibitthe

autophagic response by lowering the amountof free ER Ca2+ available for release. Such a combinedanti- and proautophagy function of Bcl-2 may help tomaintain autophagy at level that is compatible with cellsurvival, rather than death(32).

5. Mitochondria and Autophagic Cell Death

Interesting, while the central role of mitochondria inapoptosis is well established, recent evidence stronglysuggests that also in autophagy this organelles act asintracellular check points. Autophagy was recently establishedas a novel tumor suppression mechanism(33),which stimulated a wave of investigations aimedat understanding its regulatory mechanism and its importancein the development of human cancers. Autophagywas first linked to cancer following the identificationand characterization of the Beclin 1 gene. Beclin 1is a tumor-suppressor gene that is frequently monoallelicallydeleted in human sporadic cancer(9).Beclin 1 co-localizes with Bcl-2 in mitochondria and Bcl-2 inhibitsBeclin 1-dependent autophagy(34).HSpin 1 is atransmembrane protein that is localized primarly in themitochondria. HSpin 1 has been shown to bind Bcl-2and Bcl-xL (but not Bax and Bak) influencing mitochondrial
autophagy in a caspase independent manner(35).Moreover, autophagy is negatively regulated bymTOR, a key signaling protein that integrates signalsfrom nutrients and growth factors. The pathways leadingto mTOR are often altered in human cancer. Thecommon consequence of these alterations is upregulationof mTOR, which in turn inhibits autophagyand contributes to tumorigenesis. mTOR has beenshown to be partly associated with the mitochondrial OM(36),and the functional significance of this observationis currently investigated by many laboratories, includingours. Finally, recent work revealed that autophagyis regulated also by p53 as a result of its activation(37),(38). In light of the finding that in some systems autophagy and apoptosis seem to be interdependent phenomena, molecular switches are likely to link the two types of cell death.

6. ROS, Mitochondira, Aging, and Autophagy

6.1. Mitochondria and aging

Accumulation of mitochondrial DNA (mtDNA) mutations as well as a decline in mitochondrial function is a common feature in aging cells(39).Studies performed in humans reveal a decline of ATP production with age(40).Similarly, some age-related diseases are associated with mitochondrial alterations. For instance, hearing loss in aged animals is associated with mitochondrial dysfunction and mtDNA mutations (41). Mitochondria are the primary source, and a target, of reactive oxygen species (ROS), and the production of ROS is proposed to play an important role in aging(42).The mitochondria theory of aging, a variant of the free radical theory of aging, proposes that ROS production during respiration causes accumula-tion of oxidative damage in mitochondria and mtDNA, which would eventually lead to aging and death(42).Electron leakage at the electron transport chain (ETC) during respiration, through both detoxification enzymes and non-enzymatic reactions, can generate ROS. ROS cause oxidative damage to mtDNA and inhibition of the ETC, resulting in even higher levels of ROS production. This “vicious cycle” in the end leads to cellular function decline and aging(43).According to the mitochondria theory of aging, one would assume that the rate of aging would correlate with the ROS level produced at mitochondria. Additionally, reducing

ROS production or decreasing respiration might retard the aging process. Nevertheless, evidence both supports and contradicts the ROS theory of aging as shown in Table 1 and Table2. Furthermore, respiration rate and ROS production are not proportional to each other(44).Instead, ROS production is determined by the state of the ETC(39).For example, in long-lived fly mutants expressing human uncoupling protein UCP2 in mitochondria of fly neurons, there is increased respiration, enhanced oxidative stress resistance, and decreased ROS production(45).Life span extension is observed in mutants displaying both increased and decreased respiration, and mild increases in ROS are seen in longlived mutant flies and worms with partial disruption of the mitochondrial ETC(46),(47).A class of long-lived mutants, Mit, in C. elegans paradoxically display mitochondrial dysfunction, and most of the affected genes encode components of the ETC(48),(49),(50).The mechanism attributed to the life spanextension has been the subject of intense study. The life span of long-lived respiration-defective nematodes is further increased by a mutation attenuating the insulin/IGF-1 pathway(51).This additive effect on life span extension suggests that the insulin/IGF-1 signaling pathway and mitochondrial respiration machinery function in parallel in life span regulation. Several hypotheses have been proposed to explain the possible metabolic strategies that could account for the long life span. One of the intriguing hypotheses is that autophagy is required for the longevity of Mit mutants (52).In long-lived mitochondrial respiration mutants, atp-3 (ATP-synthase-3), which encodes a subunit of the mitochondrial ATP-synthase(52).and clk-1 (clock abnormal-1), defective in ubiquinone synthesis(53),(50),additional knockdown of autophagy genes, such as unc-51, bec-1, or atg18, suppresses the life span extension(54).This demonstration suggests that the life span regulation that corresponds with the mitochondrial respiration system is mediated by autophagy.

7. Mitochondria and autophagy

Mitochondrial dysfunction leads to changes in membrane potential, ROS production, ATP production, and Ca2+ homeostasis. In response to these changes, cells induce compensatory pathways, including autophagy (52). A selective elimination of damaged mitochondria by autophagy (mitophagy) might be one of the pathways utilized in response to mitochondrial dysfunction. Under nonstarvation conditions, mitophagycan be induced by mitochondrial dysfunction in yeast (25),(55). This preferential degradation of damaged mitochondria before inducing apoptotic cell death is believed to be either a mechanism for mitochondrial quality control and/or an important cytoprotectiveresponse(55).It is suggested that mitochondrial ROS production acts as a redox signal to regulate autophagy(56).Although ROS-dependent oxidative damage is associated with age-related disorders, at low concentration ROS function as signaling molecules in a number of biological processes, such as defense against invasion of microorganisms, and cell growth(57).ROS damage may induce mitochondrial permeability transition (MPT) and provide a signal leading to induction of autophagy(58).Accumulating evidence suggests that the outcomes ofautophagy induction, including those mediated by ROS, can either promote cell survival or may be associated with cell death(59).In the latter case, however, it may be that the autophagic response was insufficient to prevent cell death, which thus occurs with "autophagic features." ROS formation following starvation, rapamycintreatment, or ischemia/reperfusion conditions is essential for autophagy

induction(60),(61),(56).Several studies suggest that ROS act as a positive signal to regulate autophagy. A recent finding shows that ROS are required to induce autophagy under starvation conditions, and treatment with antioxidativeagents abolish the subsequent autophagosome formation (56). Nutrient starvation leads to mitochondrial H2O2 accumulation partially through a class III PtdIns3K. A direct target for oxidation by H2O2 is Atg4, a protease essential for autophagy. Oxidation of this protein leads to inhibition of its deconjugation activity (56), which allows the accumulation of phosphatidylethanolamine- conjugated Atg8 on the autophagosome membrane, an important step in autophagosome formation(62).Based on this observation, a model for the regulation of Atg4 activity is that the H2O2 generated from mitochondria forms a gradient which results in a corresponding gradient of Atg4 activity; Atg4 closer to the mitochondria is less active compared with Atg4 further away in the cytosol, thus regulating the lipidation status of Atg8 and autophagosome formation(63).Additionally, ROS are suggested to act as a signal for mitophagy. In response to rapamycin or NGF deprivation, ROS generation and mitochondrial lipid peroxidation are suggested to induce mitophagy(64),(65).Treatment with the caspase inhibitor zVAD promotes necrotic cell death when induced by lipopolysaccharides or cell death ligands(66),(67).Autophagy is induced in macrophages challenged with lipopolysaccharides when caspase activation is inhibited. ROS production is increased in these macrophages, and the induction of autophagy results in caspase-independent cell death(67).It is possible that, in this event, ROS might be involved in autophagy induction, because superoxide treatment results in cell death with autophagic features in these same cells.In another cell line, murine L929 fibrosarcoma, a similarevent occurs when caspases are inhibited by zVAD(35).Unexpectedly, catalase is selectively degraded by autophagy uponzVAD treatment, leading to ROS accumulation in mitochondria, and ultimately cell death(68).In contrast, a recent finding suggests that autophagy plays a protective role preventing zVADinducedcell death in L929 cells(69).In this study, autophagy induced byrapamycin or serum deprivation protects against z-VAD-induced cell death. In addition to caspase inhibition, zVAD is able to inhibit lysosomalcathepsin and calpain activities, which are required for autophagy in mammalian cells(70).The cell death reported previously might be due to the block of autophagy at a late stage through inhibition of calpainand/or other additional unknown effects of zVAD(69).Mitochondrial dysfunction can be induced by mitochondrial ETC inhibitors. For instance, the ETC complex I and II inhibitors, rotenone and TTFA, induce cell death with autophagic features in transformed/cancer cell lines through ROS production (60).In contrast, the same ETC inhibitors fail to induce ROS generation and cell death with autophagicfeatures in non-transformed primary mouse astrocytes (60), (27), suggesting that induction of autophagic cell death may be a strategy for cancer treatment especially for those tumors where the cells are resistant to apoptosis. Indeed, several antitumor drugs may rely on this approach to kill cancer cells. For instance, an anti-cancer drug currently in phase II clinical trials, FK866, which targets a NAD biosynthesis enzyme to deplete intracellular NAD, is able to induce autophagiccell death in neuroblastoma(71),(72),(73),(74).Additionally, sodium selenite was demonstrated to induce cell death in several types of cancer through cell death with autophagic features, including malignant gliomas, which are resistant to apoptotic therapies.

Sodium selenite can cause mitochondria membrane potential collapse, high superoxide anion generation, and induction of mitophagy(75).This cell death was blocked by overexpression of Cu-ZnSOD or MnSOD but not catalase. The fact that superoxide generator treatment alone is sufficient to induce autophagic cell death in gliomas(75).supports the idea that mitochondria could regulate autophagy through ROS production. The antitumor effect of another chemical, arsenic trioxide (As2O3), has been shown through upregulatingBNIP3 and induced autophagic cell death in malignant glioma cells(76).BNIP3 is a BH3 domaincontainingpro-apoptotic Bcl-2 family protein. Augmentation of the levels of either BNIP3 or another BH3 domain-containing pro-apoptotic protein, Bax, induces autophagic cell death(77).Autophagic cell death induced by overepxression of BNIP3 or Bax is dependent on the MPT and ROS accumulation(77).All together, these data suggest that through ROS generation mitochondria regulate autophagy activity. Autophagy and mitophagy induction may contribute to both cell survival and cell death, however, it is not clear what factors lead to which fate. Understanding the mechanisms that underlie the decision for life or death mediated by autophagy would provide potential therapeutic value in treatments for cancer as well as mitochondrial-associated disease. Finally, it is important to note in some of the above situations that, although we refer to autophagic cell death, it has generally not been determined whether cell death is actually due to autophagy, and if so these situations are generally non-physiological in that they rely on various inhibitors and/or the absence of apoptosis.

8. Mitophagy

Mitochondria are degraded through autophagy and can be detected within double-membrane cytosolic vesicles(78).However, there have been some questions as to whether a selective form of mitochondrial degradation (mitophagy) actually occurs. The evidence for selectivemitophagy comes primarily from yeast. A mitochondrial outer membrane protein, Uth1, and Aup1, a mitochondrial intermembranespace phosphatase related to mammalian PP2C, are apparently required for specific delivery of mitochondria into the yeast lysosome analog, the vacuole(79),(80),(81).Serum withdrawal promotes mitophagy of dysfunctional mitochondria or those with mtDNA mutations but spares the normal organelles in mammalian cells(82).Direct experimental evidence comes from the selective removal of depolarized mitochondria after laser-induced photodamage in living hepatocytes(83).“Giant” mitochondria are commonly observed in aged cells, especially in long-lived post-mitotic cells, such as cardiomyocytes and skeletal muscle fibers (84). Elimination of dysfunctional mitochondria through mitophagy is proposed to retard aging(82).These senescent mitochondria are often enlarged with mtDNA mutations and protein alterations due to oxidative damage accompanied by a lack of turnover. Through fusion and fission, mitochondria can complementthe damaged unit by exchange of content and restore their function, which is thought to be a defense mechanism against mitochondrial dysfunction(85).However, depolarized mitochondria fail to undergo fusion and fission events and are targeted for autophagic elimination(86).Interestingly, inhibition of autophagy results in a lower inner membrane potential, which blocks mitochondrial fusion, thus increasing the giant mitochondria accumulated in rat myoblasts and human fibroblasts(87).These findings imply that mitochondria removal by autophagy is a key mechanism for quality control. What is the reason for the failure in mitochondria turnover by

autophagy in aged cells? One explanation is the age-associated decline in mitophagy(88).The decreasing autophagy activity may provide a cellular environment allowing the accumulation of dysfunctional mitochondria. This idea is supported by the ability of anti-lipolytic agents, which mimic nutrient starvation, to stimulate autophagy, to rescue aging cells from the accumulation of dysfunctional mitochondria in rat liver(89),(90).Additionally, injection of anti-lipolytic agents in mice has an anti-aging effect by decreasing plasma insulin, free fatty acids, and glucose, and increasing autophagic activity in vivo(91).Another possibility is that some dysfunctional mitochondria fail to be recognized by the autophagy machinery and thereby accumulate with age(92).What signals induce mitophagy for mitochondrial turnover? Some evidence suggests that the mitochondrial permeability transition caused by ROS damage initiates mitophagy as discussed above. Along this line, certain mtDNA mutations may make the organelle less prone to mitophagy degradation because the respiration defect leads to a decrease in ROS production and less oxidative damage, which might result in a decrease in the recognition signals for mitophagy(93).The MPT describes the phenomenon of the opening of nonspecific high-conductance permeability transition pores in the mitochondrial inner membrane. It is proposed that ROS cause oxidative damage to mitochondrial membrane proteins or mtDNA mutation resulting in abnormal protein synthesis, which then leads to defects in protein folding, aggregate formation, and the formation of nonspecific aqueous pores(92).(93).The opening of the pores leads to the MPT and may induce autophagy(94).At low concentration, ROS may cause mild oxidativedamage and the MPT, inducing both autophagy andmitophagy and promoting cell survival(94).One example is the starvation-induced mitophagy in rat hepatocytes, which is mediated by the MPT(95).In response to hypoxia, cells repress mitochondrial respiration by mitophagy. When MEFs are cultured under hypoxic conditions, the increasing generation of ROS by the mitochondria is necessary to activate the transcription factor, hypoxia-inducible factor 1 (HIF-1)(96).HIF-1 then induces BNIP3 expression and subsequent mitophagy induction(97).BNIP3 augmentation could increase the free Beclin 1 level for autophagy induction by competing for binding to Bcl-2(98),which inhibits autophagy by directly interacting with Beclin 1(34).Similar phenomena are also observed in lungs and the lung epithelial cells of mice(98).Moreover, mitophagyalso plays an essential role during development. Forexample, the complete elimination of mitochondria by mitophagy is required for the maturation of reticulocytes. A recent study shows that BNIP3L (NIX) is required for selective sequestration of mitochondriaintoautophagosomes(99),(100).Similarly, BNIP3L is also involved in mitophagy during terminal erythroiddifferentiation. BNIP3L knockout mice develop anemia and show mitochondrial retention, and the red blood cells have a shorter life span in vivo(99).Both BNIP3 and BNIP3L are able to induce mitochondrial depolarization, cytochrome c release, and apoptosis when overexpressed(101),(102),(103).However, BNIP3Lmediated mitophagyin reticulocytes may not depend on mitochondrial depolarization since it is independent of Bax or Bak and is not blocked by an MPT inhibitor(99),(100).Mitophagy can be induced when apoptosis is blocked. As mentioned above, in various transformed cells that are apoptosis deficient or upon inhibition of caspase activities, mitophagy induction results in cell death(104),(105).Among those cases, ROS generation plays a role in mitophagy induction. It is proposed that the ROS level can control the balance between life and death(57).Additionally, the extent of MPT resulting from oxidative damage may lead to different cellular responses, including autophagy, apoptosis, and necrosis(25),(93).At low concentration of ROS, the stress level is just enough to lead to MPT; autophagy and mitophagy may be induced as a repair system for damaged mitochondrial removal. As the oxidative damage increases, mitochondria may no longer release factors that

promote survival, and instead molecules that trigger apoptosis may leak out. Last, since the extreme oxidative stress causes severe MPT, neither autophagy nor apoptosis can progress due to ATP depletion. Thus necrosis may take place. Whether autophagy/mitophagy leads to survival or contributes to death probably depends on the severity of mitochondrial damage, the length of ROS exposure, or whether the autophagy machinery is overwhelmed by the organelle damage.

9. Metabolic control of mitophagy

The overall mitochondrial mass within a cell is likely regulated by a balance between biogenesis and degradation. When mitochondria are excessive, or become aged or defective, organelle clearance is thought to occur primarily through autophagy, a process termed mitophagy. The removal of mitochondria can be either random or selective. During bulk autophagy, mitochondrial degradation is included as part of a generalized autophagy program activated by the metabolic state of the cell. In other cases, mitophagy is a culling process that selectively degradesonly defective mitochondria, thereby maintaining the overallhealth of the mitochondrial population. Although there is widespread interest in mitophagy as a potential quality control process for mitochondria, it should be noted that in vivo evidence for the importance of mitophagy in mitochondrial homeostasis remains sparse. In particular, studies on the Pink1/Parkin system (described in the next paragraph) have usually relied on overexpressing Parkin and stressing the cells with an uncoupler. Although such approaches are extremely valuable in dissecting the biochemical pathway, further studies are required to determine the in vivo function of mitophagy. The recent development of a mouse reporter for tracking mitophagyin vivo(106),will be helpful in this regard. The best-studied mitophagy pathway involves *Pink1*and *Parkin*, genes responsible for some cases of familial Parkinson's disease. Pink1, a mitochondrially localized kinase, is normally imported and degraded within the organelle. Because protein import is dependent on mitochondrial membrane potential, depolarization results in accumulation of Pink1 on the outer membrane(107),(108).The accumulated Pink1 phosphorylates numerous proteins, including ubiquitin, to recruit and activate Parkin, an E3 ligase(109).Activated Parkinresults in widespread ubiquitination of mitochondrial outer- membrane proteins, whose degradation by the 26S proteasome (110),(111), is required for targeting of the mitochondrion to autophagic membranes. Because Parkin is selectively enriched on dysfunctional mitochondria, healthy organelles are spared from autophagicdegradation. Pink1 and Parkin have also been implicated in another mitochondrial quality control pathway, distinct from autophagy, in which vesicles bud off from mitochondria and are trafficked to the late endosome and lysosome(112),(113).Mitophagy can be activated under certain cellular stresses. With energy stress, activation of AMPK results in phosphorylation of ULK1 and ULK2, mammalian protein kinases that are orthologues of the autophagy gene ATG1(114).ULK1 and ULK2 promote autophagy, including the degradation of mitochondria. AMPK also inhibits the growth-promoting mTORC pathway, which normally inhibits ULK function. These interlinked mechanisms couple mitophagy to the nutrient status of the cell. Hypoxic conditions are also able to trigger mitophagy via a distinct pathway. Activation of the mitochondrial phosphatase PGAM5 results in dephosphorylation of the mitochondrial autophagy receptor, FUNDC1. The dephosphorylation event promotes the interaction of FUNDC1 with ATG8 (also known as LC3), stimulating formation of the autophagic membrane(115),(116).It is not clear how hypoxia activates PGAM5, and whether this mechanism is selective for individual mitochondria. Finally,

metabolic conditions that promote increased mitochondrial function are also associated with increased mitophagy(117). Under glucose-free (oxidative) conditions, mitochondrial OXPHOS is up-regulated, and bulk mitophagy is also enhanced. The small GTPaseRheb is proposed to be involved, as it partially localizes to the outer mitochondrial membrane under oxidative conditions and interacts with the mitochondrial autophagy receptor, Nix. The relocalizationof Rheb promotes the recruitment of LC3 molecules, thereby enhancing mitophagy. The molecular signals that recruit Rhebare currently unclear. The promotion of mitophagy during oxidative conditions, when mitochondrial function is increased, appears to contrast with previous mitophagy models in which dysfunctional organelles are cleared. However, enhanced respiratory chain activity may promote damage to mitochondria (e.g., through increased production of reactive oxygen species), and it is possible that Rheb is specifically responding to these damaged mitochondria. Alternatively, this mechanism may be in place to increase bulk turnover of the population during conditions of increased functional demand. In either case, theincreased mitophagic flux promotes overall energetic efficiency of the organelle population.

10. Autophagy, Inflammation and Damaged Mitochondria

An emerging concept in recent years tightly associates autophagy with inflammation regulation mechanisms. Autophagy can regulate different aspects of the immune response: it is involved in the degradation of bacteria/virus engulfed by the cell, it regulates Pattern Recognition Receptors (PRRs) and can act as their effector, it can process Antigens for MHC presentation and finally autophagy can regulate inflammasome activation and secretion of different cytokines(118).The first three mechanisms have been extensively described by Deretic (2013)(119),and will be briefly discussed here. Autophagy can be envisaged as a mechanism to clear the cytosol from invading intracellular pathogens, either bacteria or viruses, which are engulfed in autophagic membranes and targeted to lysosomes to be degraded. This process is facilitated by sequestosome 1/p62-like receptors (SLRs) that recognize pathogens and facilitate their encasement in autophagosomes, possessing an LC3 interacting region. Furthermore, autophagy could be an effector for PRRs, degrading targets marked by toll-like receptors (TLRs) or it can help in delivering ligands to TLRs, as for TLR7(119).Antigen processing for MHC II presentation represents another important mechanism regulated by autophagy. Indeed, autophagy is crucial for viral immunosurvelliance and is also inhibited by HIV-1 by upregulatingmTOR in dendritic cells(120),(121).Moreover, autophagy is required for positive selection of naïve T cells in thymus, whereas knocking-out key autophagy proteins results in autoimmune syndromes(122).How autophagy regulates inflammasome activation is currently a subject of numerous studies and a general consensus has not been yet reached. However, two hypotheses are at the moment explored: a first one suggesting that autophagy regulates processing of IL-1b and other pro-inflammatory molecules; the second proposing that autophagy removes damaged mitochondria, thus dampening NALP3 activation. It is possible that those two processes are not mutually exclusive and act in parallel to maintain the inflammatory status in "inactive" condition. Direct regulation of IL-1b processing by inflammasomes has been demonstrated in macrophages and *in vivo* where treatment with rapamycin (autophagy inducer) decreases its secreted and circulating levels(123). Further confirmation came from the work of Shi and co-authors (2012), in which they demonstrate how inflammasome activation induces autophagy and autophagosomes formation containing inflammasomes components such as ASC or absent in melanoma 2 (AIM2), in a self-limiting process. IL-1b does not possess a "canonical" secretory signature and it is translated in the

cytosol, via polyribosomes linked to the cytoskeleton(124),(125),(126).Later it was demonstrated that secretion of IL-1b is dependent on its localization in lysosome-associated vesicles and agents that regulates autophagy can modulate its release(123),(127).These works clearly show the regulation of IL-1b processing by autophagy. Other studies put autophagy upstream of inflammasome activation. Reactive oxygen species (ROS) can activate NALP3 inflammasome and damaged mitochondria are the main source for ROS. Mitophagy, a specialized form of autophagy, constantly removes damaged mitochondria, thus lowering ROS levels. If cells are challenged with 3-methyladenine, a blocker of mitophagy, NALP3 activation is increased and redistributed near mitochondria-ER contact points, working as a sensor for mitochondrial damage(128).Moreover, mitochondrial DNA (mtDNA) can work as NALP3 activation inducer, thus sensing mitochondrial damage and 3-MA could increase NALP3 activation caused by mtDNA(129),(130).These data show a possible pipeline: decreased mitophagy leading to increased ROS and mtDNA in cytosol, leading to increased NALP3 activation. The two mechanisms described above are not necessarily self-exclusive. It is then possible that autophagy machinery collaborates in dampening inflammation and a disturbance in mitochondria homeostasis could lead to exacerbating inflammation. In MKD is it then possible that a disturbance in autophagy mechanisms causes damage in mitochondria, impairing their recycling and thus increasing ROS levels; this, in turn, increases activation of inflammation. This chain of events still remains to be verified; however exploring this mechanism could represent a novel strategy to fight this debilitating disease.

11. Signaling Pathways Regulating Autophagy

The Ras/PKA pathway—The Ras/cAMP-dependent protein kinase A (PKA) signaling pathway plays an important role in glucose sensing from yeast to mammals. Yeast PKA contains a heterotetramer composed of the regulatory subunit Bcy1 and three apparently redundant catalytic subunits Tpk1, Tpk2, and Tpk3. In nutrient-rich conditions, the small GTPases Ras1 and Ras2 are active and enhance cAMP generation by the adenylyl cyclase. Elevated cAMP binds to Bcy1 and releases its inhibitory effect on PKA. Constitutive activation of the Ras/PKA pathway suppresses autophagy induced by TOR inhibition in yeast(131),(132), suggesting that the Ras-PKA pathway downregulates autophagy in parallel with the TORTap42 pathway. Autophagy inhibition by Ras/PKA may be mediated through regulation of Atg1, which is identified as a phosphorylation substrate of PKA(133).In the presence of nutrients, PKA phosphorylation causes Atg1 to be largely cytosolic and dissociated from the PAS, whereas during starvation, Atg1 is dephosphorylated and localized to the PAS. It should be noted that Atg1 may not be the sole PKA target because a hyperactive Ras mutant, Ras2G19V, which constitutively activates PKA signaling, is still able to inhibit autophagy in cells expressing an Atg1 variant lacking the PKA phosphorylation sites. In addition to PKA, the protein kinase Sch9, the closest yeast homolog to the mammalian protein kinase B (PKB)/Akt as well as to the TOR target S6 kinase (S6K), is involved in nutrient sensing(134).Simultaneous inactivation of PKA and Sch9 induces autophagy, which can be further increased by inactivation of TORC1(135),suggesting that autophagy is negatively regulated by at least three parallel pathways in yeast, TORC1, Ras/PKA, and Sch9.Ras/PKA and Sch9 may regulate autophagy at the transcriptional level, as the transcription factors Msn2/4 and the Rim15 kinase are required for autophagic flux induced by inactivation of both PKA and Sch9, but not for that induced by inactivation of TOR(135).

12. Insulin/Growth Factor Pathways

When growth factors are withdrawn from the extracellular milieu, in spite of sufficient nutrients, autophagy is induced and is indispensable for maintaining cellular functions and energy production(136).In higher eukaryotes such as *Drosophila* and mammalian cells, the pathways through which hormones regulate autophagy are different from those of nutrients, but both converge on TOR. Insulin and insulin-like growth factors regulate mTOR through the class I PtdIns3K. Upon insulin binding, autophosphorylation of the insulin receptor on tyrosine residues results in the recruitment and phosphorylation of IRS1 and IRS2 (insulin receptor substrate 1 and 2), which creates a docking scaffold that allows binding of adaptor proteins, including subunits of the class I PtdIns3K such as p85. Generation of PIP3 (phosphatidylinositol (3,4,5)-trisphosphate; Figure 2a, red circles in the membrane) by the class I PtdIns3K increases membrane recruitment of both protein kinase B (PKB)/Akt and its activator PDK1 (phosphoinositide-dependent protein kinase 1), leading to phosphorylation and activation of PKB/Akt by PDK1(137),(138).The 3′-phosphoinositide phosphatase PTEN reverses PIP3 production, decreases the downstream PKB/Akt signaling and thus positively regulates autophagy (3). Activated PKB/Akt promotes phosphorylation of the protein encoded by the TSC2 tumor suppressor gene that is mutated in the tuberous sclerosis complex (TSC) tumorsyndrome. The phosphorylation blocks TSC2 interaction with TSC1 and prevents formation of the TSC1/2 complex(139),which causes Rheb to exist in the active GTP-bound form(140),(141),and allows it to directly bind and activate mTORC1(142).When hormones are absent, mTOR is inactivated, which releases the inhibitory effect on autophagy. Besides TOR, Ras signaling also plays a role in autophagy regulation by growth factors. Ras transduces signals from growth factor receptor tyrosine kinases to intracellular effectors, such as Raf-1/MAP (mitogen-activated protein) kinases and the class I PtdIns3K. In NIH3T3 mouse embryonic fibroblasts (MEFs), activated Ras suppresses autophagy through the class I PtdIns3K, but not through Raf-1(143).In contrast to PtdIns3K, the Ras effector Raf-1 is an amino acid sensor and positively regulates autophagy in HT-29 human colon cancer cells (144).In this situation, amino acids target and inhibit the activity of the Raf-1 kinase, which downregulates the downstream MEK1/2 [MAPK(mitogen-activated protein kinase)/ERK kinase 1/2] and ERK1/2 (extracellular signal-regulated kinase 1/2) kinases and autophagy activity. Amino acid deprivation reverses this inhibition and induces ERK1/2 and autophagy. As a consequence, two downstream effector cascades of Ras, the Ras-PtdIns3K and Ras-Raf-1- ERK1/2 pathways, are likely to oppose each other in autophagy regulation through signaling in response to growth factors versus the absence of amino acids. It is also possible that the genetic differences between normal and cancer cell lines determine how Ras signaling controls autophagy.

13. Transcriptional and Epigenetic Regulation of Autophagy

13.1. Transcription

Autophagy genes are regulated at a transcriptional level in response to stress. For example, under starvation conditions, transcription of the autophagosome marker Atg8/LC3 is rapidly

upregulated in yeast and certain mammalian cells. However, not much is known about the underlying transcriptional machinery. FoxO (Forkhead box transcription factor class O) is the first transcription factor that is shown to be necessary and sufficient to induce autophagy in the *Drosophila* larval fat body(145).For mammalian cells, discovery of a transcription dependent mechanism via FoxO3 comes from studies on protein degradation during muscle atrophy(146),(147).FoxO3 induces the transcription of multiple autophagy genes, includin. FoxO3 directly binds to the promoters of *LC3B, Gabarapl1, atg12, Bnip3l*, and *Bnip3* to activate gene transcription. Constitutively active FoxO3 is sufficient to induce autophagosome formation in adult mouse skeletal muscle, which promotes lysosomal proteolysis and leads to muscle wasting. Importantly, FoxO3 functions in parallel to the mTOR pathway, whilst both pathways are downstream of IGF-1/insulin-PtdIns3K-PKB/Akt signaling. Thus, autophagy is regulated by two different mechanisms: nontranscriptional inhibition by mTOR and transcriptiondependentupregulation through FoxO3. Nevertheless, transcriptional mechanisms that physiologically regulate expression of autophagy genes in tissues other than myotubes have not been characterized. Autophagy induction inversely correlates with active protein synthesis. In the presence of abundant nutrients, proteins are synthesized and autophagy suppressed, whereas in response to stress stimuli, cells trigger translational arrest and autophagy induction. Recent studies suggest that translational initiation factors, for example, eIF4GI, that specifically activate translation of genes controlling cell growth and proliferation, block the induction of autophagy downstream of TOR(148),whereas signaling pathways that induce translational arrest, such as the eEF-2 (eukaryotic elongation factor-2) kinase and the eIF2α kinase signaling pathway, positively regulate autophagy(149),(150).Phosphorylation of eIF2α at Ser51 by the conserved eIF2α protein kinase family is essential for the global translational arrest and selective upregulation in the translation of transcriptional activators (such as GCN4) that stimulate transcription of starvation-induced genes. Yeast has one eIF2α kinase, Gcn2 (general control nonderepressible-2), which is negatively regulated by Tor (74), whereas mammals have four known eIF2α kinases, GCN2, PKR, PERK, and HRI (heme-regulated inhibitor), activated by amino acid starvation, viral infection, ER stress, and heme deprivation, respectively 3a). In both yeast and MEFs, eIF2α phosphorylation by GCN2 is required for starvationinduced autophagy, and in MEFs, PKR is required for autophagy induced by viral infection(149).The downstream effect of eIF2α phosphorylation on autophagy is likely to be transcriptional, as transcription of Atg12 is upregulated by phosphorylated eIF2α in polyQ72- loaded mammalian cells(151).In yeast, selective translation of Gcn4 (rather than the global translational arrest) by eIF2α phosphorylation is essential for autophagy, consistent with a report identifying several autophagy genes, including ATG1, ATG13, and APE1, as downstream transcription activation targets of Gcn4(152).However, the study is largely based on microarray analysis, and independent assays are needed to test the functions of transcriptional activators in yeast autophagy.

14. Conclusion

Autophagy is a major mechanism for the removal of damaged cellular structures and is critical to maintain cell survival. As demonstrated by various studies, autophagy genes are important for long-term viability.Mitochondria play an important role in aging and regulate autophagy through ROS generation and the MPT (131). Autophagy induction signaled from mitochondria can result in enhanced survival or may possibly contribute to cell death.However, the

significance of defects in autophagy for disease and ageing is apparentfrom growing evidence linking mutation or loss of function of key autophagy genes in cancer,neuropathies, heart disease, auto-immune disease and other conditions.The coordination of Atg proteins with other subcellular components, including the cytoskeleton, the secretory pathway, oncogenes and tumor suppressors, and immunoproteins, is crucial for correct autophagy induction. It is clear, however, that the organelle is responsive to numerous types of metabolic stimuli, and this likely has resulting effects on the health and function of the organelle population.

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