Supporting Online Material for

Mitochondria and the Autophagy–Inflammation–Cell Death Axis in Organismal Aging

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Supplemental Discussion.

Mitochondrial turnover. Individual mitochondrial proteins can be subjected to turnover by proteolytic cleavage. Indeed, mitochondria contain proteases (including the lon peptidase 1, LONP1 and other AAA proteases, as well as a matrix-located proteasome) that may degrade misfolded or oxidized proteins. Most proteins in mitochondria are organized in polyprotein assemblies, called supercomplexes or respirasomes, which may exclude misfolded proteins (*1*). However, it is unknown whether such supercomplexes undergo reversible assembly and disassembly or whether they must be turned over as a whole when one single component is oxidized.

Although some turnover of outer mitochondrial membrane proteins may involve extramitochondrial proteasomes (*2*), most of the mitochondrial turnover is mediated by autophagy. According to one plausible scenario, mitochondria may undergo fission in an asymmetric fashion to generate one functional organelle and another dysfunctional one (with a dissipated inner transmembrane potential). Only the latter one would then be destroyed by mitophagy (*3*). The mechanisms of mitophagy have been discussed in the main text.

The stimulation of general autophagy (for instance by rapamycin or caloric restriction) can reduce mitochondrial alterations in animal models of neurodegeneration (*4-6*), in line with the idea that even general autophagy (as opposed to mitophagy) may lead to the specific elimination of aberrant mitochondria. It is possible that general autophagy is not completely unselective and may preferentially degrade proteins and structures that are on the "verge" of aggregation or damage (*7*). This concept, which remains to be proven, predicts that an increase in general autophagy would reset the threshold of quality control so that organelles that exhibit only minor alterations (and that normally would not be removed by autophagy)

are subject to autophagic turnover. Theoretically, this concept may explain why induction of general autophagy may "purge" cells of dysfunctional mitochondria.

Involvement of the mitochondrial permeability transition in mitophagy. The term mitochondrial permeability transition (MPT) is commonly used to indicate an abrupt increase in the permeability of the inner mitochondrial membrane to ions and small solutes that is near-to-simultaneously followed by the dissipation of the mitochondrial transmembrane potential ($\Delta\psi_m$), and hence by the cessation of all $\Delta\psi_m$-dependent mitochondrial functions like ATP synthesis, as well as by a massive, osmotically-driven entry of water into the mitochondrial matrix. In turn, this leads to the osmotic swelling of the mitochondrial matrix and, eventually, to mitochondrial outer membrane permeabilization (MOMP) (*8, 9*). MPT is mediated by a supramolecular protein complex that is assembled at the junctions between the inner and outer mitochondrial membranes, the co-called "permeability transition pore complex" (PTPC). In healthy cells, the components of the PTPC reportedly mediate the exchange of small metabolites between the cytosol and the mitochondrial matrix. Conversely, in response to a wide array of lethal stimuli including the overgeneration of reactive oxygen species (ROS) and cytosolic Ca^{2+} overload, the PTPC adopt a high-conductance conformation that provokes MPT (*8, 9*). The exact molecular composition of the PTPC remains elusive, but it appears that its scaffold structure includes proteins that are embedded in the outer mitochondrial membrane, such as the voltage-dependent anion channel (VDAC), inner membrane proteins, like the adenine nucleotide translocase (ANT), as well as mitochondrial matrix proteins, such as cyclophilin D (CYPD) (*8, 9*). Moreover, it has been suggested that PTPC functions are modulated by a large number of mitochondrial and cytosolic interactors, including both pro- and anti-apoptotic

members of the BCL-2 protein family (see below) (*10-12*). Recent genetic manipulations have demonstrated that the most critical component of the PTPC would be CYPD, as only the knockout of the CYPD-encoding gene *Ppif* (*13-15*), but not that of the genes coding for all known VDAC and ANT isoforms (*16, 17*), consistently prevents MPT and exerts cytoprotective effects *in vivo*, in murine models of ischemic disease (but notably, not apoptosis).

Autophagy has long been suggested to mediate the removal of old, damaged or ectopic mitochondria, but the first experimental confirmation of this hypothesis lagged until it was elegantly demonstrated that laser-induced photo-damage (leading to immediate MPT) of selected mitochondria inside living hepatocytes result in their quick removal by the autophagic machinery (*18, 19*). Similarly, it was shown that nutrient deprivation stimulates mitophagy, together with general autophagy, by increasing the incidence of spontaneous MPT by 5-fold, up to a rate of approximately 1% mitochondria/hour (*18, 19*). Both these instances of mitophagy could be suppressed by the CYPD and MPT inhibitor cyclosporin A (CsA) (*18, 19*). There are several additional examples in which CsA inhibits mitophagy. This applies to rat hepatocytes cultured in the absence of serum and the presence of glucagon (to mimic starvation) (*20*), the livers from mice expressing mutant α1-antitrypsin (*21*), primary fibroblasts from coenzyme Q-deficient patients (*22*), nicotinamide-treated fibroblasts (*23*), heat-shocked human cancer cells (*24*), and nutrient-starved HL-1, neonatal rat or adult mouse cardiomyocytes (*25*). In starving rat hepatocytes, it has been shown that a non-immunosuppressive CsA analogue, N-methyl-4-isoleucine cyclosporine (NIM811), also inhibits mitochondrial depolarization and mitophagy (*19*). Moreover, starvation failed to induce autophagy in cardiomyocytes from *Ppif*$^{-/-}$ mice (*25*). Altogether these results suggest that MPT can induce changes in mitochondria that ultimately lead to their autophagic destruction. Although this has not been formally proven, it is tempting to speculate that the

MPT-driven $\Delta\psi_m$ dissipation suffices to stimulate mitophagy.

As a caveat, it should be noted that animals lacking CYPD (and MPT) (*13-15*) do not display the perinatal lethality associated with defects in autophagy, nor the degenerative effects of tissue-specific deletion of autophagy genes, and therefore it is unlikely that these animals have a general defect in autophagy. Further, CsA is not a universal inhibitor of autophagy, and there are examples showing that CsA can induce general autophagy, for instance in rat pancreatic acinar cells *in vivo* (*26*), in primary human renal tubular cells *in vitro* (*27*), and in rat kidneys *in vivo* (*27*), as well as in the muscle of mice deficient for collagen VI (*28*). The mechanisms through which CsA may induce autophagy have not been studied in detail, but it is possible that they involve the endoplasmic reticulum stress response (*27*).

Impact of Bcl-2 proteins on mitochondria and autophagy. Members of the BCL-2 protein family can be classified into three main groups based on structural and functional information. First, multidomain anti-apoptotic BCL-2 proteins, such as BCL-2, BCL-X_L and MCL-1, include BCL-2 homology (BH) motifs 1 to 4 as well as a transmembrane domain that mediate their insertion into intracellular membranes. Second, two multidomain pro-apoptotic BCL-2 proteins, BAX and BAK, are characterized by BH1 to 3 domains but are devoid of a TM motif. Third, BH3-only proteins, including BID, BIM and BBC3/PUMA, in most cases solely contain a BH3 domain. BH3-only proteins have been shown to operate as sensors that relay lethal signals to mitochondria by directly activating or de-repressing the pore-forming function of BAX and BAK, the two major effectors of MOMP (*29*). On the other hand, BCL-2, BCL-X_L and MCL-1 exert cytoprotective effects by sequestering their

pro-apoptotic counterparts into inactive complexes (*30, 31*) as well as by other functions (*e.g.*, by modulating Ca^{2+} fluxes at the ER) (*32*).

The discovery that the essential autophagic modulator Beclin 1 contains a conserved BH3 domain that is both necessary and sufficient for its interaction with BCL-2 and BCL-X_L (*33-37*), suggested that BCL-2 proteins also regulate autophagy. Since then, several lines of evidence have accumulated in support of this hypothesis. Overexpression of BH3-only proteins can disrupt the inhibitory interaction between Beclin 1 and BCL-2/BCL-X_L, as this has been reported for BAD (*34, 38*) and truncated BID (tBID) (*34*). Some BH3 only proteins, such as NIX and BNIP3, have been specifically associated with the induction of mitophagy (*39, 40*). Importantly, induction of autophagy by NIX and BNIP also occurs in apoptosis-incompetent BAX- and BAK-deficient cells (*41, 42*), suggesting that the pro-autophagic and pro-apoptotic functions of BH3-only proteins are disconnected from each other. The pro-autophagic signaling kinase death-associated protein kinase (DAPK) has been shown to phosphorylate Beclin 1 at Thr119, a critical residue within its BH3, hence favoring the dissociation of Beclin 1 from inhibitory interaction with BCL-2/BCL-X_L (*43*). Thus, BCL-2 proteins constitute a complex network that operates at the crossroad between cell death and autophagy regulation (*31*).

Pharmacological agents that mimic the action of BH3-only proteins, so called BH3 mimetics (which in general occupy the BH3-binding groove of anti-apoptotic proteins such as BCL-2, BCL-X_L or MCL-1, though with differential specificities), efficiently induce autophagy. In the case of ABT737, this effect has been attributed to the competitive disruption of the inhibitory interaction between Beclin 1 and BCL-2/BCL-X_L (*38*), and this has been confirmed for another BH3 mimetic, gossypol (*44*). In contrast, Obatoclax, a broad-spectrum BH3 mimetic, has been reported to induce LC3 lipidation (which constitutes a sign

of autophagy) in a Beclin 1-independent fashion, although this phenomenon was not accompanied by ultrastructural signs of autophagy (*45*), underscoring the possibility that such agents have Beclin 1-independent (off-target?) effects. ABT737 can restore mitophagy in NIX-deficient erythroblasts (*39*), and peptides corresponding to the BH3 domains of BNIP3 and NIX (but not mutated peptides) are sufficient to promote a modest but reproducible autophagic response (*46*). The data by Bellot *et al.* should be interpreted with caution, because several studies have demonstrated that the transmembrane domains of both NIX and BNIP3 are essential for at least their pro-apoptotic function (*47, 48*). Nonetheless, altogether these observations suggest that BNIP3- and NIX-mediated autophagy involves its BH3 domain. In strict contrast, it appears that the mutation of the BH3 domain of NIX does not affect its capacity to target depolarized mitochondria for autophagic destruction. However, this capacity is lost when the C-terminal mitochondrial localization domain of NIX is deleted or when a domain interacting with LC3-like protein (such as LC3 or its homolog GABARAP) is mutated (*49-51*). BNIP3 has been show to induce MPT, and it has been thought that this effect would contribute to BNIP3-induced autophagy (*52*). However, a recent report affirms that BNIP3-stimulated autophagy can be inhibited neither by the knockout of CYPD nor by its pharmacological inhibition with CsA (*53*).

Altogether, the available data suggest that in some (but not all) cases autophagy can be stimulated by BH3-only proteins (and indeed *Caenorhabditis elegans* lacking its unique BH3-only protein, Egl1, are autophagy incompetent) (*38*) and that BH3-only proteins can disrupt inhibitory interactions between Beclin 1 and BCL-2/BCL-X_L, thus allowing Beclin 1 to activate the lipid kinase HVPS34 and engage autophagy. Although Beclin 1 possesses a BH3 domain, it fails to suppress the anti-apoptotic function of BCL-2(*54*), perhaps because the interaction between Beclin 1 and BCL-2 has a particularly low affinity.

Reduced autophagy in aging. Multiple reports indicate that in multiple tissues autophagy decreases with age (*55-58*), although systematic studies on autophagy competence in aging tissues are elusive. There are multiple possible reasons for the age-associated decline in autophagy and mitophagy. First, the expression of essential proteins required for autophagy may decline with advancing age due to reduced transcription of *ATG5*, *ATG7* and *BECN1* (the gene that encodes Beclin 1) and other autophagy-related genes in the brain (*59, 60*). With increasing age, the lysosomal activity decreases and autophagy becomes inefficient due to the accumulation of lipofuscin, a brown granular pigment that consists of crosslinked proteins and lipids resulting from incomplete lysosomal digestion (*61*). It has also been proposed that the reduction of proteins involved in mitochondrial fission (*62*) might explain reduced mitochondrial clearance by autophagy (*63, 64*). These changes, as well as deficient autophagy, may explain the accumulation of enlarged (often referred to as 'giant') or highly interconnected mitochondria in aging cells (*65-67*). While the literature on age-associated failing autophagy is scarce, there is clear evidence that inhibiting autophagy reduces lifespan while inducing autophagy by genetic or pharmacological manipulations can increase longevity in multiple model organisms including yeast, nematodes, flies and mice (**Supplemental Table 1**).

Autophagy, mitophagy and cancer. A link between defects in autophagy and cancer has been recognized and speculated upon since the realization that Beclin 1, which is often lost in breast cancer, is an essential component of the initiation complex for autophagy (as discussed in the main text) (*68*). Mice heterozygous for the *Becn1* null allele display

spontaneous carcinogenesis (*69, 70*), leading to the idea that autophagy functions as a tumor suppressor mechanism.

It nevertheless remained possible that Beclin 1 exerts oncosuppressive functions independent of autophagy. However, more recent studies have shown that cells heterozygous for *BECN1* display a genomic instability that is also observed in transformed epithelial cells lacking the essential autophagy component ATG5 (*71*), and such deficiencies also promote transformation in a breast cancer model (*72*). Such genomic instability is associated with accumulation of damaged mitochondria and protein aggregates, generation of ROS, and DNA damage, and can be reduced by ROS inhibitors (*71, 73*). These observations support the view that defects in autophagy can promote the accumulation of damaged mitochondria, which in turn generate ROS and cause DNA damage, inciting mutagenesis that can lead to cellular transformation.

This idea is further supported by the observation that *PARK2*, the gene that encodes Parkin, is frequently deleted in colorectal carcinoma, especially in the form that is associated with adenomatous polyposis coli (*APC*) deficiency (*74, 75*). *Park2*$^{+/-}$ mice crossed to APC^{min} mice show dramatically accelerated intestinal adenoma development (*75*). Mutations or deletions in *PARK2* have also been described in hepatocellular carcinoma (*76*), glioblastoma (*77*), ovarian (*78*) and lung cancer (*79*). Since Parkin is involved in at least one mechanism of mitophagy (see main text), it is tempting to draw a connection between defective mitophagy and oncogenesis.

There are alternatives (or, at least, more complex scenarios). Defects in mitophagy or autophagy, as we have seen, can promote inflammation, which in turn contributes to tumorigenesis. Indeed, a genetic polymorphism in the autophagic gene *ATG16L* is associated with Crohn's disease (*80*), an inflammatory condition that can predispose for

colon carcinoma. Similarly, mice lacking an allele of *Becn1* (*69*) or with a liver-specific knockout of *Atg7* (*81*) display liver damage and/or hepatosteatosis, in turn favoring inflammation and the development of hepatocellular carcinoma. Thus, the links that we have drawn between autophagy, mitochondria, inflammation, and cell death in aging may also impact on cancer risk.

Supplemental Table 1: Examples of autophagy-dependent lifespan-prolonging and anti-aging effects in animals.

Manipulation	Phenotype	Relationship to autophagy	Ref.
Administration of rapamycin to *Caenorhabditis elegans*	Increased lifespan.	Loss-of-function mutations of *atg-1* or *atg-7* abolish lifespan extension.	(*82*)
Administration of rapamycin to *Drosophila melanogaster*	Increased lifespan.	Loss of the longevity phenotype upon *atg-5* RNAi.	(*83*)
Administration of rapamycin to mice.	Extension of maximum lifespan by up to 14% in males and females accompanied by MTOR inhibition.	The cause-effect relationship between autophagy and lifespan extension remains elusive.	(*84*)
Administration of resveratrol to *C. elegans*	Increased lifespan.	Loss of the longevity phenotype upon *bec-1* RNAi.	(*85*)
Administration of spermidine to *C. elegans*	Increased lifespan.	Loss of the longevity phenotype upon *bec-1* RNAi.	(*86*)
Administration of spermidine to *D. melanogaster*	Increased lifespan.	Loss-of-function mutations of *atg-7* abolish lifespan extension.	(*85*)
daf-2 (Insulin/IGF-1 receptor) loss-of-function mutation in *C. elegans*	Increased lifespan.	Loss-of-function mutations of *bec-1* abolish lifespan extension.	(*87*)
eat-2 (ad1113) dietary restriction mutation in *C. elegans*	Increased lifespan.	Loss of the longevity phenotype upon *bec-1* and *atg-7* RNAi.	(*88*)
Knockin mutation of Huntingtin, causing the deletion of the polyglutamine stretch ($Htg^{\Delta Q/\Delta Q}$), in mice.	Increased lifespan.	The cause-effect relationship between autophagy and lif span extension remains elusive. The $Htg^{\Delta Q}$ protein may induce autophagy *in vitro*.	(*89*)
Transgenic expression of Atg8a in the brain of *D. melanogaster*	Counteracts the age-associated loss of Atg8a. Increased lifespan (up to 56% in females), reduced accumulation of insoluble ubiquitinylated and carbonylated proteins, increased resistance against H_2O_2.	Direct increase in neuronal autophagy.	(*90*)
Transgenic expression of LAMP2 by means of an inducible, hepatocyte-specific construct in mice.	Reduced abundance of oxidized proteins, polyubiquitinylated protein aggregates and $TUNEL^+$ cells.	Restoration of chaperone-mediated autophagy and macroautophagy in the liver of aged animals.	(*91*)
Transgenic overexpression of sirtuin 1 in *C. elegans*	Increased lifespan.	Loss of the longevity phenotype upon *bec-1* RNAi.	(*92*)

Abbreviations: LAMP2, lysosomal-associated membrane protein 2; MTOR, mechanistic target or rapamycin; TUNEL, terminal deoxynucleotidyl transferase (TDT)-mediated dUTP nick-end labeling.

Supplemental Table 2: Examples of mitochondrial and autophagy-related perturbations that have been associated with neurodegenerative diseases.

Disease	Defects		Ref.
Alzheimer disease	Autophagic defects	Presenilin-1 (whose loss-of-function mutation can cause hereditary Alzheimer disease) acts as a chaperone for one the subunits of the lysosomal proton pump, meaning that Alzheimer-related Presenilin-1 mutations result in defective lysosomal acidification and a consequent block in autophagosome clearance that reflects an increase in autophagic vacuolization.	(*93*)
		Tau, a tubulin-interacting protein that is involved in Alzheimer disease, functions as an inhibitor of HDAC6, resulting in the inhibition of quality control-related autophagy.	(*94*)
		The expression of Beclin 1 is decreased in affected brain regions of patients with Alzheimer disease early in the development of the pathology.	(*95*)
	Mitochondrial defects	β-amyloid impairs OXPHOS, in particular at the level of respiratory complex IV.	(*96*)
		Mitochondrial fission and fusion are impaired in Alzheimer disease.	(*97*)
		Presenilins, which when mutated cause familial Alzheimer disease, are highly enriched in ER MAMs.	(*98*)
Huntington disease	Autophagic defects	Age at onset is influenced by the V471A polymorphism in *ATG7*.	(*99*)
		Mutant Huntingtin (the single etiological determinant of Huntington disease) aggregates are in part degraded by and hence overwhelm the autophagic machinery.	(*100*)
		Mutant Huntingtin interacts with Beclin 1 and impairs the Beclin 1-mediated turnover of long-lived proteins.	(*59*)
		Transgenic expression of mutant Huntingtin in mice results in deficient sequestration of autophagic cargo.	(*101*)
	Mitochondrial defects	In mice, a Huntington-like syndrome can be induced by the administration of the mitochondrial toxin 3-NPA.	(*102*)
		Mutant huntingtin perturbs mitochondrial functions at multiple levels, including organelle trafficking, fission and fusion.	(*103*)
Parkinson disease	Autophagic defects	Overexpression of α-synuclein, the main component of Lewy bodies, impairs macroautophagy.	(*104*)
		DJ-1, loss-of-function mutations of which cause autosomal recessive parkinsonism, works in parallel to the PINK1/Parkin pathway to activate autophagy maintain mitochondrial function in the presence of an oxidative environment.	(*105, 106*)
		Parkin loss-of-function mutations cause autosomal recessive parkinsonism. Such mutations compromise its recruitment to mitochondria or it capacity to ubiquitinylate mitochondrial outer membrane proteins, and hence to mitophagy.	(*107, 108*)
		PINK1, loss-of-function mutations of which cause autosomal recessive parkinsonism, is required for the recruitment of Parkin to mitochondria. Moreover, PINK interacts with Beclin 1 and promotes autophagy.	(*109, 110*)

Mitochondrial defects	In mice and primates, a Parkinson-like syndrome can be induced by the administration of the mitochondrial toxins MPTP and rotenone.	(*102*)

Abbreviations: 3-NPA, 3-nitropropionic acid; ER, endoplasmic reticulum; HDCA6, histone deacetylase 6; LAMP2, lysosomal-associated membrane protein 2; MAMs, mitochondria-associated membranes; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MTOR, mechanistic target of rapamycin; OXPHOS, oxidative phosphorylation.

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