Chemistry & Biology
Article

Mitochondrial Electron Transport Chain Complex III Is Required for Antimycin A to Inhibit Autophagy

Xiuquan Ma,[1,4] Mingzhi Jin,[1,4] Yu Cai,[1] Hongguang Xia,[1] Kai Long,[1] Junli Liu,[1] Qiang Yu,[2,*] and Junying Yuan[1,3,*]
[1]State Key Laboratory of Bioorganic and Natural Products Chemistry, Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences, 345 Ling-ling Road, Shanghai 200032, China
[2]Department of Pharmacology, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 555 Zuchongzhi Road, Shanghai 201203, China
[3]Department of Cell Biology, Harvard Medical School, 240 Longwood Avenue, Boston, MA 02115, USA
[4]These authors contributed equally to this work
*Correspondence: qyu@sibs.ac.cn (Q.Y.), junying_yuan@hms.harvard.edu (J.Y.)
DOI 10.1016/j.chembiol.2011.08.009

SUMMARY

Autophagy is a cellular lysosome-dependent catabolic mechanism mediating the turnover of intracellular organelles and long-lived proteins. We show that antimycin A, a known inhibitor of mETC complex III, can inhibit autophagy. A structural and functional study shows that four close analogs of antimycin A that have no effect on mitochondria inhibition also do not inhibit autophagy, whereas myxothiazol, another mETC complex III inhibitor with unrelated structure to antimycin A, inhibits autophagy. Additionally, antimycin A and myxothiazol cannot inhibit autophagy in mtDNA-depleted H4 and mtDNA-depleted HeLa cells. These data suggest that antimycin A inhibits autophagy through its inhibitory activity on mETC complex III. Our data suggest that mETC complex III may have a role in mediating autophagy induction.

INTRODUCTION

Autophagy is a cellular lysosome-dependent catabolic mechanism mediating the turnover of intracellular organelles and long-lived proteins (Baehrecke, 2005; Codogno and Meijer, 2005; Klionsky et al., 2008; Levine and Klionsky, 2004; Levine and Yuan, 2005). Autophagy plays a very important role in normal development and is involved in multiple human diseases, including bacterial and viral infections, neurodegenerative disorders, cardiovascular disease, acute pancreatitis, cancer, aging, obesity, and type II diabetes (Goldman et al., 2010; Hashimoto et al., 2008; Helin et al., 1980; Jin and White, 2008; Shintani and Klionsky, 2004; Zhang et al., 2009). The formation of autophagosomes is mediated by a set of approximately 30 *ATG* genes that were first identified in yeast *Saccharomyces cerevisiae* and, subsequently, found to be conserved during evolution, including in mammals (Cao and Klionsky, 2007; Klionsky et al., 2003; Levine and Klionsky, 2004; Reggiori and Klionsky, 2002).

The mitochondrion is known to play an important role in integrating signals regulating cell survival. Recently, the outer membrane of mitochondria in mammalian cells has been suggested as a new source of autophagosomal membranes under starvation conditions (Hailey et al., 2010; McEwan and Dikic, 2010), but the detailed mechanism is unclear.

Antimycin A (AMA) is a chemical compound produced by *Streptomyces kitazawensis* (Nakayama et al., 1956). AMA is known to bind to the Qi site of cytochrome *c* reductase in the mitochondrial complex III to inhibit the oxidation of ubiquinol in the electron transport chain, which blocks the mitochondrial electron transfer between cytochrome *b* and *c* (Alexandre and Lehninger, 1984; Campo et al., 1992; Maguire et al., 1992; Pham et al., 2000; Xia et al., 1997). The inhibition of electron transport causes a collapse of the proton gradient across the mitochondrial inner membrane, leading to the loss of the mitochondrial membrane potential ($\Delta\Psi m$) (Balaban et al., 2005; Campo et al., 1992; Pham et al., 2000). The consequences of inhibiting complex III include an increase in the production of ROS (Balaban et al., 2005; Panduri et al., 2004) and a reduction in the cellular levels of ATP (Campo et al., 1992; Maguire et al., 1992; Pham et al., 2000).

In this study we identified an unexpected activity of mitochondrial electron transport chain (mETC) complex III inhibitor AMA in inhibiting autophagy. Using AMA as a tool, we explored the role of mitochondria in mediating autophagy. Our results suggest that mETC complex III may have a role in mediating autophagy.

RESULTS

AMA Inhibits Autophagy

To explore the potential role of mitochondria in regulating autophagy, we used AMA, the mETC complex III inhibitor, to treat a human glioblastoma H4 cell stably expressing LC3-GFP (Sarkar et al., 2007; Zhang et al., 2007). The treatment of AMA inhibited the increase in the levels of autophagosomes induced by rapamycin, as indicated by the LC3-GFP+ puncta (Figure 1A). Imaging analysis showed that AMA significantly reduced the number of LC3-GFP+ puncta (Figure 1A). Similarly, the treatment of AMA reduced both basal and rapamycin-induced autophagy in MEF cells (Figure 1B), and serum starvation induced autophagy in HeLa cells (Figure 1C). Furthermore, the inhibition of autophagy by AMA was dose and time dependent (Figures 1D and 1E). AMA began to inhibit autophagy at 5 ng/ml (~9.5 nM), and inhibited autophagy obviously after 2 hr treatment at 10 ng/ml in MEF cells. AMA also inhibited autophagy in Bcap-37 cells (see Figure S1A available online), which indicated that AMA effect is not cell specific.

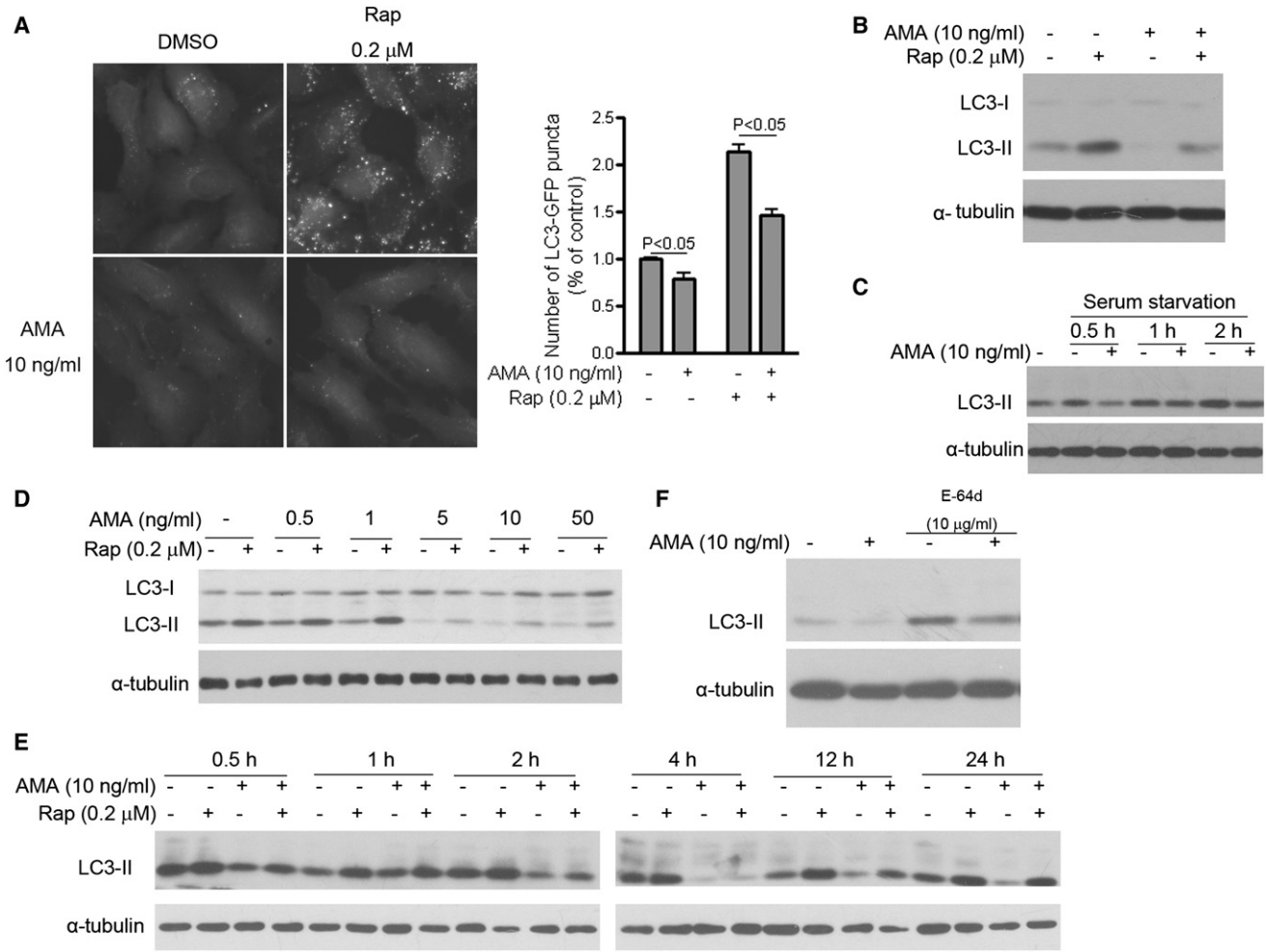

Figure 1. AMA Inhibits Autophagy

(A) The effect of AMA on rapamycin (Rap)-induced autophagy in H4-LC3-GFP cells. H4-LC3-GFP cells were treated with AMA (10 ng/ml) in the presence or absence of Rap (0.2 μM) for 6 hr, and the images were collected and analyzed with a HCS Reader.

(B and C) The effect of AMA on the levels of LC3II. (B) MEF cells were treated with AMA (10 ng/ml) in the presence or absence of Rap (0.2 μM) for 8 hr, and the cell lysates were subjected to western blotting using anti-LC3 antibody. Anti-α-tubulin was used as a loading control. (C) HeLa cells were treated with AMA (10 ng/ml) with or without serum starvation for indicated times, and the cell lysates were subjected to western blotting using anti-LC3 antibody. Anti-α-tubulin was used as a loading control.

(D) The dose effect of AMA on the levels of LC3II. MEF cells were treated with AMA at different concentrations for 6 hr, and the cell lysates were subjected to western blotting using anti-LC3 and anti-α-tubulin antibodies.

(E) The effect of AMA on the levels of LC3II. MEF cells were treated with indicated compounds for different times, and the cell lysates were subjected to western blotting using anti-LC3 and anti-α-tubulin antibodies.

(F) The effect of AMA on the levels of LC3II induced by E-64d. H4 cells were treated by indicated compounds for 4 hr, and the cell lysates were subjected to western blotting using anti-LC3 and anti-α-tubulin antibodies.

See also Figure S1.

LC3II is degraded by lysosomal hydrolases during autophagy (Tanida et al., 2005). Consistently, the treatment of AMA also reduced the levels of LC3II in the presence of E-64d, which blocks the lysosomal degradation (Figure 1F). Taken together, these results indicate that AMA can inhibit autophagy in mammalian cells.

AMA-Mediated Inhibition of Autophagy Is Independent of ATP and ROS Reduction

AMA specifically inhibits mitochondrial electron transport between cytochrome *b* and *c*, which leads to a loss of intracellular ATP (Campo et al., 1992; Maguire et al., 1992; Pham et al., 2000). Because autophagy pathway contains several energy-dependent steps (Plomp et al., 1989), one might postulate that a reduction in the levels of ATP induced by AMA may lead to autophagy inhibition. To test this possibility, we determined whether ATP reduction induced by AMA contributes to autophagy inhibition. We tested the effects of a glycolytic inhibitor 2-deoxy-D-glucose (2DG) (Dennis et al., 2001), which inhibits glycolysis, and AICAR, which activates AMPK (Meley et al., 2006), on autophagy in MEF cells (Liang et al., 2007). The treatment of MEF cells with 2DG significantly reduced the levels

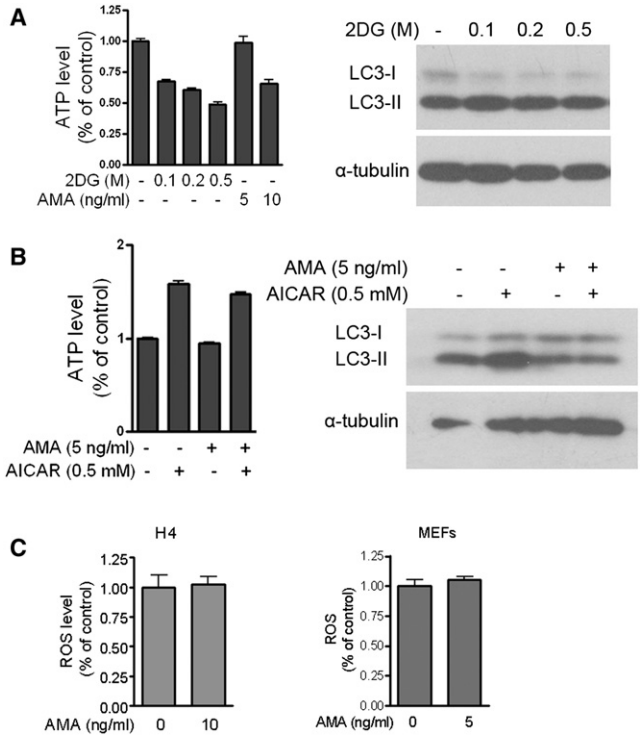

Figure 2. AMA-Mediated Inhibition of Autophagy Is Independent of ATP and ROS Reduction

(A) The effect of 2DG and AMA on ATP and autophagy. MEF cells were treated for 8 hr with 2DG or AMA at indicated concentrations, and the cell lysates were subjected to ATP assay or western blotting using anti-LC3 and anti-α-tubulin antibodies.

(B) The effect of AICAR on AMA reduced ATP and autophagy. MEF cells were pretreated for 1 hr with the AMPK activator AICAR (0.5 mM), and then treated with AICAR for another 4 hr in the presence or absence of AMA (5 ng/ml), and the cell lysates were subjected to ATP assay or western blotting using anti-LC3 and anti-α-tubulin antibodies.

(C) The effect of AMA on ROS generation. H4 or MEF cells were treated with AMA at indicated concentrations for 2 hr before incubating with DHE (1 μg/ml) for 20 min; the fluorescent images were then collected and analyzed with a HCS Reader.

See also Figure S2.

of ATP but had no effect on autophagy (Figure 2A), and AMA inhibited autophagy at 10 ng/ml, which had a lesser effect on ATP than that of glycolytic inhibitor 2DG (Figure 2A). The result indicated that AMA inhibition of autophagy was not due to a reduction in the levels of ATP. Additionally, we found that the treatment of MEF cells with AMA at 5 ng/ml for 8 hr, which was sufficient to inhibit autophagy (Figure 1D), had a minimum effect on the cellular levels of ATP at this time point (Figure 2A), suggesting that reducing ATP levels is not required for AMA to inhibit autophagy.

On the other hand, the treatment of AMPK activator AICAR induced autophagy in MEF cells as reported (Liang et al., 2007). However, cotreatment of AICRA and AMA increased the ATP level but did not rescue the inhibition of autophagy by AMA (Figure 2B). These results suggest that the inhibition of autophagy by AMA may be disconnected from its effect on intracellular ATP.

Production of reactive oxygen species (ROS) has been suggested to play an important role in the induction of autophagy (Lipinski et al., 2010; Scherz-Shouval and Elazar, 2007; Scherz-Shouval et al., 2007). Therefore, we tested the effect of AMA on intracellular levels of ROS using dihydroethidium (DHE) as a probe. Our results showed that the treatment of AMA had no obvious effect on ROS in H4 and MEF cells under the conditions that inhibit autophagy (Figure 2C). At higher concentrations, AMA increased ROS in H4 cells and decreased ROS in MEF cells (Figures S2F and S2I). Because AMA inhibited autophagy in both H4 and MEF cells (Figures 1B and 1C), AMA inhibition of autophagy was unlikely to be caused by ROS reduction.

The loss of $\Delta\Psi m$ has been reported to induce autophagy (mitophagy) (Elmore et al., 2001). Thus, it is unlikely that AMA should inhibit autophagy by reducing mitochondrial $\Delta\Psi m$. Specifically, FCCP, an uncoupling agent that dissipates the proton gradient across mitochondrial inner membrane to abolish $\Delta\Psi m$ (Gottlieb et al., 2000), has been reported to induce autophagy in reticulocytes (Sandoval et al., 2008). To confirm this result, we tested the effect of FCCP on autophagy. Our results showed that the treatment of FCCP increased LC3-GFP+ puncta in H4-LC3-GFP cells (Figure S2A), and induced LC3II increase in H4 and MEF cells (Figure S2B). This evidence supports our hypothesis that AMA inhibition of autophagy is not mediated through $\Delta\Psi m$ reduction.

mETC Complex III Is Required for AMA to Inhibit Autophagy

We postulate that AMA inhibition of autophagy may depend on its known bioactivity on mETC. To test this hypothesis, we carried out a structural and functional analysis of AMA analogs to examine if their activity on mitochondrial respiration may be required for them to inhibit autophagy. Studies with a number of derivatives and synthetic analogs showed that the dilactone ring and the phenolic hydroxyl group are indispensable for the activity of AMA on its respiration (Batra et al., 1971). We modified an ester group on AMA dilactone ring to acylamide (AA01), or methylated phenolic hydroxyl group (MeO-AA). The two compounds are closely related to AMA in structure but cannot inhibit mitochondrial respiration as measured by their effects on the levels of ATP even at 10 μM (data not shown), whereas AMA reduced the intracellular levels of ATP to about 30% at about 19 nM in MEF cells (Figure 2A). Other mitochondrially inactive analogs, including AA01, MeO-AA, AA02, and AA10, also had no effect on autophagy in MEF cells after treatment for 6 hr at 5 μM (Figure 3A), which is ~500-fold more than the required concentration of AMA for inhibiting autophagy (~10 nM). Because these close derivatives of AMA had no effect on mitochondrial respiration or autophagy, we suggest that AMA inhibition of autophagy may depend on its inhibition on mETC complex III.

To further test the role of mETC complex III on autophagy, we used myxothiazol, another mETC complex III inhibitor. The results showed that similar to that of AMA, the treatment of myxothiazol also inhibited LC3-GFP+ spot counts in H4-LC3-GFP cells (Figure 3B), and reduced the levels of LC3II in both H4 and MEF cells (Figure 3C). Myxothiazol-mediated inhibition of autophagy is also ROS and ATP independent because the treatment of myxothiazol increased ROS in H4 cells (Figure 3D), and decreased ATP to a level lower than that of glycolytic inhibitor 2DG (Figures 3D and 2A), which does not have any effect on the levels of LC3II.

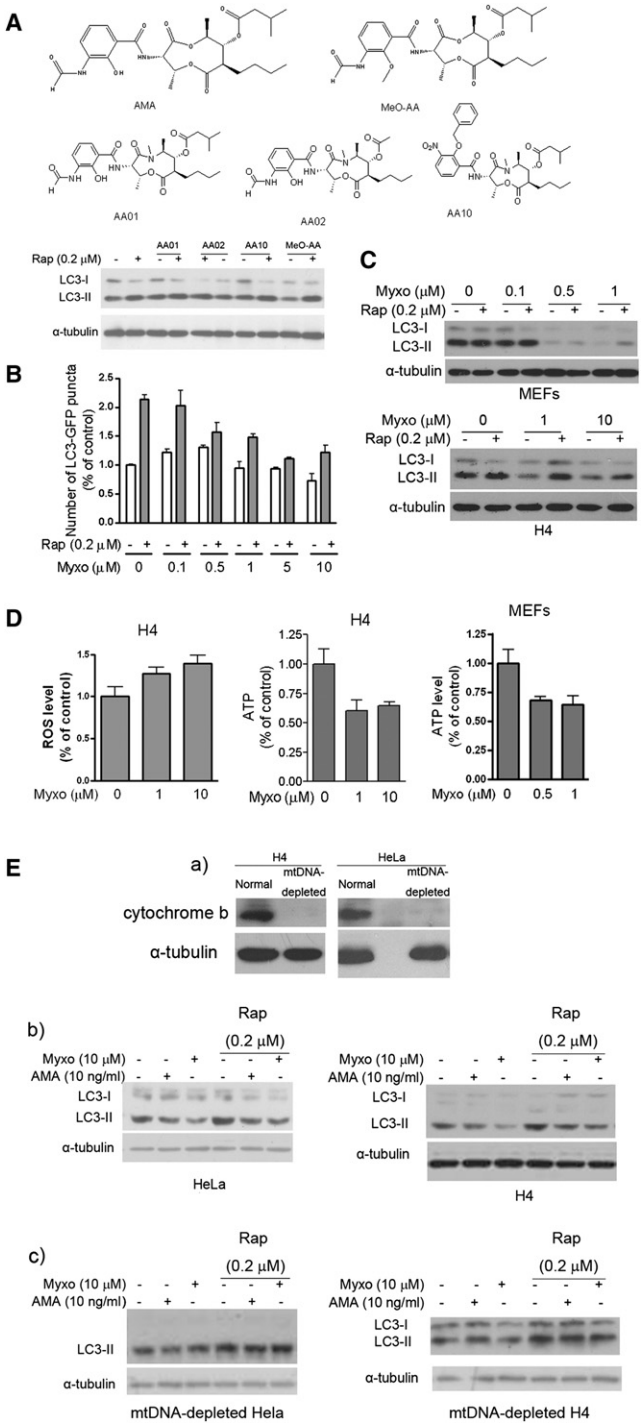

Figure 3. mETC Complex III Is Required for AMA to Inhibit Autophagy

(A) The structure of AMA and its analogs and the effects of AMA analogs on autophagy. MEF cells were treated with AMA analogs in the presence or absence of Rap (0.2 μM) for 6 hr at 5 μM, and the cell lysates were subjected to western blotting using anti-LC3 and anti-α-tubulin antibodies.

(B) The effect of myxothiazol (Myxo) on autophagy. H4-LC3-GFP cells were treated with Myxo at different concentrations in the presence or absence of Rap (0.2 μM) as indicated for 4 hr, and the images were collected and analyzed with a HCS Reader.

To further test the role of mitochondria, we examined the effects of AMA and myxothiazol in mtDNA-depleted cells. Treatment of cultured vertebrate cells with low amounts of EB results in rapid and selective depletion of mtDNA (King and Attardi, 1989, 1996). We established mtDNA-depleted HeLa and H4 cell lines after EB (50 ng/ml) treatment for 12 days. Cytochrome *b*, a mitochondrial marker, was not detectable in mtDNA-depleted cells as assessed by western blotting, suggesting the success of depleting mtDNA (Figure 3Ea). The levels of LC3II were increased by rapamycin treatment in mtDNA-depleted HeLa and mtDNA-depleted H4 cells, indicating that the autophagy pathway was intact in mtDNA-depleted cells (Figure 3Ec). However, neither AMA nor myxothiazol can inhibit the basal or rapamycin-stimulated LC3II after mtDNA depletion (Figure 3Ec). In contrast, AMA and myxothiazol obviously reduced the levels of the basal as well as rapamycin-stimulated LC3II in normal control HeLa and H4 cells (Figure 3Eb).

AMA showed no toxicity at the concentrations required for inhibiting autophagy. There was no apparent cell death of MEF or H4 cells exposed to 10 ng/ml AMA for more than 40 hr, and cell growth was only slightly inhibited after 24 hr incubation with AMA (Figures S1B and S1C). In fact AMA inhibits autophagy in MEF cells at 5 ng/ml. These results suggest that AMA inhibition of autophagy is not caused by cell death.

These results further support our hypothesis that the inhibition of mETC complex III leads to inhibition of autophagy. Finally, we attempted to examine the involvement of the complex I, II, and IV using inhibitors of other mETC complexes (complex I, II, IV). After treatment for 4 hr with rotenone, TTFA, or sodium azide (NaN_3), LC3II was reduced in MEF cells (Figure S2C). Conversely, rotenone enhanced autophagy in H4 cells after treatment for 4 hr, and TTFA and NaN_3 had no obvious effect on the levels of LC3II (Figure S2D). Under the conditions used for autophagy, the complex I, II, and IV inhibitors affected parameters of mitochondrial function (MMP, ROS, or ATP) in both H4 and MEF cells (Figure S2). Thus, unlike that of the complex III, the inhibitors of mETC complex I, II, IV do not provide a consistent effect on autophagy. Further studies are needed to address the roles of the complexes I, II, and IV in autophagy.

DISCUSSION

In this manuscript we show that AMA, a mETC complex III inhibitor, inhibits the basal and induced autophagy in normal cells.

(C) H4 or MEF cells were treated with Myxo in the presence or absence of Rap (0.2 μM) as indicated for 4 hr, and the cell lysates were subjected to western blotting using anti-LC3 and anti-α-tubulin antibodies.

(D) H4 or MEF cells were treated with Myxo at indicated concentrations for 4 hr and analyzed with ROS or ATP assay.

(E) The effects of AMA and Myxo in normal HeLa and normal H4 cells and mtDNA-depleted HeLa and mtDNA-depleted H4 cells. (a) Parent H4 cells and HeLa cells were cultured in the presence of EB (50 ng/ml), pyruvate (100 μg/ml), and uridine (50 μg/ml) for 12 days. Both parent and EB-treated cell lysates were subjected to western blotting using anti-cytochrome *b* and anti-α-tubulin antibodies. (b and c) Normal HeLa and normal H4 cells (b), mtDNA-depleted HeLa cells, and mtDNA-depleted H4 cells (c) were treated by indicated compounds for 4 hr, and the cell lysates were subjected to western blotting using anti-LC3 and anti-α-tubulin antibodies.

See also Figure S2.

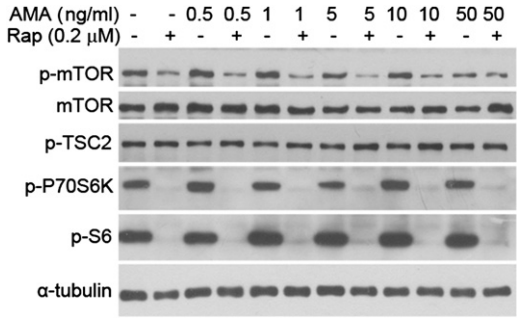

Figure 4. AMA Does Not Reactivate Class I PI3K-Akt-mTOR Signal Pathway

MEF cells were treated for 6 hr with AMA at different concentrations as indicated, and the cell lysates were subjected to western blotting using indicated antibodies.

This effect requires a functional mitochondrial respiration chain because AMA has no effect in mtDNA-depleted cells. Additionally, AMA analogs that do not inhibit mitochondrial respiratory chain cannot inhibit autophagy even at 500-fold excess of AMA concentration required for it to inhibit autophagy. Another mETC complex III inhibitor myxothiazol with a structure unrelated to that of AMA also inhibits autophagy. These results suggest that the mETC complex III may play a specific role in autophagy regulation. Furthermore, because AMA shows inhibition of autophagy at a dose of 5 ng/ml, which has a minimum effect on the cellular levels of ATP, a significant reduction in the cellular levels of ATP is not required for AMA to inhibit autophagy. On the other hand, because autophagy serves as a recycling mechanism for energy and biomaterial under stress conditions, inhibition of autophagy by AMA may contribute to its effect on ATP. In addition, AMA has no obvious effect on MMP and ROS under the conditions that inhibit autophagy, which indicates that partial inhibition of complex III is enough for inhibition of autophagy.

We used AMA as a tool to explore the mechanism of mETC complex III in autophagy induction. We found that the treatment of AMA reduced the levels of LC3II but had no effect on known regulators of the pathway, such as the class I PI3K/Akt/mTOR signal transduction pathway (Figure 4), the levels of proteins in class III PI3K complex including Vps34 and Beclin1, and also Atg12-Atg5 conjugate (Figure 5).

How might the mitochondrial complex III be involved in autophagosome initiation? One possibility is that the complex III regulates a signaling event in autophagy. The complex III has been proposed to regulate the activity of HIF (Guzy et al., 2005; Tormos and Chandel, 2010), and because HIF-1 has been shown to regulate the expression of BNIP3, which in turn promotes autophagy, future experiments could examine the possibility for the involvement of HIF-1 and BNIP3 in AMA-mediated autophagy inhibition. Another possibility is the source of autophagic membrane. Mitochondria have been suggested to donate lipids to autophagic membrane in mammalian cells (Hailey et al., 2010). The membranes of isolation membrane and autophagosomes are of the thin type (6–7 nm), like the membranes of the endoplasmic reticulum (ER), *cis*-Golgi, nuclear envelope, and the inner and outer membranes of mitochondria. It is possible that different membrane pools may contribute to the isolation membrane (Juhasz and Neufeld, 2006; Reggiori and Klionsky, 2005). It has not been demonstrated whether mitochondria have provided lipids for autophagic membranes in mammalian cells, except starvation-induced autophagy (Hailey et al., 2010). Recently, ER and Golgi have been suggested as the origin of autophagosomal membranes in mammalian cells (Axe et al., 2008; Geng et al., 2010; Hayashi-Nishino et al., 2009; Takahashi et al., 2011; van der Vaart et al., 2010; van der Vaart and Reggiori, 2010; Ylä-Anttila et al., 2009), but the detailed mechanism is unknown. However, no evidence indicates that ER or Golgi is the only origin for autophagic membranes in mammalian cells.

The outer membrane of mitochondria in mammalian cells has been suggested as a new source of autophagosomal membranes under starvation conditions (Hailey et al., 2010; McEwan and Dikic, 2010). Mitochondria may also supply lipids for autophagosomal membranes with a different mechanism under other conditions. Whether or not mitochondria in mammalian cells contribute to the autophagic membrane under other conditions except starvation remains to be seen. We propose that AMA and other complex III inhibitors might provide a tool for such studies.

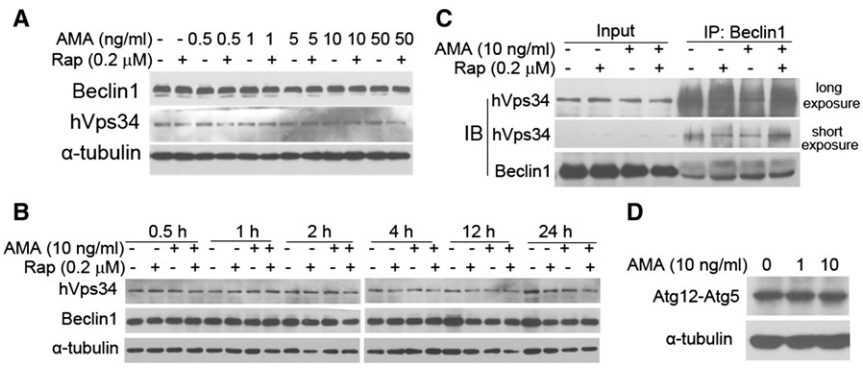

Figure 5. AMA Has No Effect on Class III PI3K (hVps34), Beclin1 Protein Levels and Their Complex, and Atg12-Atg5 Conjugate System

(A and B) The effect of AMA on the levels of hVps34 and Beclin1 proteins. MEF cells were treated with AMA for 6 hr at different concentrations (A) or with AMA at 10 ng/ml in the presence or absence of rapamycin (Rap) for different times as indicated (B), and the cell lysates were subjected to western blotting using anti-Beclin1, anti-hVps34, and anti-α-tubulin antibodies.

(C) The effect of AMA on endogenous hVps34/Beclin1 complex. MEF cells were treated by indicated compounds for 4 hr. Beclin1 was immunoprecipitated from the lysates with a monoclonal antibody. The immunoprecipitates were subjected to western blotting using polyclonal anti-Beclin1 and anti-hVps34 antibodies.

(D) H4 cells were treated by AMA at 1 or 10 ng/ml for 4 hr, and the cell lysates were subjected to western blotting using Atg12 and anti-α-tubulin antibodies.

SIGNIFICANCE

Autophagy is a cellular lysosome-dependent catabolic mechanism mediating the turnover of intracellular organelles and long-lived proteins. Dysfunction of autophagy has been implicated in multiple human diseases. Identification of autophagy factors in mammalian cells is important to understand how this complex cellular pathway responds to a broad range of challenges. Here, we report that mitochondrial electron transport chain (mETC) complex III plays a role in autophagy induction. We show that antimycin A, a known inhibitor of mETC complex III, can inhibit both rapamycin and serum starvation-induced autophagy. A structural and functional study shows that four close analogs of antimycin A that have no effect on mitochondria inhibition also do not inhibit autophagy, whereas myxothiazol, another mETC complex III inhibitor with unrelated structure to antimycin A, inhibits autophagy. Additionally, antimycin A and myxothiazol cannot inhibit autophagy in mtDNA-depleted H4 and mtDNA-depleted HeLa cells. These data suggest that antimycin A inhibits autophagy through its inhibitory activity on mETC complex III. Our data suggest that mETC complex III may have a role in mediating autophagy induction. Two mechanisms are supposed for mitochondrial complex III in regulating autophagosome initiation. One possibility is that the complex III regulates a signaling event in autophagy. The complex III has been proposed to regulate the activity of HIF, and because HIF-1 has been shown to regulate the expression of BNIP3, which in turn promotes autophagy, future experiments could examine the possibility for the involvement of HIF-1 and BNIP3 in AMA-mediated autophagy inhibition. Another possibility is the involvement of mitochondria as a source of autophagic membrane. Mitochondria have been suggested to donate lipids to autophagic membrane. Whether mitochondria in mammalian cells contribute to the autophagic membrane under normal nutritional conditions remains to be seen. We propose that AMA and other complex III inhibitors might provide a tool for such studies.

EXPERIMENTAL PROCEDURES

Plasmids, Antibodies, Cell Reagents, and Cell Lines

The following compounds were used: rapamycin (Sigma-Aldrich; R0395); AMA (Sigma-Aldrich; A8674); rotenone (Sigma-Aldrich; R8875); TTFA (Sigma-Aldrich; T27006); MG132 (Sigma-Aldrich; C2211); E-64d (Sigma-Aldrich; E8640); 2DG (Sigma-Aldrich; D8375); AICAR (Sigma-Aldrich; A9978); and DHE (Sigma-Aldrich; D7008). AMA derivatives were provided by Professor Dawei Ma. NaN_3 was from AMRESCO (0639). GFP-LC3-expressing constructs were as previously described (Shibata et al., 2006). ATP bioluminescent assay kit was from Promega (V6713). Monoclonal anti-α-tubulin was from Sigma-Aldrich (T6074). Polyclonal anti-LC3 was from Sigma-Aldrich (L7543) or Novus (NB600-1384). HRP-conjugated goat anti-mouse IgG antibody and HRP-conjugated goat anti-rabbit IgG antibody were from Chemicon (AP307P, AP308P). H4 and HeLa cell lines were the same as previously described (Pan et al., 2008).

Cell Culture and Transfection

The cells used for the experiments were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin/streptomycin, and 2 mM L-glutamine at 37°C, 5% CO_2. Transfection of H4 cells was done using Lipofectamine 2000 reagent (Invitrogen; 18324-012). For starvation, cells were washed with phosphate-buffered saline (PBS) and incubated in serum-free DMEM. MtDNA-depleted H4 and mtDNA-depleted HeLa cells were established as follows: parental H4 cells and HeLa cells were cultured in the presence of EB (50 ng/ml), pyruvate (100 μg/ml), and uridine (50 μg/ml). After 12 days, EB treatment cells were grown in the same medium without EB, and mtDNA-depleted cell lines were established.

Image Analysis

H4-LC3-GFP cells were seeded in 96-well plate and cultured in the presence of compounds for the time as indicated. Images data were collected and analyzed with a Thermo Scientific ArrayScan VTI HCS Reader. DMSO and rapamycin were used as negative or positive control, respectively. GFP-LC3-labeled vacuoles are denoted as dots in all figures.

Immunoprecipitation and Immunoblotting

Cell lysates were prepared in a lysis buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100, 1 mM phenylmethanesulfonyl fluoride, 1 mM Na3VO4, and protease inhibitor cocktail). The lysates were clarified by centrifugation at 14,000 rpm for 15 min and were subjected to immunoprecipitation using specific antibodies in combination with protein G-agarose (Invitrogen). Precipitated immunocomplexes were washed four times in wash buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100) and boiled in sample buffer. Samples were subsequently separated by SDS-PAGE and transferred to Immobilon-P polyvinylidene difluoride membranes (Millipore). Immunoblotting analysis was performed with indicated antibodies and visualized with SuperSignal West Pico Chemiluminescent Substrate (Pierce Chemical).

ATP and ROS Assays

ATP was measured using a luciferin-luciferase assay kit (Promega) by following the manufacturer's instruction. The levels of intracellular ROS were measured by using a fluorescence probe DHE. The cells were treated with AMA for 2 hr and then incubated with 1 μg/ml DHE at 37°C for 20 min in HBSS. Fluorescent images were collected and analyzed with Thermo Scientific ArrayScan VTI HCS Reader.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and two figures and can be found with this article online at doi:10.1016/j.chembiol.2011.08.009.

ACKNOWLEDGMENTS

This work was supported in part by an NIH Director's Pioneer Award US (to J.Y.), by grants from the National Institute on Aging (US) PO1 AG027916 (to J.Y.), grants from the National Natural Science Foundation of China 31129004 (to J.Y.) and 31000619 (to Q.Y.), the Shanghai Science and Technology Research Grants 08DZ1971403 (to Q.Y.) and 09JC1416700 (to Q.Y.), and a grant from The China National Science & Technology Key Project 2012CB910704 (to Q.Y.).

Received: December 30, 2010
Revised: August 1, 2011
Accepted: August 15, 2011
Published: November 22, 2011

REFERENCES

Alexandre, A., and Lehninger, A.L. (1984). Bypasses of the antimycin a block of mitochondrial electron transport in relation to ubisemiquinone function. Biochim. Biophys. Acta *767*, 120–129.

Axe, E.L., Walker, S.A., Manifava, M., Chandra, P., Roderick, H.L., Habermann, A., Griffiths, G., and Ktistakis, N.T. (2008). Autophagosome formation from membrane compartments enriched in phosphatidylinositol

3-phosphate and dynamically connected to the endoplasmic reticulum. J. Cell Biol. *182*, 685–701.

Baehrecke, E.H. (2005). Autophagy: dual roles in life and death? Nat. Rev. Mol. Cell Biol. *6*, 505–510.

Balaban, R.S., Nemoto, S., and Finkel, T. (2005). Mitochondria, oxidants, and aging. Cell *120*, 483–495.

Batra, P.P., Harbin, T.L., Howes, C.D., and Bernstein, S.C. (1971). A study of the relationship of structure and activity of antimycin A in the induction of carotenoid synthesis in *Mycobacterium marinum*. J. Biol. Chem. *246*, 7125–7130.

Campo, M.L., Kinnally, K.W., and Tedeschi, H. (1992). The effect of antimycin A on mouse liver inner mitochondrial membrane channel activity. J. Biol. Chem. *267*, 8123–8127.

Cao, Y., and Klionsky, D.J. (2007). Physiological functions of Atg6/Beclin 1: a unique autophagy-related protein. Cell Res. *17*, 839–849.

Codogno, P., and Meijer, A.J. (2005). Autophagy and signaling: their role in cell survival and cell death. Cell Death Differ. *12* (*Suppl 2*), 1509–1518.

Dennis, P.B., Jaeschke, A., Saitoh, M., Fowler, B., Kozma, S.C., and Thomas, G. (2001). Mammalian TOR: a homeostatic ATP sensor. Science *294*, 1102–1105.

Elmore, S.P., Qian, T., Grissom, S.F., and Lemasters, J.J. (2001). The mitochondrial permeability transition initiates autophagy in rat hepatocytes. FASEB J. *15*, 2286–2287.

Geng, J., Nair, U., Yasumura-Yorimitsu, K., and Klionsky, D.J. (2010). Post-Golgi Sec proteins are required for autophagy in *Saccharomyces cerevisiae*. Mol. Biol. Cell *21*, 2257–2269.

Goldman, S., Zhang, Y., and Jin, S. (2010). Autophagy and adipogenesis: implications in obesity and type II diabetes. Autophagy *6*, 179–181.

Gottlieb, E., Vander Heiden, M.G., and Thompson, C.B. (2000). Bcl-x(L) prevents the initial decrease in mitochondrial membrane potential and subsequent reactive oxygen species production during tumor necrosis factor alpha-induced apoptosis. Mol. Cell. Biol. *20*, 5680–5689.

Guzy, R.D., Hoyos, B., Robin, E., Chen, H., Liu, L., Mansfield, K.D., Simon, M.C., Hammerling, U., and Schumacker, P.T. (2005). Mitochondrial complex III is required for hypoxia-induced ROS production and cellular oxygen sensing. Cell Metab. *1*, 401–408.

Hailey, D.W., Rambold, A.S., Satpute-Krishnan, P., Mitra, K., Sougrat, R., Kim, P.K., and Lippincott-Schwartz, J. (2010). Mitochondria supply membranes for autophagosome biogenesis during starvation. Cell *141*, 656–667.

Hashimoto, D., Ohmuraya, M., Hirota, M., Yamamoto, A., Suyama, K., Ida, S., Okumura, Y., Takahashi, E., Kido, H., Araki, K., et al. (2008). Involvement of autophagy in trypsinogen activation within the pancreatic acinar cells. J. Cell Biol. *181*, 1065–1072.

Hayashi-Nishino, M., Fujita, N., Noda, T., Yamaguchi, A., Yoshimori, T., and Yamamoto, A. (2009). A subdomain of the endoplasmic reticulum forms a cradle for autophagosome formation. Nat. Cell Biol. *11*, 1433–1437.

Helin, H., Mero, M., Markkula, H., and Helin, M. (1980). Pancreatic acinar ultrastructure in human acute pancreatitis. Virchows Arch. A Pathol. Anat. Histol. *387*, 259–270.

Jin, S., and White, E. (2008). Tumor suppression by autophagy through the management of metabolic stress. Autophagy *4*, 563–566.

Juhasz, G., and Neufeld, T.P. (2006). Autophagy: a forty-year search for a missing membrane source. PLoS Biol. *4*, e36.

King, M.P., and Attardi, G. (1989). Human cells lacking mtDNA: repopulation with exogenous mitochondria by complementation. Science *246*, 500–503.

King, M.P., and Attardi, G. (1996). Isolation of human cell lines lacking mitochondrial DNA. Methods Enzymol. *264*, 304–313.

Klionsky, D.J., Cregg, J.M., Dunn, W.A., Jr., Emr, S.D., Sakai, Y., Sandoval, I.V., Sibirny, A., Subramani, S., Thumm, M., Veenhuis, M., and Ohsumi, Y. (2003). A unified nomenclature for yeast autophagy-related genes. Dev. Cell *5*, 539–545.

Klionsky, D.J., Abeliovich, H., Agostinis, P., Agrawal, D.K., Aliev, G., Askew, D.S., Baba, M., Baehrecke, E.H., Bahr, B.A., Ballabio, A., et al. (2008). Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. Autophagy *4*, 151–175.

Levine, B., and Klionsky, D.J. (2004). Development by self-digestion: molecular mechanisms and biological functions of autophagy. Dev. Cell *6*, 463–477.

Levine, B., and Yuan, J. (2005). Autophagy in cell death: an innocent convict? J. Clin. Invest. *115*, 2679–2688.

Liang, J., Shao, S.H., Xu, Z.X., Hennessy, B., Ding, Z., Larrea, M., Kondo, S., Dumont, D.J., Gutterman, J.U., Walker, C.L., et al. (2007). The energy sensing LKB1-AMPK pathway regulates p27(kip1) phosphorylation mediating the decision to enter autophagy or apoptosis. Nat. Cell Biol. *9*, 218–224.

Lipinski, M.M., Zheng, B., Lu, T., Yan, Z., Py, B.F., Ng, A., Xavier, R.J., Li, C., Yankner, B.A., Scherzer, C.R., et al. (2010). Genome-wide analysis reveals mechanisms modulating autophagy in normal brain aging and in Alzheimer's disease. Proc Natl Acad Sci USA *107*, 14164–14169.

Maguire, J.J., Kagan, V.E., and Packer, L. (1992). Electron transport between cytochrome c and alpha tocopherol. Biochem. Biophys. Res. Commun. *188*, 190–197.

McEwan, D.G., and Dikic, I. (2010). Not all autophagy membranes are created equal. Cell *141*, 564–566.

Meley, D., Bauvy, C., Houben-Weerts, J.H., Dubbelhuis, P.F., Helmond, M.T., Codogno, P., and Meijer, A.J. (2006). AMP-activated protein kinase and the regulation of autophagic proteolysis. J. Biol. Chem. *281*, 34870–34879.

Nakayama, K., Okamoto, F., and Harada, Y. (1956). Antimycin A: isolation from a new Streptomyces and activity against rice plant blast fungi. J. Antibiot. *9*, 63–66.

Pan, H., Yu, J., Zhang, L., Carpenter, A., Zhu, H., Li, L., Ma, D., and Yuan, J. (2008). A novel small molecule regulator of guanine nucleotide exchange activity of the ADP-ribosylation factor and Golgi membrane trafficking. J. Biol. Chem. *283*, 31087–31096.

Panduri, V., Weitzman, S.A., Chandel, N.S., and Kamp, D.W. (2004). Mitochondrial-derived free radicals mediate asbestos-induced alveolar epithelial cell apoptosis. Am. J. Physiol. Lung Cell. Mol. Physiol. *286*, L1220–L1227.

Pham, N.A., Robinson, B.H., and Hedley, D.W. (2000). Simultaneous detection of mitochondrial respiratory chain activity and reactive oxygen in digitonin-permeabilized cells using flow cytometry. Cytometry *41*, 245–251.

Plomp, P.J., Gordon, P.B., Meijer, A.J., Høyvik, H., and Seglen, P.O. (1989). Energy dependence of different steps in the autophagic-lysosomal pathway. J. Biol. Chem. *264*, 6699–6704.

Reggiori, F., and Klionsky, D.J. (2002). Autophagy in the eukaryotic cell. Eukaryot. Cell *1*, 11–21.

Reggiori, F., and Klionsky, D.J. (2005). Autophagosomes: biogenesis from scratch? Curr. Opin. Cell Biol. *17*, 415–422.

Sandoval, H., Thiagarajan, P., Dasgupta, S.K., Schumacher, A., Prchal, J.T., Chen, M., and Wang, J. (2008). Essential role for Nix in autophagic maturation of erythroid cells. Nature *454*, 232–235.

Sarkar, S., Perlstein, E.O., Imarisio, S., Pineau, S., Cordenier, A., Maglathlin, R.L., Webster, J.A., Lewis, T.A., O'Kane, C.J., Schreiber, S.L., and Rubinsztein, D.C. (2007). Small molecules enhance autophagy and reduce toxicity in Huntington's disease models. Nat. Chem. Biol. *3*, 331–338.

Scherz-Shouval, R., and Elazar, Z. (2007). ROS, mitochondria and the regulation of autophagy. Trends Cell Biol. *17*, 422–427.

Scherz-Shouval, R., Shvets, E., Fass, E., Shorer, H., Gil, L., and Elazar, Z. (2007). Reactive oxygen species are essential for autophagy and specifically regulate the activity of Atg4. EMBO J. *26*, 1749–1760.

Shibata, M., Lu, T., Furuya, T., Degterev, A., Mizushima, N., Yoshimori, T., MacDonald, M., Yankner, B., and Yuan, J. (2006). Regulation of intracellular accumulation of mutant Huntingtin by Beclin 1. J. Biol. Chem. *281*, 14474–14485.

Shintani, T., and Klionsky, D.J. (2004). Autophagy in health and disease: a double-edged sword. Science *306*, 990–995.

Takahashi, Y., Meyerkord, C.L., Hori, T., Runkle, K., Fox, T.E., Kester, M., Loughran, T.P., and Wang, H.G. (2011). Bif-1 regulates Atg9 trafficking by

mediating the fission of Golgi membranes during autophagy. Autophagy *7*, 61–73.

Tanida, I., Minematsu-Ikeguchi, N., Ueno, T., and Kominami, E. (2005). Lysosomal turnover, but not a cellular level, of endogenous LC3 is a marker for autophagy. Autophagy *1*, 84–91.

Tormos, K.V., and Chandel, N.S. (2010). Inter-connection between mitochondria and HIFs. J. Cell. Mol. Med. *14*, 795–804.

van der Vaart, A., and Reggiori, F. (2010). The Golgi complex as a source for yeast autophagosomal membranes. Autophagy *6*, 800–801.

van der Vaart, A., Griffith, J., and Reggiori, F. (2010). Exit from the Golgi is required for the expansion of the autophagosomal phagophore in yeast *Saccharomyces cerevisiae*. Mol. Biol. Cell *21*, 2270–2284.

Xia, D., Yu, C.A., Kim, H., Xia, J.Z., Kachurin, A.M., Zhang, L., Yu, L., and Deisenhofer, J. (1997). Crystal structure of the cytochrome bc1 complex from bovine heart mitochondria. Science *277*, 60–66.

Ylä-Anttila, P., Vihinen, H., Jokitalo, E., and Eskelinen, E.L. (2009). 3D tomography reveals connections between the phagophore and endoplasmic reticulum. Autophagy *5*, 1180–1185.

Zhang, L., Yu, J., Pan, H., Hu, P., Hao, Y., Cai, W., Zhu, H., Yu, A.D., Xie, X., Ma, D., and Yuan, J. (2007). Small molecule regulators of autophagy identified by an image-based high-throughput screen. Proc. Natl. Acad. Sci. USA *104*, 19023–19028.

Zhang, Y., Goldman, S., Baerga, R., Zhao, Y., Komatsu, M., and Jin, S. (2009). Adipose-specific deletion of autophagy-related gene 7 (atg7) in mice reveals a role in adipogenesis. Proc. Natl. Acad. Sci. USA *106*, 19860–19865.