Autophagy exacerbates caspase-dependent apoptotic cell death after short times of starvation

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Abstract

Autophagy is generally regarded as a mechanism to promote cell survival. However, autophagy can occasionally be the mechanism responsible of cell demise. We have found that a concomitant depletion of glucose, nutrients and growth factors provoked cell death in a variety of cell lines. This death process was contingent upon caspase activation and was mediated by BAX/BAK proteins, thus indicating its apoptotic nature and the engagement of an intrinsic pathway. In order to abrogate autophagy, 3-methyladenine (3-MA), BECLIN-1 siRNA and *Atg5* knock-out (Tet-Off type) approaches were alternatively employed. Irrespective of the procedure, at short times of starvation, we found that the ongoing autophagy was sensitizing cells to the permeabilization of the mitochondrial outer membrane (MOMP), caspase activation and, therefore, apoptosis. On the contrary, at longer times of starvation, autophagy displayed its characteristic pro-survival effect on cells. As far as we know, we provide the first experimental paradigm where time is the only variable determining the final outcome of autophagy. In other words, we have circumscribed in time the shift transforming autophagy from a cell death to a protection mechanism. Moreover, at short times, starvation-driven autophagy exacerbated the apoptotic cell death caused by several antitumor agents. In agreement with this fact, their apoptotic effects were greatly diminished by autophagy inhibition. The implications of these facts in tumor biology will be discussed.

Chemical compounds

3-methyladenine (Pubmed CID 1673)

Camptothecin (Pubmed CID 24360)

Etoposide (Pubmed CID 36462)

7-bromoindirubin-3-oxime (Pubmed CID 11846148)

Necrostatin-1 (Pubmed CID 282833)

Q-VD-OPh (Pubmed CID 11237609)

2-phenylethynesulfonamide (Pubmed CID 327653)

Staurosporine (Pubmed CID 44259)

Abbreviations: 3-MA, 3-methyladenine; 7BIO, 7-bromoindirubin-3-oxime; PES, 2-phenylethynesulfonamide; AVOs, acidic vesicular organelles; AO, acridine orange; AIF, apoptosis-inducing factor; ATG, autophagy-related; baf A₁, bafilomycin A₁; CPT, camptothecin; cyt C; cytochrome C; COX IV, cytochrome oxidase IV; DEVD, Ac-Asp-Glu-Val-Asp-AFC; DKO, double knockout; Eto, etoposide; LC3, microtubule-associated protein 1 light chain 3; MEFs, mouse embryonic fibroblasts; MOMP, mitochondrial outer membrane permeabilization; NB, Naphthol blue; PES, 2-phenylethynesulfonamide; p62, p62/SQSTM1; PtdInsKC3, phosphatidylinositol 3-kinase class III; PI, propidium iodide; RIP1, receptor-interacting protein 1; siRNA, small interference RNA; SM, starvation medium; STP, staurosporine.

Keywords: apoptosis; autophagy; short-term starvation; mitochondrial permeabilization; caspases.

1. Introduction

Insufficiently vascularized solid tumors commonly suffer from transient periods of nutrient, growth factors and oxygen restriction. An analogous situation is found in myocardial and brain tissues after ischemia. These severe forms of starvation impart a strong metabolic stress, which is partially alleviated through the induction of macroautophagy [1]. Macroautophagy (hereafter referred as autophagy) is an exquisitely orchestrated catabolic process whereby portions of the cytoplasm are sequestered into double-membrane vesicles called autophagosomes. The fusion of these vesicles with lysosomes, that supply their hydrolytic machinery, will result in the formation of the autophagolysosomes. These degradative organelles will fuel the cellular metabolism through the recycling of biomolecules and, indirectly, contributing to the bioenergetic management. Severe forms of starvation employ autophagy as a restorative system whereby cells enter in a “self-cannibalism” process aimed to supply the cell with the metabolic intermediaries needed to survive. Consistently, starved cells undergo a reduction of size, which can be partially attributed to the autophagic process [2].

Canonical autophagy is driven by a series of sequential steps controlled by a group of “autophagy-related” proteins (ATGs), which are highly conserved from yeast to humans [3]. Classically, the process is divided in four steps: initiation, nucleation, elongation and closure. The formation of autophagic vesicles, or nucleation, is regulated by the phosphatidylinositol 3-kinase class III (PtdInsKC3; Vsp34 in yeast), which is the target of 3-methyladenine (3-MA), one of the most employed pharmacological inhibitors of autophagy. This kinase forms a complex with BECLIN-1, the mammalian ortholog of the yeast ATG6, a reported haploinsufficient tumor suppressor [4]. The elongation of the autophagosomes relies on the proper activity of two ubiquitin-like protein conjugation systems: the protein light chain 3 (LC3, also known as ATG8) and ATG12. These systems

are interdependent since the complex ATG12-ATG5-ATG16 is in charge of transferring molecules of phosphatidyl-ethanolamine to LC3-I, a cleaved form of LC3. In this way, the lipidated form of LC3-I (LC3-II) will be properly located at the growing autophagosomes [5]. Alternatively, autophagosomes can be assembled independently of proteins such ATG5 or BECLIN-1, thus indicating the existence of non-canonical forms of autophagy [6,7]. LC3-II has been proposed to function as a receptor for a selective substrate, p62/SQSTM1, which is degraded within the autophagolysosomes. In addition, the p62/SQSTM1 ubiquitin-binding domain is proposed to act as a receptor for ubiquitinated proteins and inclusion bodies, which will be directed to the autophagosomes [8]. Both LC3-II and p62/SQSTM1 are powerful markers to study the dynamics of the autophagic process also known as “autophagic flux” [9].

The role of autophagy in regards to the final fate of a cell is a subject of intense debate. In 1972, type II or autophagy was considered one of the three types of cell death, defined essentially on morphological grounds. For a while, the mere observation of a dying cell with a vacuolated cytoplasm was sufficient to conclude that this cell was dying by autophagy. Nonetheless, growing amount of data evidenced that autophagy cooperated or enabled alternative subroutines of cell death [10]. Therefore, the detection of autophagic features is not sufficient to discard the involvement of other pathways of cell death. Recent findings have established that autophagy, as a subroutine of cell death, is constrained to a few context- and cell-specific circumstances. Under these precise situations, the Nomenclature Committee of Cell death (NCCD) is favoring the use of the term “autophagic cell death” instead of the misleading, autophagy. Finally, it is worth to mention that autophagy is generally accepted as a pro-survival program triggered in response to unfavorable cellular environments [11]. Altogether, it seems that autophagy can lead cells to a broad range of cell fates. The specific molecular situations that will determine the consequences of activating autophagy are under study. Apoptosis is one of the most

frequent subroutines of death engaged by starvation in cultured cells [1,12,13]. Intrinsic or mitochondrial apoptosis is marked by the mitochondrial outer membrane permeabilization (MOMP), which results in the release of cytochrome c (cyt C), the apoptosis-inducing factor (AIF) and other proteins from the inter-membrane space. Unleashed cyt C triggers the assembly of the apoptosome and the subsequent activation of the initiator caspase-9 upstream of the executioner caspase-3/7 [14]. On the other hand, AIF can promote forms of caspase-independent cell death [15]. Mitochondrial or intrinsic type apoptosis relies on BAX and BAK proteins and, therefore, the abrogation of these proteins is a good strategy to interrogate the involvement of this type of apoptosis in the cell death [16,17]. Last but not least, necrotic cell death has also been observed in specific cell lines or apoptotic-deficient ones undergoing starvation-driven autophagy [18,19]. Mounting evidences support the existence of diverse regulated forms of necrosis [20], one of which requires the kinase activity of RIP1 (receptor-interacting protein 1) and RIP3 [20,21]. Necrostatin-1, an inhibitor of the kinase RIP1, is a broadly employed tool to ascertain the participation of necroptosis in a specific lethal context [22].

We sought for a near-physiological manner to elicit autophagy such as the restriction of glucose, nutrients and growth factors. This model resembles the “*in vivo*” context of rapidly growing and insufficiently irrigated tumor. Under these restrictive settings, the role of autophagy on the final fate of cultures was interrogated. We found that a series of tumor and non-tumor cell lines, permanently deprived of glucose, nutrient and growth factor, underwent caspase-dependent mitochondrial-driven apoptosis. At short times, starvation-elicited autophagy was driving the mitochondrial permeabilization, the activation of caspases and cell death. On the other hand, at longer times, autophagy was mitigating cell lethality. Thus, these observations pull together the pro-survival and pro-death functions of autophagy under the same trigger, being the temporal frame the only determinant of the

autophagic outcome. Moreover, we discovered that short-term starvation-driven autophagy sensitized cells to undergo apoptosis in response to several anticancer agents.

2. Materials and Methods

2.1 Cells and cell cultures

Immortalized *Bax*^{-/-}*Bak*^{-/-} MEFs (DKO) and their wild type (WT) counterparts have their origin at late Prof. S.J. Korsmeyer's laboratory. *ATG5* Tet-Off MEFs m5-7 were gently supplied by Dr. Codogno's laboratory (originated at Prof. Mizushima's laboratory). PC3 and DU145 were kindly provided by Dr. Lupold's laboratory (James Buchanan Brady Urological Institute, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA). SH-SY5Y, HeLa, MCF7 cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). SH-SY5Y, MCF7, HeLa and MEFs were maintained in DMEM and PC3 and DU145 in RPMI. All media were purchased from Lonza (Rockland, ME, USA) and contained a 5% volume of FCS (Biochrom AG, Berlin, Germany). 5 µg/ml PlasmocinTM (InvivoGen, San Diego, CA, USA) was used as the media antibiotic. General culturing conditions were 37°C and a water-saturated, 5% CO₂ atmosphere. Culture dishes and other plastic disposable tools were supplied by VWR (Radnor, PA, USA) and Becton Dickinson (Franklin Lakes, NJ, USA).

2.2 Suppression of autophagy in *Atg5* Tet-Off MEFs

To suppress autophagy, *Atg5* Tet-Off MEFs m5-7 [2] were maintained for 4 days in regular DMEM plus 40 ng/ml doxycycline hydrochloride (Sigma-Aldrich, St. Louis, MO, USA). Control populations of *Atg5* Tet-Off MEFs m5-7 undergoing autophagy were obtained by restoring the *ATG5* expression in the former populations. Briefly, after subjecting the cultures to a regular growth medium plus Dox for 3 days, cultures were washed three times with phosphate-buffer saline (PBS) and cultured in Dox-free medium for an

additional 24 h period.

2.3 Transfection of BECLIN-1 siRNA (siBec) or control siRNA (siC)

WT MEFs were subjected to two-rounds of reverse-transfection with the Lipofectamine® RNAiMAX Transfection Reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. Briefly, 3 µl of transfection reagent was used to reverse transfect 100 nM of siBecn1 (5'-CAGUUUGGCACAAUCAUA-3') or of non-targeting siC (5'-UAAGGCUAUGAAGAGAUAC-3') from Sigma-Aldrich (St. Louis, MO, USA) diluted in Opti-MEM™ media (Life Technologies, Carlsbad, CA, USA) into 130 000 WT MEFs (22 mm diameter cell culture plate) growing in regular growing medium supplemented with 2.5 % FBS in the absence of antibiotics. To perform a second round of transfection, the already transfected MEFs were harvested and subjected to another reverse-transfection following the previously described protocol. 24 h into the second round of transfection, FBS was increased to 5 % in the presence of the transfection reagents and cells were allowed to recover for an additional 24 h period. Next, cells were plated to reach a 90% confluence at the time of starvation. To control the efficiency of siBecn1, whole cell extracts of WT MEFs transfected with siC or siBecn1 and subjected to starvation medium (SM), were obtained.

2.4 Cell starvation and treatment

To undergo the glucose, nutrient and growth factor starvation, WT, DKO and *Atg5* Tet-Off MEFs were plated in fresh media at 320 cells/mm² to reach a 90% confluence 24 h later. To be in the same conditions, HeLa cells were plated at 265 cells/mm². Owing to the great susceptibility of MEFs to detach from plastic, cell culture plates were pre-treated with 6 µg/ml of poly-L-Lysine (Sigma-Aldrich, St. Louis, MO, USA) for 1 h. Glucose, nutrient and growth factor starvation was performed by rinsing cells once with PBS and incubating them in SM for the time specified in the figure legend. Composition of SM is 137 mM NaCl, 5.4 mM KCl, 0.25 mM NaH₂ PO₄, 0.44 mM KH₂PO₄, 1.3 mM CaCl₂, 1 mM MgSO₄, 4.2 mM

NaHCO₃, 25 mM HEPES and 15.2 mg/L Phenol red. To pharmacologically inhibit autophagy, 3-MA (Calbiochem®, part of Merck KGaA, Darmstadt, Germany) diluted in SM at a final concentration of 10 mM, was applied to cell cultures for the times specified in the figure legends. To inhibit apoptosis, cell cultures were exposed to (3S)-5-(2,6-Difluorophenoxy)-3-[[[(2S)-3-methyl-1-oxo-2-[(2-quinolinylcarbonyl)amino]butyl]amino]-4-oxo-pentanoic acid hydrate (Q-VD-OPh) from Sigma-Aldrich (St. Louis, MO, USA) diluted in SM at the final concentration indicated in the figure legend. To inhibit necroptosis, cultures were exposed to necrostatin-1 (Tocris Bioscience, Ellisville, MI, USA) diluted in SM. WT and *Atg5* Tet-Off MEFs maintained either in regular full media or SM were further challenged with etoposide and staurosporine purchased from Calbiochem®, part of Merck KGaA (Darmstadt, Germany) or camptothecin and DMSO (as a control) from Sigma-Aldrich (St. Louis, MO, USA). To assay the effects of the necrotic inducers, 7-BIO and PES from Calbiochem® part of Merck KGaA (Darmstadt, Germany) were tested on WT MEFs. Unless otherwise stated, the non-listed reagents were from Sigma-Aldrich (St. Louis, MO, USA).

2.5 Western blot analysis

Cells were challenged with SM in the absence or presence of baf A₁ (Cayman Chemicals, Ann Arbor, MI, USA) for the times indicated in the figure legend. Whole cell extracts were obtained by lysing cells in a buffer containing 100 mM Tris/ClH pH 6.8, 1% SDS, 1 mM EDTA and the protease Inhibitor cocktail set III Calbiochem®, part of Merck KGaA (Darmstadt, Germany). Protein extracts were then boiled at 100°C and sonicated for 20 seconds before being clarified by centrifugation. For cyt C subfractioning, cells treated as indicated in the figure legends, were lysed on ice for 5 min with a buffer containing 50 mM Tris pH 6.8, 150 mM NaCl, 1 mM EDTA, the protease Inhibitor cocktail set III and digitonin (Life Technologies, Carlsbad, CA, USA). Digitonin was adjusted to reach a final 0,04 %

(MEFs) or 0,016 % (HeLa). Protein extracts were centrifuged at 4° for 5 min at 16 000 g. Supernatant (cytosolic subfraction) was collected. After eliminating the traces of the cytosolic subfraction, pellet was lysed for 20 min on ice using a buffer containing 50 mM Tris pH 7.2, 150 mM NaCl, 0.5% NP40, 5 mM EDTA and the protease Inhibitor cocktail set III. Protein extracts were then clarified by centrifugation (pellet subfraction) and quantified by means of the DC Protein Assay (BioRad, Hercules, CA, USA). Volumes were calculated to equalize the protein load in SDS 12%-polyacrylamide gel electrophoresis. Following electrotransfer to 0.45 µm PVDF membranes (EMD Millipore part of Merck KGaA, Darmstadt, Germany), the following antibodies were applied: anti-caspase 3 (#9665) from Cell Signaling, anti-LC3B (L7543) and anti-p62 (P0068) from Sigma-Aldrich (St. Louis, MO, USA), anti-BECLIN-1 (NB110-87318) from Novus Biologicals (Littleton, CO, USA), anti-cyt C (sc-13156) from Santa Cruz Biotech (Dallas, TX, USA), and anti-COX IV (A21348) from Molecular Probes, part of Life Technologies (Carlsbad, CA, USA). Immunoblots were finally developed with the Immobilon™ reagent (Millipore, part of Merck KGaA, Darmstadt, Germany). Chemiluminiscence was recorded and densitometric analysis was performed by means of a Chemidoc™ apparatus and the Image Lab version 4.0.1 software from Bio-Rad (Hercules, CA, USA). Loading of each sample was assessed by staining the membranes during 5 min in a solution containing 10% methanol, 2% acetic acid and 0.1% of Naphthol blue black (Sigma-Aldrich, St. Louis, MO, USA). Then, membranes were de-stained in a 10% methanol and 2% acetic acid solution during 10 min. Membranes were allowed to dry and scanned. Western-blot quantification was the result of referring the densitometric signal of a given sample to the control stated in the figure legend and thus, obtaining the relative content. Graphics represent the average and standard error of the mean (S.E.M) of at least three independent western-blots.

2.6 Cell death and apoptosis assays

To determine cell death, cells were collected by trypsinization and centrifuged 3 min at 1500 r.p.m. Pelleted cells were resuspended in 5 µg/ml propidium iodide (PI) from Sigma-Aldrich (St. Louis, MO, USA) diluted in PBS. Following 15 min incubation at room temperature, cells were subjected to flow cytometry analysis. For subG1 analysis, treated cells were washed in PBS, fixed in 70% cold ethanol and incubated for a minimum of 24h at -80°C. Before staining, fixed cells were rinsed in PBS and resuspended in a PBS solution of 5 µg/ml PI and 100 µg/ml RNase A (Boehringer Ingelheim, Ingelheim am Reim, Germany). After 15 min incubation at room temperature, subG1 fractions were assessed by flow cytometry.

2.7 Caspase activation by DEVD-directed caspase activity

Caspase activity (DEVDase activity) was obtained by quantifying the fluorescence released from Ac-DEVD-afc (Enzo Life Sciences, Farmingdale, NY, USA) substrate after incubation at 37°C in the lysed cultures. This method has been validated and described in our previous work [23]. Values after the subtraction of the basal DEVDase activity from an untreated control were plotted.

3. Results

3.1 Cells under glucose, nutrient and growth factor starvation show autophagic markers and caspase-dependent cell death.

Cell death and autophagy are two common features of severely starved cultures. For this reason, we were interested in unraveling the relationship between cell death and autophagy under these conditions. A time course of cell death was elaborated using a

series of ontogenically divergent tumor-derived cell lines (the breast cancer MCF7, the neuroblastoma SH-SY5Y, the cervix cancer HeLa and the prostate adenocarcinoma PC3 and DU145) subjected to Hank's balanced salt solution without glucose and fetal calf serum, hereafter referred to as starvation medium (SM). Of note, this buffer was partially mimicking "in vitro" the conditions of a tumor under an insufficient or null vascularization. Regardless of the time-dependent disparities, all the cell lines exposed to SM engaged into cell death (Fig. 1A). We focused our attention on HeLa cells because greater levels of cytotoxicity were evident at shorter times of SM treatment when compared to the other cell lines. To interrogate levels of autophagy in HeLa cells, we turned to commonly used markers of the autophagic flux, which are able to discriminate between blockage and induction of autophagy [24]. In this case, we explored the diminution of p62 and the levels of lipidated LC3 (LC3-II) in the presence or absence of the autophagic flux inhibitor bafilomycin A₁ (baf A₁). Baf A₁, an inhibitor of the vacuolar H⁺ ATPase, was preferred over other lysosomotropic agents because of its lower cytotoxicity and nanomolar IC₅₀. The LC3-II increase in the presence of baf A₁ and the p62 reduction without baf A₁ evidenced that, from 6 h up to 16 h of starvation, HeLa cells presented greater levels of autophagy than in basal conditions (Fig. 1B). To corroborate our data through a third independent method, the acidic vesicular organelles (AVOs) were evidenced by acridine orange (AO). As expected, SM gradually increased the incidence of AO-stained orange-red AVOs in cultures of SM-treated HeLa cells while triggering a significant reduction of cell size (data not shown). Overall, these facts were strongly indicating the presence of active autophagy in HeLa cells. Cell death evaluation through propidium iodide (PI) staining revealed the presence of discrete amounts of cell death after 12 h of SM treatment, which sensibly increased to 35.6 ± 1.4 % at 24 h and reached a maximum of 87.7 ± 3 % at 48 h. Starvation can lead to both apoptotic and necrotic subroutines of cell death. To distinguish between these two possibilities, we assessed the protective effects of Q-VD-OPh, a broad

inhibitor of caspases and necrostatin-1, an inhibitor of necroptotic cell death, on SM-driven cell death. While necrostatin-1 did not exert any overt protective effect (data not shown), the inhibition of caspases by Q-VD-OPh resulted in a significant protection of HeLa cells exposed to SM (Fig. 1C). Altogether, these results were suggesting that cells exposed to SM were undergoing an apoptotic cell death. According to the exposed role of caspases mediating starvation-driven apoptotic cell death, the activation of executioner caspases (caspase-3/7) in response to SM was interrogated throughout a 24 h period by measuring the DEVDase activity. HeLa cells responded to SM by inducing a significant activation of the executioner caspases already evident at 8h of treatment (Fig. 1D). Therefore the coexistence of the apoptotic and autophagic programs were again confirmed. In parallel, we evidenced the cleavage of caspase-3 by western-blot, corroborating the results of the enzymatic activities (arrow, Fig. 1E). Finally, we detected that SM was triggering the appearance of subG1 DNA fragmentation in HeLa cells and that Q-VD-OPh was able to suppress it (Fig. 1F).

3.2 Glucose, nutrient and growth factor starvation leads to BAX/BAK-mediated apoptosis.

Bax/Bak^{-/-} double knockout (DKO) mouse embryonic fibroblasts (MEFs) are characterized by a disabled mitochondrial apoptotic pathway and, hence, they are protected facing most apoptotic stimuli, including serum deprivation, loss of attachment and growth factor withdrawal [16]. DKO MEFs are a valuable and broadly used tool to determine the involvement of mitochondrial or intrinsic apoptotic cell death in an experimental paradigm. DKO MEFs and their WT counterparts were subjected to SM and autophagy was assessed in a time-dependent fashion. In WT MEFs, the autophagic flux by LC3-II was quickly increased after 6 h of SM treatment and remained higher than in basal conditions up to the latest time explored (16 h, Fig. 2A). Though with a small delay, p62 levels

confirmed the results obtained by LC3-II (Fig. 2A). Although apoptosis-deficient cells are known to display a more robust response to autophagic triggers [25], DKO MEFs manifested equivalent levels of autophagic flux to their WT counterparts by LC3-II and p62 analysis (Fig. 2A vs Fig. 2B). Likewise, levels of AO-stained orange-red AVOs in DKO MEFs confirmed its equivalency to their WT MEFs counterparts (data not shown). Next, DKO MEFs and their WT counterparts were exposed to SM before interrogating the cell cytotoxicity by PI staining. In WT MEFs, mild but distinctive levels of cell death were evident after 12 h of treatment (23.1 ± 4.1 %, Fig. 2C), reaching a maximum after 24 h of continual starvation (84.1 ± 1.1 %, Fig. 2C). As previously reported in HeLa cells, the pharmacological inhibition of caspases by Q-VD-OPh conferred a significant protection to WT MEFs exposed to SM (Fig. 2C). On the other hand, necrostatin-1 did not confer any protective effect (data not shown). DKO MEFs were amply refractory to cell death by SM (Fig. 2C) and remained alive even after 48 h of treatment (data not shown). To further confirm that cell death was engaging the apoptotic machinery, WT and DKO MEFs were subjected to SM and the caspase activation was assessed through time by the DEVDase activity assay (Fig. 2D). As expected, WT MEFs responded to SM by triggering an early activation of caspases (Fig. 2D). The maximum activation for WT MEFs was reported after 16 h of SM treatment while DKO MEFs exhibited no sign of caspase activation at the interrogated times (Fig. 2D). In the same line, we reported the presence of caspase-3 cleavage fragments in WT MEFs subjected to SM (Fig. 2E) whereas no fragments were seen in starved populations of DKO MEFs (data not shown). Finally, we proved that WT MEFs displayed an increase of the SubG1 fraction rescued by the addition of Q-VD-OPh (Fig. 2F). Altogether, these results were strongly evidencing that HeLa and MEF cells responded to SM by inducing autophagy and caspase-dependent cell death requiring from BAX/BAK-driven mitochondrial permeabilization.

3.3 Glucose, nutrient and growth factor starvation triggers mitochondrial outer membrane permeabilization suppressed by the pharmacological inhibition of autophagy

Our experiments were indicating that the mitochondrial outer membrane permeabilization (MOMP) was a pivotal element in the SM-driven apoptotic cell death. However, we ignored the contribution of SM-driven autophagy in the MOMP. Mitochondrial or intrinsic apoptosis is characterized by the release of pro-apoptotic proteins such as cyt C. To perform these experiments, 3-MA was used as an inhibitor of autophagy. Cellular subfractions of cytosolic soluble proteins (cytosolic) and the remaining cytosolic insoluble proteins (pellet) were obtained at short and long times of SM-treatment in the presence or absence of 3-MA. Purity of the cell subfractions was corroborated by the presence of the mitochondrial cytochrome oxidase IV (COX IV) in the pellet. Protein extracts from HeLa cells and MEFs subjected to long periods of starvation (22 and 18h, respectively) were interrogated for the presence of cyt C in the cytosolic and pellet subfractions (Fig. 3A and 3B). At late times, SM-treated HeLa cells and MEFs exhibited a great translocation of cyt C to the cytosol. The comparison of these cultures in the presence or absence of 3-MA revealed no obvious differences regarding the cytosolic content of cyt C. However, if cyt C levels were observed in the pellet subfraction, 3-MA was decreasing it (Pellet, Fig. 3A and 3B). These results were supporting that the inhibition of SM-driven autophagy was indeed increasing the release of cyt C. Alternatively, we assessed the levels of cyt C using protein extracts of HeLa cells and MEFs subjected to short periods of starvation (8 and 7h, respectively). To our surprise, the inhibition of autophagy by 3-MA triggered a reduction of the cytosolic cyt C. This phenomenon correlated with an increase of its content in the pellet subfraction (Fig. 3A and 3B). Therefore, SM-driven autophagy was triggering the release of cyt C from the mitochondria and the inhibition of autophagy was preventing it. Executioner caspases are terminal proteases in charge of dismantling the cellular physiology during the apoptotic

pathway. Release of cyt C from mitochondria is an upstream event in the activation of caspase-3/7. Therefore, we surmised that the suppression of autophagy would limit the activity of caspase-3/7. As predicted, HeLa cells and MEFs subjected to SM in the presence of the autophagy inhibitor 3-MA, displayed lower levels of caspase activity (Fig. 3C and 3D respectively). In WT MEFs, this trend was maintained up to late times, when the caspase activation of cultures in the presence or absence of 3-MA reached equivalent levels (Fig. 3D).

Owing to the unspecific effects of drugs such as 3-MA, we thought of using an alternative method to block the nucleation step of autophagy. BECLIN-1 associates to PtdInsKC3, becoming a crucial protein for the induction of canonical autophagy and a common target of inhibitory genetic strategies. To reach the greatest diminution of BECLIN-1 protein, MEFs subjected to full growth medium underwent two rounds of transfection with a *Beclin-1* siRNA (siBecn1) or a control siRNA (siC) over a 4 days time period. These conditions resulted in a dramatic reduction of BECLIN-1 protein (Fig. 3E) and the blockage of SM-driven autophagy, as shown by the absence of p62 reduction after 6 h of starvation (Fig. 3E). In regard to the activation of caspases, MEFs transfected with a siC and subjected to SM presented greater levels of active caspases than MEFs with reduced levels of BECLIN-1 (Fig. 3F). These results were evidencing, by a non-pharmacological approach, that canonical autophagy was indeed regulating SM-driven apoptotic cell death.

3.4 Glucose, nutrient and growth factor starvation requires Atg5-dependent canonical autophagy to prompt an early permeabilization of mitochondria

Canonical autophagy relies on proteins such as ATG5 for the elongation and closure of the autophagosomes and consequently, the *Atg5* Tet-Off MEFs m5-7 is an inestimable tool to study it. This cell line was generated from *Atg5* ^{-/-} MEFs and offers the possibility of transiently knocking-down an exogenous form of Atg5 by a 4 days doxycycline (Dox)

treatment [2]. We first assessed the autophagic flux of *Atg5* Tet-Off MEFs subpopulations maintained or not in Dox over a 16 h period. As expected, the presence of Dox abrogated the autophagic flux detected by LC3-II and p62 levels (Fig. 4A). The study of cyt C localization evidenced that at long times of treatment, the Dox-treated population of *Atg5* Tet-Off MEFs released greater levels of cyt C to the cytosol than the populations kept in media without Dox (16 h, Fig. 4B). A concomitant decrease of cyt C in the pellet subfractions was reported (Fig. 4B). These findings were compatible with autophagy having a protective effect over the mitochondrial permeabilization. Remarkably, we found that at short times of treatment, this behavior was again reversed. Cytosolic extracts from Dox-treated *Atg5* Tet-Off MEFs exhibited lower cytosolic levels of cyt C than *Atg5* Tet-Off MEF not exposed to Dox (4 h, Fig. 4B). Accordingly, for the first 16 h of SM treatment, the activity of executioner caspases was significantly lower in Dox-treated *Atg5* Tet-Off MEFs than in cultures not exposed to Dox (Fig. 4C). Altogether, these findings were corroborating that, at short times, canonical autophagy (ATG5-, BECLIN-1- and PtdInsKC3-dependent) exhibited a relevant activity promoting the mitochondrial permeabilization and caspase activation.

3.5 SM-driven autophagy triggers time-dependent opposite effects on cell death

In an attempt to clarify the effects of these time-depending events on the final fate of cultures, cell cytotoxicity was monitored from 12 h to 24 h in the presence or absence of 3-MA. MEFs are characterized by completing cell death in response to SM in a briefer time frame than HeLa cells (Fig. 1C compared to Fig. 2C). Owing to this behavior, they were selected for these studies. At 12 h, SM-mediated cell cytotoxicity was prevented by the inhibition of autophagy (Fig. 5A). However, starting at 16 h, the presence of 3-MA enhanced cell toxicity in response to SM (46 ± 2.47 vs 78.65 ± 1.4 , Fig. 5A). Since 3-MA is reported to promote autophagy in nutrient-rich conditions [26], its inhibitory activity under

starved conditions was reassessed. As shown in Fig. 5B, 3-MA was able to significantly block the p62 decrease, indicating autophagy was impeded. Once the shift had been clearly established in WT MEFs, we attempted to demonstrate the reproducibility of this phenomenon in the remaining cell lines. First, for each cell line we selected an early time, where caspase activity was reduced after inhibiting autophagy and, a later time, where caspase activity was independent of the autophagic status. A growing body of evidence supports that the ability of autophagy to inhibit or promote apoptosis is highly cell line- and context-dependent [27,28]. However, at early times, inhibition of autophagy provoked a reduction of the cell death in all the cell lines tested irrespective of the inhibitory approach used (Fig. 5C). On the contrary, as previously reported by other groups [1,29], the continuous suppression of autophagy for long periods of time (16 h or longer for the studied cell lines) maximized the cell cytotoxicity in response to SM (Fig. 5D). Notably, the complete protection DKO MEFs exposed to SM in the presence or absence of 3-MA for 24h (4.9 ± 2.6 % vs 7.9 ± 1.2 %, Fig. 5C) was indicating that, after inhibiting autophagy, cell death also required the mitochondrial permeabilization via BAX/BAK. These results were reaffirming the previously established role of autophagy in survival of long-term starved cell cultures but, interestingly, they were also highlighting a role of autophagy promoting early forms of caspase-dependent BAX/BAK-dependent apoptotic cell death.

3.6 Short times of SM-driven autophagy potentiates caspase-dependent apoptosis of several anticancer agents

Insufficiently irrigated solid tumors naturally undergo autophagy in response to transient periods of starvation [18]. We speculated about the contribution of autophagy to the cell lethality facing a series of antitumor agents (data not shown). Among them, we selected the compounds that were eliciting $\geq 20\%$ of PI positive cells in the presence of SM for a 10h period of time, precisely etoposide (Eto) and camptothecin (CPT). In addition, we

included staurosporine (STP) as a standard inducer of intrinsic apoptosis that was also triggering ≥ 20 % cell death at 10h in SM. Short starvation periods greatly increased the sensitivity of cultures to all the assayed pro-apoptotic drugs (Fig. 6A), supporting that severe starvation primes cells to die by caspase-dependent mechanisms. Then, MEFs maintained in SM in the presence or absence of 3-MA were further treated with 0.150 μ M STP, 50 μ M Eto and 10 μ M CPT for 10 h before assessing cell death by PI staining. In each case, cell death was significantly inhibited whenever autophagy was blocked by 3-MA (Fig. 6C). Similarly, the addition of Q-VD-OPh prevented the pharmacologically-elicited apoptotic cell death of the starved cultures (Fig. 6C). In most cases, the protection conferred by the general inhibition of caspases was equivalent to the one provided by the suppression of autophagy. These findings were supported by the caspase activity assays. Whenever MEFs were impeded to undergo autophagy (Fig. 6E), reduced levels of caspase activation were found, even in the presence of the anticancer drugs. To discard an unspecific effect of 3-MA on caspase activation, we reproduced these results in the *Atg5* Tet-Off MEFs. We observed that the simple introduction of cells in SM, primed them to undergo caspase-dependent apoptotic cell death in response to Eto, STP and CPT (Fig 6B). The suppression of ATG5 reduced the sensitivity of these cultures for engaging into apoptotic cell death (Fig. 6D). Cell demise was prevented by the general inhibitor of caspases Q-VD-OPh (Fig. 6D), proving again that we were facing a caspase-dependent apoptotic cell death subroutine. To further reassert these findings, the caspase activity of cultures under the former conditions was evaluated by DEVDase activity assay. Autophagy deficient subpopulations of *Atg5* Tet-Off MEFs (Fig. 6F) exhibited reduced levels of caspase activity in SM when compared to the same cells undergoing autophagy. Addition of STP, CPT and Eto to the deprived medium increased the caspase activity in comparison to the levels reached in SM by itself ("C", Fig. 6F). Nonetheless, the autophagy deficient subpopulations reached significantly lower levels of caspase activity

for each of the used drugs (Fig. 6F). Next, we wondered whether the lethal actions of autophagy would be restricted to the context of apoptotic drugs or, on the contrary, would be also evident when applying necrotic triggers. To perform these experiments, 7-bromo-indirubine-3-oxime [30] (7BIO) and 2-phenylethynesulfonamide [31] (PES), two caspase-independent necrotic inducers were employed. Remarkably, WT MEFs exposed to SM in the presence of 3-MA were as sensitive to the lethal effects of 7BIO and PES as the autophagic-competent cells (Fig. 7). We concluded that 3-MA protective actions were a singularity of caspase-dependent apoptotic inducers. Altogether, these results were strongly proving that in the context of starvation, autophagy was accelerating the naturally occurring or pharmacologically-imparted caspase-dependent apoptotic cell death.

4. Discussion

Severe forms of starvation are reported to elicit both autophagy and cell death [1,12]. The interplay between autophagy and cell death processes is a matter of controversy. Here we show how nutrient, glucose and growth factor deprivation elicit cell death in a variety of ontogenically divergent cell lines. At short times, starvation-driven caspase-dependent apoptosis relies on the machinery of autophagy and requires the caspase activation and the mitochondrial permeabilization via BAX/BAK. The ongoing SM-driven autophagy relies on ATG5, BECLIN-1 and the activation of PtdInsKC3, as proven by 3-MA. On the contrary, at longer times of starvation, the cell death is characterized by its independency from the autophagy machinery. Moreover, at this time, the inhibition of autophagy is promoting cell survival. When focusing on short-term starvation, autophagy-driven caspase-dependent cell death is enhancing the apoptotic cell death in response to several pro-apoptotic antitumor drugs. Accordingly, either the inhibition of autophagy or the blockage of executioner caspases, translate into a strong protection facing these antitumor drugs.

Growing tumors are naturally exposed to periods of insufficient or null irrigation [4,18]. Similarly, ischemia is a pathological situation characterized by a sudden interruption of blood supply [12]. These cellular contexts share a common trait, a shortage of oxygen, nutrients, glucose and trophic factors. Several approaches have intended to mimic these environments *in vitro* by combining the depletion of single amino acids, glucose, growth factors, oxygen and serum. Starvation is a powerful signal to activate macroautophagy, a bulk form of autophagy, triggered to cope with the energetic and metabolic demands of a deprived cell. During the completion of autophagy, the lysosome-mediated degradation of cytoplasmic material will supply cells with energy and metabolites that cannot be synthesized *de novo*. Moreover, anaplerotic reactions taking place during starvation, will sustain the necessary biosynthesis of proteins and nucleic acids. In addition, cataplerotic pathways will efficiently intertwine the metabolic intermediates as needed. Sufficient levels of energy will be obtained, for instance, through cataplerotic reactions and the beta-oxidation of fatty acids. Therefore, privation of one single nutrient, sugar or lipid can create a physiological compensation that can alter the real effect of starvation. Our work was performed with a medium depleted of all these components to avoid any kind of artifact. It is worth to mention the controversy over the role of glucose withdrawal in the induction of autophagy [19,32,33]. Interestingly, a recent publication shows that the simple glucose withdrawal triggers cell death by either apoptosis or necrosis without an apparent induction of the autophagic flux [19]. However, we proved that the composition of our buffer and, specifically, the absence of glucose, did not impede a series of ontogenically divergent cell lines to undergo autophagy (Fig. 1B, 2A, 2B). In agreement with their findings, we observed that the reintroduction of glucose into our complete starvation media (SM) slightly increased the autophagic flux of WT MEFs (data not shown), thus supporting glucose is positively regulating the autophagic flux.

Here, we provide evidence that mitochondria play a seminal role in the clearance of starved mammalian cells. In agreement with our data, interleukine-3 (IL-3) withdrawal evidenced the relevance of BAX/BAK-regulated mitochondrial homeostasis in the apoptotic demise of IL-3 dependent bone marrow-derived cells [1]. Similarly, primary neonatal rat cardiac myocytes under glucose and serum withdrawal were cleared through the mitochondrial apoptotic pathway of cell demise [12]. Interestingly, the pharmacological inhibition of caspases with zVAD-fmk prevented the terminal features of apoptosis without restoring the cellular morphology and the contractile capability of these myocytes, thus suggesting that energy depletion and caspase-independent events might be also important players in this process. In line with these findings, the general inhibition of caspases is not fully protecting HeLa and WT MEFs from SM-driven cell death. For instance, cell cytotoxicity in HeLa cells (48 h) and WT MEFs (24h) subjected to SM in the presence of Q-VD-OPh is $47 \pm 4.5 \%$ and $61 \pm 5.2 \%$, (Fig. 1C and 2C, respectively). In addition, only BAX/BAK deficient cells become refractory to long-term SM-driven cell death (Fig. 2C). It would then appear that the early induction of intrinsic apoptosis would lead starved cells into cell death. In this sense, we have observed an early release of AIF from mitochondria (data not shown), which added to the release of other pro-death proteins from this organelle, would justify why BAX/BAK deficient MEFs surpass Q-VD-OPh in their protecting capacity. Others have reported that the type of cell death subroutine engaged in response to starvation is cell-line dependent rather than stimulus-dependent. For example, under glucose starvation Rh4 rhabdomyosarcoma cells undergo necrotic cell death while HeLa cells and MEF, apoptotic cell death [19]. We cannot predict whether all the cell lines under SM would be consistently undertaking the same subroutine of cell death. In this sense, density could be playing a determinant role in the type of cell death, necrotic or apoptotic, in response to serum withdrawal [34]. Therefore we cannot rule out if changes in

the 90% density, the standard cell confluency of our experiments, will alter the type of cell death.

Despite the early anti-apoptotic effects achieved through inhibition of autophagy, we demonstrate that long-term starved cultures, unable to undergo autophagy, exhibit a greater cytotoxicity. Thus indicating that after long times of starvation, autophagy acts as a protective mechanism. To perform these experiments, autophagy was suppressed for a long period of time and thus, one possible explanation for the increased death is the inefficiency of our approaches to block autophagy. This hypothesis is especially interesting in the context of a chemical inhibitor such as 3-MA. This drug presents opposite effects regulating autophagy under nutrient-rich (promotion) and nutrient-poor (inhibition) conditions caused by disparities in the kinetics of class I and III PtdIns 3-kinase inhibition [26]. Owing to these discrepancies, the inhibitory effects of 3-MA at long times of starvation were confirmed by analyzing the levels of p62 by western blot (Fig. 5B) and AO-positive vesicles by fluorescent microscopy (data not shown). In addition to the genetic strategies employed (siBecn1 and ATG5 deficient cells), we thought of using a second chemical inhibitor of autophagy, spautin-1 [35]. Unfortunately, while spautin-1 was able to block the caspase activation at short times of starvation, it presented a greater lethal profile than 3-MA when maintained for long times in starved cultures (results not shown). As a second hypothesis, we propose the activation of a second wave of ATG5-, Beclin-1- and Vps34-independent form of non-canonical autophagy. However, the preservation of p62 content and the diminished levels of AO-positive vesicles by 3-MA argue against this idea (data not shown). The third hypothesis has to do with mitochondria and energy. Only the absence of BAX/BAK confers a full protection (up to 48h) facing the inhibition of autophagy, pointing out to a prominent role of the mitochondrion or mitochondrial-related phenomena in the induction of cell death. In this sense, a time-dependent decrease in the energy production is a natural response to the glucose withdrawal and ensuing glycolytic

production of ATP. Under these circumstances, the beta-oxidation of fatty acids and the respiration of metabolic intermediates originated after the degradation of cytoplasmic material within the autophagolysosomes, might be partially compensating the shortage of ATP. However, the inhibition of autophagy might be diminishing these alternative pathways of energy production. In this sense, autophagy is reported to act as a protective mechanism against bioenergetic catastrophe [36]. It is then possible to hypothesize that, either the exhaustion of ATP resources or the decrease of ATP levels under a hypothetical threshold, might be playing a relevant role in the death ensuing SM-driven autophagy inhibition. To illustrate this situation, we find the case of immortalized interleukin-3 (IL-3) dependent bone marrow cell lines from *Bax*^{-/-}*Bak*^{-/-} mice, which are protected from apoptosis in response to IL-3 withdrawal. In this settings, inhibition of autophagy by 3-MA or chloroquine is eliciting cell death. However, the supplementation of a permeable form of pyruvate intended to produce NADH and ATP restores the viability of these cells [1].

An expanding body of evidence sustains that autophagy is governing apoptotic cell death under specific developmental circumstances. For example, it is known that autophagy drives caspase-dependent and independent cell death necessary for the regression of *Drosophila melanogaster* salivary glands [37]. However, the truth is that *Atg5*, *Atg7* and *Beclin-1* knockout mice do not present deficiencies in the developmental forms of programmed cell death [38]. Notwithstanding these discrepancies, multiple findings support that, in adult cells, autophagy does regulate other subroutines of cell death under context-specific circumstances. Putting aside those models where autophagy seems to be by itself the ongoing subroutine of death, all gathered into the common term “autophagic cell death”, it is noteworthy the existence of autophagy-dependent apoptotic cell death models [39–46]. Our findings fall into this last scenario since, at short times of starvation, autophagy is regulating apoptotic cell death. For example, overexpression of ATG1 in fly exacerbates autophagy, resulting in increased apoptosis [39]. In rat striatal neurons, over-

stimulation of kainic acid (KA) receptors activates autophagy-driven intrinsic apoptosis, thus placing autophagy upstream of KA-driven excitotoxicity [40]. A similar case is found in cortical neurons since STP-elicited autophagy is driving the apoptotic cell death [41]. Similarly, clearance of lymphoblastic leukemia cells exposed to the glucocorticoid dexamethasone requires the induction of autophagy prior to the mitochondrial apoptotic death [42]. A similar response is observed in multiple myeloma cells treated with dexamethasone [43]. Inhibition of autophagy prevents the apoptosis ensuing TNF α treatment of sarcoma cells with an inhibited NF- κ B pathway [44], ER stress in non-transformed fibroblast [45], and serum and potassium deprivation of cerebellar granule cells [46]. Unfortunately, there is no consensus on the precise mechanism intertwining autophagy and apoptosis. A growing number of distinct pathways with key proteins are being proposed [13,17,47–52]. For example, one of these proteins is FoxO1. The protein by itself and its localization are key factors in determining the induction of autophagic and apoptotic cell death in HCT116 and HeLa cells subjected to serum starvation or hydrogen peroxide [47]. Yet another protein that links the autophagic and apoptotic phenomena is ATG5. The calpain-dependent cleavage of ATG5 into a 24-kDa cleavage fragment uncovers a “BH3-only” function of this protein [48]. Notably, these findings are in agreement with calpain being required for the induction of macroautophagy in mammalian cells [49]. Although in our experimental paradigms the suppression of ATG5 reduces the short-term SM-driven apoptosis, in the same settings, the inhibition of calpain by the pharmacological inhibitor calpeptin did not confer any protection facing SM (data not shown). Thus, we don’t favor calpain and ATG5 cleavage as key regulators in the induction of autophagy-driven apoptosis by SM. Interestingly, ATG5 is also a validated link between autophagy and the extrinsic pathway of apoptosis. ATG5 forms a complex with the death receptor adaptor protein FAS-associated death domain (FADD) and caspase-8, bringing this complex in proximity to p62/SQSTM1. These three proteins participate in the

activation of caspase-8 and the ensuing apoptotic cell death [50]. Another possible mechanism to link autophagy and apoptosis is the covalent assembly of the ATG12-ATG3 complex occurring during starvation. While the proper assembly of this complex triggers intrinsic apoptotic cell death, its disruption elicits the enlargement of the mitochondrial mass and a concomitant increase of the anti-apoptotic Bcl-2 proteins [51]. Alternatively, HCT116 colon cancer cells subjected to nutrient-serum starvation display a concomitant upregulation of PUMA and p21. While p21 presents unknown protective effects at the mitochondrial level, PUMA is in charge of eliciting the intrinsic apoptosis [13]. Yet in another system, the simple overexpression of PUMA or BAX causes a form of selective mitophagy accompanied by cyt C release and apoptotic cell death. In these experimental paradigms, down-regulation of several ATGs has protective effects facing apoptosis, suggesting that autophagy contributes to the apoptotic response [17]. Moreover, in support of our findings, the same group concluded that autophagy might be contributing to the efficient release of cyt C and so, to the induction of apoptosis. Other authors propose the disturbance of an autophagy-driven epigenetic program as the key element to shift autophagy from a pro-survival to a pro-death mechanism. Namely, the down-regulation of the histone acetyltransferase hMOF, the ensuing reduction in H4K16 acetylation level and the transcriptional regulation of autophagy-related genes are steps necessary for the proper progression of autophagy and its pro-survival effects [52]. We ignore the relevance of each of these pathways in our experimental paradigm. We propose future high throughput studies to address this issue.

Our work underscores the paramount role of autophagy inducing caspase-dependent apoptotic cell death in short-term starved cells and, under the same circumstances, enhancing the caspase-dependent apoptotic cell death in response to a series of apoptotic stressors. In this sense, autophagic cells in core areas of multicellular spheroids, which mimic the three dimension structure of a tumor, undergo glucose shortage, which make

them particularly sensitive to the mitochondrial dysfunction [36]. In our studies, we used a series of apoptotic drugs that are known to elicit the intrinsic apoptotic pathway and hence, that trigger the mitochondrial failure. We observe that, in comparison to the same cells in full media, a short-term starvation is strongly favoring the apoptotic cell demise (Fig. 6A and 6D). In the same line, HCT116 cells up-regulating PUMA in response to nutrient-serum starvation, display an increased sensitivity to the Bcl-X_L inhibitors [13]. Alternatively, the abrogation of ATG3-ATG12 complex protects cells from mitochondrial pro-apoptotic triggers [51]. On the opposite, our results prove that cell death in response to drugs that don't require the BAX/BAK pro-apoptotic gateway (7BIO and PES, Fig. 7), remains unaffected by starvation.

In conclusion, our manuscript introduces the first model in cell culture characterized by time being the only determinant of the modulation of apoptosis by autophagy. The shift from apoptosis promotion to inhibition has been circumscribed temporally, thus simplifying the research of molecular events governing it. Moreover, the shift has translational implications because it becomes a crucial factor determining the outcome of chemotherapy in solid tumors. In addition, it remarks the heterogeneity present in a tumor mass, not only of genotypic or phenotypic nature but chrono-biological as well. In other words, concerning cell death mechanisms, time is a coordinate to be taken into account in the starvation conditions imposed by the solid tumor biology.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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FIGURE LEGENDS

Figure 1. Severe starvation upregulates autophagic markers and triggers caspase-dependent cell death **A.** Tumor-derived cell lines were maintained in starvation media (SM) for the times indicated in the figure. Cell death was analyzed by PI incorporation. Values are expressed as mean $\pm$ SD (n=3) **B.** Protein extracts of HeLa cells challenged with SM for the indicated times, were analyzed by western blot. LC3-II and p62 levels in the presence (+) or absence (-) of 100 nM baf A₁ were assessed. *Naphthol blue* (NB) stained membrane served as a loading control. Histograms represent p62 and LC3-II “Relative content” referred to untreated cells at time 0. Relative content is expressed as mean $\pm$ SEM of at least 3 independent experiments **C.** HeLa cells were subjected to SM for the indicated times in the presence or absence of 10 μ M Q-VD-OPh (QVD). Cell death was measured as in A and expressed as mean $\pm$ SD (n=3); Student’s t-test * P < 0.01, *** P < 0.001 **D.** Time course of caspase activation (DEVDase activity) using HeLa cells treated with SM for the times indicated at the figure. Activity is expressed as mean $\pm$ SD (n=3) and measured as arbitrary units of fluorescence (a.u.f.) **E.** Processing of caspase-3 in HeLa cells challenged with SM for the indicated times, was analyzed by western blot. Black arrow indicates cleaved caspase-3. Plot below represents the processed caspase-3 “Relative content” referred to untreated cells at time 0. Relative content is expressed as mean $\pm$ SEM of at least 3 independent experiments **F.** HeLa cells were subjected to SM (-QVD) or SM plus Q-VD-OPh (+QVD) for the indicated times. Apoptosis (indicated by sub-G1 DNA content) were measured by flow cytometry after PI staining and cell permeabilization. Values are expressed as mean $\pm$ SD (n=3); Student’s t-test *** P < 0.001.

Figure 2. Severe starvation leads to mitochondrial-dependent apoptosis **A.** Protein extracts from WT MEFs and **B.** DKO MEFs subjected to SM for the indicated times, were analyzed by western blot. LC3-II and p62 levels in the presence (+) or absence (-) of 100 nM baf A₁ were assessed. *Naphthol blue* (NB) stained membrane served as a loading control. Histograms represent p62 and LC3-II “Relative content” referred to untreated cells at time 0. Relative content is expressed as mean $\pm$ SEM of at least 3 independent experiments **C.** DKO MEFs and WT MEFs were challenged with SM in the presence or absence of 50 μ M Q-VD-OPh for the time indicated. Cell death was analyzed by PI incorporation. Data are expressed as mean $\pm$ SD (n=3); Student’s t-test ** P< 0.005, *** P< 0.001 **D.** WT and DKO MEFs were treated with SM for the indicated times and caspase activation (DEVDase activity) was determined at time points indicated. Activity is measured in arbitrary units of fluorescence (a.u.f.). Data are expressed as mean $\pm$ SD (n=3) **E.** Processing of caspase-3 in WT MEFs challenged with SM for the indicated times, was analyzed by western blot. Black arrow indicates cleaved caspase-3. Plot below represents the processed caspase-3 “Relative content” referred to untreated cells at time 0. Relative content is expressed as mean $\pm$ SEM of at least 3 independent experiments **F.** WT MEFs were subjected to SM (-QVD) or SM plus Q-VD-OPh (+QVD) for the indicated times. Apoptosis (indicated by subG1 DNA content) were measured by flow cytometry after PI staining and cell permeabilization. Values are expressed as mean $\pm$ SD (n = 3); Student’s t-test ** P < 0.005, *** P < 0.001.

Figure 3. SM-driven autophagy triggers early permeabilization of mitochondria and caspase activation **A.** HeLa cells and **B.** WT MEFs were treated with SM in the absence or presence of 10 mM 3-MA for the indicated times. Following a complex sub-fractioning protocol, cytosolic and pellet subfractions were analyzed by western blot. Bands immunoreactive with anti-cyt C and anti-COX IV antibodies are shown. *Naphthol blue* (NB) stained membrane served as a loading control. Histograms represent cyt C “Relative

content". In the cytosolic subfraction, the unit value of reference is the amount of cyt C released by cells at the longest time of starvation in the absence of 3-MA. In the pellet subfraction, the unit value of reference is the amount of cyt C in mitochondria of untreated cells at time 0. Relative content is expressed as mean $\pm$ SEM of at least 3 independent experiments **C.** HeLa cells and **D.** WT MEFs were treated with SM in the presence or absence of 10 mM 3-MA and caspase activity (DEVDase activity) was quantified through time. Activity is measured in arbitrary units of fluorescence (a.u.f.); Student's t-test * $P < 0.01$, ** $P < 0.005$, *** $P < 0.001$ **E.** WT MEFs subjected to two rounds of transfection with an siRNA against *Beclin-1* (siBecn1, #3) or an siRNA control (siC, #2) were challenged with SM for the indicated times. As a control, untransfected WT MEFs (#1) are shown. Whole protein extracts were processed for western blot and analyzed with anti-p62 and anti-BECLIN1 antibodies. *Naphthol blue* (NB) stained membrane was used as a loading control. Histograms represent p62 and BECLIN-1 "Relative content" referred to untreated cells at time 0 (#1). Relative content is expressed as mean $\pm$ SEM of at least 3 independent experiments **F.** Time course of caspase activation (DEVDase activity) using WT MEFs transfected with siBecn1 or siC and treated with SM. Activity is measured in arbitrary units of fluorescence (a.u.f.) and data are expressed as mean $\pm$ SD of two independent experiments with 3 independent measurements per condition; Student's t-test *** $P < 0.001$.

Figure 4. SM-driven ATG5-dependent autophagy triggers early permeabilization of mitochondria and caspase activation **A.** **B.** and **C.** *Atg5* Tet-Off MEFs in the absence (+Dox) or presence of ATG5 (-Dox) were challenged with SM for the stated times. When indicated, 100 nM baf A₁ was added for the last 3h of treatment. **A.** Protein extracts were analyzed by western blot using anti-LC3 and anti-p62 antibodies. *Naphthol blue* (NB)

stained membrane served as a loading control. Histograms represent p62 and LC3-II “Relative content” referred to untreated cells in the presence of ATG5 (-Dox) at time 0. Relative content is expressed as mean $\pm$ SEM of at least 3 independent experiments **B**. Following a subfractioning protocol, cytosolic and pellet subfractions were analyzed by western blot. Bands immunoreactive with anti-cyt C and anti-COX IV antibodies are shown. *Naphthol blue* (NB) stained membrane served as a loading control. Histograms represent cyt C “Relative content”. In the cytosolic subfraction, the unit value of reference is the amount of cyt C released by cells in the presence of ATG5 (-Dox) at the longest time of starvation. In the pellet subfraction, the unit value of reference is the amount of cyt C in mitochondria of untreated cells in the presence of ATG5 (-Dox) at time 0. Relative content is expressed as mean $\pm$ SEM of at least 3 independent experiments **C**. Time course of caspase activity (DEVDase activity). Activity is measured in arbitrary units of fluorescence (a.u.f.). Data are expressed as mean $\pm$ SD (n=3); Student’s t-test ** $P < 0.05$, *** $P < 0.001$.

Figure 5. SM-driven autophagy triggers time-dependent opposing effects on cell death **A**. WT MEFs were challenged with SM over a 24 h period in the presence or absence of 10 mM 3-MA. A time course of cell death was obtained by PI incorporation. Values are expressed as mean $\pm$ SD (n=3); Student’s t-test * $P < 0.01$, ** $P < 0.005$, *** $P < 0.001$ **B**. Whole cell extracts from WT MEFs subjected to SM in the presence or absence of 10 mM 3-MA were analyzed by western blot with a p62/SQSTM1 antibody. *Naphthol blue* (NB) stained membrane was used as a loading control. Plot represents p62 “Relative content” referred to 3-MA-treated cells after a 12 h period of starvation. Relative content is expressed as mean $\pm$ SEM of at least 3 independent experiments **C**. and **D**. *Atg5* Tet-Off MEFs in the absence (+Dox) or presence of ATG5 (-Dox) were subjected to SM. In parallel, HeLa cells and WT MEFs were subjected to SM in the presence or absence of 3-MA **B**. Cell death at 10 h of starvation was measured as in A. Values are expressed as

mean $\pm$ SD (n=3); Student's t-test * $P < 0.01$, ** $P < 0.005$ **C.** Cell death at 18h (*Atg5* Tet-Off MEFs) or 24 h of starvation (WT MEFs and HeLa) was measured as in A. In addition, cell death of DKO MEFs starved for 24 h in the presence or absence of 10 mM 3-MA was plotted. Values are expressed as mean $\pm$ SD (n=3); Student's t-test * $P < 0.01$, ** $P < 0.005$.

Figure 6. SM-driven canonical autophagy sensitizes to apoptosis by several anticancer agents **A.** WT MEFs and **B.** *Atg5* Tet-Off MEFs in the absence of Dox were maintained in full growth medium (FM) or starvation-medium (SM) and treated with vehicle (C, DMSO), staurosporine (STP, 0.250 μ M), camptothecin (CPT, 10 μ M) or etoposide (Eto, 50 μ M) for 10h. Cell death was measured by PI incorporation. Values are expressed as mean $\pm$ SD (n=3) **C.** WT MEFs and **D.** *Atg5* Tet-Off MEFs in SM were further subjected to treatments with 3-MA, Q-VD-OPh (QVD) and Dox as indicated. Then, they were subjected to a treatment with the same drugs used in A. Cell death was obtained by PI incorporation. Values are expressed as mean $\pm$ SD (n=3) Student's t-test ** $P < 0.005$, *** $P < 0.001$ **E.** WT MEFs in the presence or absence of 3-MA **F.** *Atg5* Tet-Off MEFs in the presence or absence of Dox were further treated with vehicle, STP, CPT or Eto for the times indicated in the figures. Caspase activation (DEVDase activity) was determined. Activity is measured in arbitrary units of fluorescence (a.u.f.) and expressed as mean $\pm$ SD (n=3); Student's t-test ** $P < 0.005$, *** $P < 0.001$.

Figure 7. Short times of starvation do not increase cytotoxicity of WT MEFs in response to necrotic inducers. WT MEFs exposed to SM (Apoptosis in the figure) were further challenged for 10 h with 25 μ M 7BIO and 20 μ M PES (Necrosis in the figure) in the presence or absence of 10 mM 3-MA. Cell death was then measured by flow cytometry and the count of PI-stained cells. Values are expressed as mean $\pm$ SD of two independent experiments with 3 independent measurements per condition.

Figure 1. Mattiolo, P. *et al.*

Figure 2. Mattiolo, P. *et al.*

Figure 3. Mattiolo, P. *et al.*

A

B

C

Figure 4. Mattiolo, P. *et al.*

Figure 5. Mattiolo, P. *et al.*

Figure 6. Mattiolo, P. *et al.*

Figure 7. Mattiolo, P. *et al.*