

Spermidine and resveratrol induce autophagy by distinct pathways converging on the acetylproteome

Eugenia Morselli,1,2,4 Guillermo Maríño,1,2,4 Martin V. Bennetzen,5 Tobias Eisenberg,5 Evgenia Megalou,7 Sabrina Schroeder,6 Sandra Cabrera,8 Paule Bénit,9 Pierre Rustin,9 Alfredo Criollo,1,2,4 Oliver Kepp,1,2,4 Lorenzo Galluzzi,1,2,4 Shensi Shen,1,2,4 Shoaib Ahmad Malik,1,2,4 Maria Chiara Maiuri,1,2,4 Yoshiyuki Horio,10 Carlos López-Otín,9 Jens S. Andersen,5 Nektarios Tavernarakis,7 Frank Madeo,6 and Guido Kroemer1,2,3,11,12,13

Introduction

Macroautophagy (which we refer to as autophagy) is a cellular self-cannibalistic pathway in which parts of the cytosol or cytoplasmic organelles are enveloped in double-membraned vesicles, autophagosomes, which then fuse with lysosomes (Klionsky, 2007). Autophagy plays a major role in the maintenance of cellular homeostasis, allows for the mobilization of energy reserves when external resources are limited, and is essential for the removal of damaged organelles and potentially toxic protein aggregates (Levine and Kroemer, 2008). At the organismal level, autophagy can mediate cytoprotection (for instance neuroprotection and cardioprotection in the context of ischemic preconditioning; Moreau et al., 2010) and delay the pathogenic manifestations of aging (Levine and Kroemer, 2009). Given the reactions in the cytosol and in the nucleus, respectively. Both resveratrol and spermidine were able to induce autophagy in cytoplasts (enucleated cells). Moreover, a cytoplasm-restricted mutant of SIRT1 could stimulate autophagy, suggesting that cytoplasmic deacetylation reactions dictate the autophagic cascade. At doses at which neither resveratrol nor spermidine stimulated autophagy alone, these agents synergistically induced autophagy. Altogether, these data underscore the importance of an autophagy regulatory network of antagonistic deacetylases and acetylases that can be pharmacologically manipulated.

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tophagy protects organelles, cells, and organisms against several stress conditions. Induction of autophagy by resveratrol requires the nicotinamide adenine dinucleotide–dependent deacetylase sirtuin 1 (SIRT1). In this paper, we show that the acetylase inhibitor spermidine stimulates autophagy independent of SIRT1 in human and yeast cells as well as in nematodes. Although resveratrol and spermidine ignite autophagy through distinct mechanisms, these compounds stimulate convergent pathways that culminate in concordant modifications of the acetylproteome. Both agents favor convergent deacetylation and acetylation reactions in the cytosol and in the nucleus, respectively. Both resveratrol and spermidine were able to induce autophagy in cytoplasts (enucleated cells). Moreover, a cytoplasm-restricted mutant of SIRT1 could stimulate autophagy, suggesting that cytoplasmic deacetylation reactions dictate the autophagic cascade. At doses at which neither resveratrol nor spermidine stimulated autophagy alone, these agents synergistically induced autophagy. Altogether, these data underscore the importance of an autophagy regulatory network of antagonistic deacetylases and acetylases that can be pharmacologically manipulated.

Abbreviations used in this paper: ABD, acetylation background dataset; CoA, coenzyme A; GO, gene ontology; MS, mass spectrometry; mTOR, mechanistic target of rapamycin; NGM, nematode growth medium; SILAC, stable isotope labeling with amino acids in cell culture; WT, wild type.

E. Morselli and G. Maríño contributed equally to this paper.
Correspondence to Guido Kroemer: kroemer@orange.fr; or Frank Madeo: frank.madeo@uni-graz.at

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potential health and longevity-promoting effects of autophagy, pharmacological agents that stimulate autophagy at a low level of toxicity are urgently needed.

Rapamycin and the so-called rapalogs are the most effective clinically used inducers of autophagy yet have severe immunosuppressive effects (Hartford and Ratain, 2007). Thus, alternative, nontoxic autophagy inducers (such as resveratrol or carbamazepine) are being characterized for their pharmacological profile in suitable preclinical models (Hidvegi et al., 2010; Rose et al., 2010). Nontoxic compounds, such as resveratrol and spermidine, are also being evaluated for their potential to induce autophagy in vivo (Eisenberg et al., 2009; Morselli et al., 2010). Resveratrol is a natural polyphenol found in grapes, red wine, berries, knotweed, peanuts, and other plants. The interest in this molecule rose because it was suggested to mediate the cardioprotective effects of red wine (Baur and Sinclair, 2006). Resveratrol is also a potent inducer of autophagy (Scarlatti et al., 2008a,b), and this effect is mediated through the activation of sirtuin 1 (SIRT1), a NAD+-dependent deacetylase (Morselli et al., 2010). Resveratrol has been suggested to directly activate SIRT1 (Baur and Sinclair, 2006; Lagouge et al., 2006), although indirect effects may actually be preponderant (Beher et al., 2009; Pacholec et al., 2010). Spermidine is polyamine found in citrus fruit and soybean, which has recently been shown to increase the lifespan of yeast, nematodes, and flies in an autophagy-dependent fashion (Eisenberg et al., 2009).

The transfection-enforced expression of SIRT1 is sufficient to stimulate autophagy in human cells (Lee et al., 2008). Starvation-induced autophagy (but not autophagy induced by rapamycin) requires SIRT1, both in vitro (in mammalian cells; Lee et al., 2008) and in vivo (in Caenorhabditis elegans; Morselli et al., 2010). Activated SIRT1 induces autophagy via its capacity to deacetylate acetyl lysine residues in other proteins (Lee et al., 2008). Conversely, knockdown of the acetyltransferase EP300 (Lee and Finkel, 2009), as well as inhibition of histone acetylases, potently induces autophagy (Eisenberg et al., 2009), indicating that protein deacetylation may play a general role in the initiation of the autophagic cascade. EP300 acetylates several autophagy-relevant proteins, including autophagy-related 5 (ATG5), ATG7, ATG12, and microtubule-associated protein 1 light chain 3 B (LC3; Lee and Finkel, 2009), whereas SIRT1 deacetylates ATG5, ATG7, LC3 (Lee et al., 2008), and the transcription factor forkhead box O3, which can stimulate the expression of proautophagic genes (Kume et al., 2010). As a result, protein (de)acetylation reactions influenced by sirtuins and other enzymes control autophagy at multiple levels, including the modification of autophagy core proteins and/or of transcriptional factors that control the expression of autophagic genes.

Driven by these premises and incognita, we comparatively assessed the mechanisms of autophagy induction mediated by two distinct compounds that modulate protein acetylation, namely resveratrol and spermidine. We found that both agents induce autophagy through initially distinct yet convergent pathways that culminate in the acetylation and deacetylation of hundreds of proteins, with opposed patterns in distinct subcellular compartments. Based on this characterization, we demonstrated that these agents can stimulate autophagy in a synergistic fashion, both in vitro, in cultured human cells, and, in vivo, in mice.

**Results**

**Sirtuin-dependent versus -independent autophagy induced by resveratrol and spermidine**

Spermidine and resveratrol were comparable in their autophagy stimulatory potency and induced hallmarks of autophagy with similar kinetics in human colon cancer HCT 116 cells. These signs included the redistribution of a GFP-LC3 chimera, which is usually diffuse, to cytoplasmic puncta and the lipidation of endogenous LC3, increasing its electrophoretic mobility (Fig. 1 and Fig. S1 A). In these conditions, neither spermidine nor resveratrol impaired oxidative phosphorylation (Fig. S1 B), ruling out that resveratrol might induce autophagy via mitochondrial toxicity (Dörrie et al., 2001). Knockdown of SIRT1 with a specific siRNA suppressed the proautophagic activity of resveratrol (Fig. 1, A and B) yet failed to affect spermidine-induced autophagy (Fig. 1 C). Similarly, the SIRT1 inhibitor EX527 (Peck et al., 2010) abolished autophagy induction by resveratrol but not by spermidine (Fig. 1, D–F). These results indicate that resveratrol and spermidine trigger autophagy through distinct mechanisms.

**Phylogenetic conservation of sirtuin-independent autophagy induction by spermidine**

We next investigated whether the orthologues of *sirt1* in *Saccharomyces cerevisiae* and *C. elegans* (*sir2* and *sir-2.1*, respectively) are required for the proautophagic activity of spermidine. In yeast, spermidine caused the redistribution of a GFP-Atg8p chimera from a diffuse to a vacuolar localization (Fig. 2 A), the autophagy-dependent proteolytic liberation of GFP from GFP-Atg8p (Fig. 2 B; Suzuki et al., 2004), as well as an autophagy-related increase in vacuolar AP (Fig. 2 C; Noda et al., 1995). These effects were similar in wild-type (WT) and Δ*sir2* yeast strains (Fig. 2, A–C). Moreover, spermidine significantly improved the survival of aging WT yeast cultures, a beneficial effect that was attenuated, yet remained significant, in aging Δ*sir2* yeast cultures (Fig. 2 D). Accordingly, spermidine reduced the aging-associated overproduction of reactive oxygen species (measured by assessing the conversion of nonfluorescent dihydroethidine into fluorescent ethidium) both in WT and Δ*sir2* cells (Fig. 2 E). In *C. elegans* embryos, spermidine induced the autophagy-related expression and cytoplasmic aggregation of DsRed::LGG-1 (Fig. 3, A and B; Eisenberg et al., 2009). This effect was significant in both WT and *sir-2.1* mutant nematodes, although the *sir-2.1* mutation attenuated autophagy induction by spermidine (Fig. 3, C and D). Consistently, spermidine prolonged the lifespan of WT and *sir-2.1*-deficient worms by 18 and 13%, respectively. Collectively, these results indicate that spermidine can stimulate autophagy and extend the lifespan of yeast cells and nematodes that lack SIRT1 orthologues.
Resveratrol and spermidine induce autophagy through convergent pathways
To investigate the signal transduction pathway stimulated by resveratrol and spermidine, the phosphorylation status of multiple cellular proteins was analyzed in human colon cancer HCT 116 cells by means of an antibody array. Surprisingly, spermidine and resveratrol, alone or in combination, elicited similar changes in the phosphorylation status of multiple kinases and their substrates (Fig. 4, A–C). For example, both spermidine and resveratrol mediated the dephosphorylation of the protein tyrosine kinase 2β (also known as PYK2) and the cyclin-dependent kinase inhibitor 1B (better known as p27Kip1). However, neither of the two agents had major effects on the phosphorylation levels of the regulatory subunit of AMP-dependent kinase and its substrate acetyl-coenzyme A carboxylase (CoA), which was in line with the hypothesis that the energy metabolism of the cells was normal. Moreover, spermidine and resveratrol did not affect the phosphorylation of mechanistic target of rapamycin (mTOR) nor that of its substrate ribosomal protein S6 kinase (also known as p70S6K; Fig. 4, A–C), which suggests that resveratrol and spermidine induce autophagy through AMP-dependent kinase/mTOR-independent convergent pathways. Accordingly, the administration of an optimal dose of resveratrol and spermidine (100 µM for both agents) did not result in higher levels of autophagy than that of either agent alone (Fig. 4 D). This kind of epistatic analysis confirms the suspected convergence of the proautophagic pathways elicited by both agents.

Convergent action of resveratrol and spermidine on the acetylproteome
Next, we comparatively explored the effects of resveratrol and spermidine on the acetylation patterns of cytosolic, mitochondrial, and nuclear proteins. To that purpose, we performed stable isotope labeling with amino acids in cell culture (SILAC) and then purified the lysates of HCT 116 cells transfected with a GFP-LC3–encoding plasmid, cultured in complete medium for 24 h, and left untreated or treated for 4 h with 100 µM resveratrol (Resv) or spermidine (Spd). Surprisingly, 170 proteins whose acetylation status was modified in response to resveratrol or spermidine treatment are part of the recently elucidated human autophagy protein network (Behrends et al., 2010). Many of the (de)acetylated proteins identified in our study are central to the network because 89 among them interact with at least 10 proteins in the network (Table S1). Surprisingly, 170 proteins whose acetylation status was modified in response to resveratrol or spermidine treatment are part of the recently elucidated human autophagy protein network (Behrends et al., 2010). Many of the (de)acetylated proteins identified in our study are central to the network because 89 among them interact with at least 10 proteins in the network (Table S1).}
Figure 2. The lifespan-extending and autophagy-inducing effects of spermidine in yeast are not mediated by Sir2. (A–E) EGFP-Atg8p was ectopically expressed in wild-type (WT) or Δsir2 S. cerevisiae undergoing chronological aging on small synthetic 2% glucose media with or without (Co, control) supplementation of 4-mM spermidine (Spd). (A) Representative images. EGFP-Atg8p localization (bottom) was visualized by fluorescence microscopy. Yeast cells undergoing autophagy (in which EGFP-Atg8p exhibits a prominent vacuolar localization) are indicated by arrows. Yeast morphology was monitored by differential interference contrast (DIC; top). (B) Representative immunoblots against EGFP. Free EGFP indicates the vacuolar degradation of EGFP-Atg8p fusion, thereby representing the autophagic flux. Notice that both WT and Δsir2 yeast cells show similar free EGFP levels after spermidine-mediated autophagy induction. (C) Relative alkaline phosphatase (ALP) activity indicative of autophagy. n = 3. (D) Survival data. n = 4. (E) Quantification of reactive oxygen species. Bars indicate the percentages of cells exhibiting the reactive oxygen species–mediated conversion of dihydroethidine (DHE) into ethidium (Eth; n = 4). Data represent means ± SEM; *, P < 0.001 as compared with untreated cells of the same genotype. GAPDH, glyceraldehyde 3-phosphate dehydrogenase. RFU, relative fluorescence unit.
induction by spermidine and/or resveratrol is a transcription-dependent or -independent event using cytoplasts (enucleated cells). Cytoplasts were still able to accumulate GFP-LC3 puncta in response to spermidine or resveratrol treatment (Fig. 8, A and B), indicating that nuclei (and by extension transcription) are not required for short-term autophagy stimulation by these two agents. Next, we enforced overexpression of transgenic WT SIRT1 (which although preponderantly localizes to the nucleus, has been reported to efficiently shuttle to the cytoplasm; Tanno et al., 2007) or that of a mutant SIRT1 protein with a mutation in the nuclear localization signal (which is, therefore, virtually restricted to the cytoplasm; Fig. 8 C). Interestingly, no fundamental differences were found in the consensus (de)acetylation sites that were modified in response to resveratrol or spermidine (Fig. 6 and Fig. 7). In the cytosol, resveratrol and spermidine induced convergent deacetylation more frequently than convergent acetylation, whereas in the nucleus, acetylation was dominantly triggered by both agents (Fig. 5 B, P < 0.001, χ² test). Moreover, when we analyzed the distinct biological processes associated with the observed (de)acetylated proteins after gene ontology (GO) term enrichment (Ashburner et al., 2000), deacetylated proteins often fell in the category of metabolism (which includes autophagy; Fig. S3). Therefore, we investigated whether short-term autophagy induction by spermidine and/or resveratrol is a transcription-dependent or -independent event using cytoplasts (enucleated cells). Cytoplasts were still able to accumulate GFP-LC3 puncta in response to spermidine or resveratrol treatment (Fig. 8, A and B), indicating that nuclei (and by extension transcription) are not required for short-term autophagy stimulation by these two agents. Next, we enforced overexpression of transgenic WT SIRT1 (which although preponderantly localizes to the nucleus, has been reported to efficiently shuttle to the cytoplasm; Tanno et al., 2007) or that of a mutant SIRT1 protein with a mutation in the nuclear localization signal (which is, therefore, virtually restricted to the cytoplasm; Fig. 8 C). Both constructs...
two agents at low doses (10 µM) was able to significantly up-regulate autophagic flux, the combination of spermidine and resveratrol at low doses (10 µM) was as efficient in enhancing GFP-LC3 puncta formation, LC3 lipidation, and an increase in autophagic flux as were high doses of spermidine or resveratrol (Fig. 9, A and B).

To try to extend these results to a physiological setting, we intraperitoneally injected optimal doses of resveratrol (25 mg/kg) or spermidine (50 mg/kg) into mice expressing a GFP-LC3 transgene to induce autophagy in an array of organs. One tenth of this optimal dose (2.5 mg/kg resveratrol or 5 mg/kg spermidine) had no major proautophagic effect in vivo when either compound was injected alone. However, the combination of low doses of both agents was highly efficient in triggering autophagy in vivo (Fig. 9, C and D).

Similar results were obtained when these agents were injected into WT mice, as shown by means of LC3 lipidation and p62 degradation (Fig. 9 E). In conclusion, low doses of spermidine and resveratrol can induce autophagy in a synergistic fashion.
Resveratrol can induce autophagy only in the presence of SIRT1 (Morselli et al., 2010), whereas SIRT1 (or its orthologues in yeast and nematodes) is dispensable for spermidine-stimulated autophagy. Thus, these agents clearly ignite distinct pathways across a large phylogenetic distance. In spite of the difference in the primary targets of resveratrol and spermidine, both agents activated convergent pathways in that thus far they both stimulated mTOR-independent autophagy and elicited rather similar changes in the phosphoproteome and, more importantly, in the acetylproteome. Both agents provoked multiple changes (increases or decreases) in the lysine acetylation of hundreds of proteins, and the convergent changes induced by both agents largely outnumbered discordant modifications. When combined between each other, high doses of spermidine and resveratrol did not induce higher levels of autophagy than each of the two agents alone, which is in line with the idea that the terminal pathways stimulated by these compounds overlap. Spermidine and resveratrol modulated the acetylation of >100 proteins that are part of the central network of autophagic regulators/executioners (Behrends et al., 2010). This suggests that both agents stimulate autophagy through a multipronged mechanism that involves a large number of (de)acetylation reactions.

Although resveratrol can (directly or indirectly) activate SIRT1, a deacetylase (Baur and Sinclair, 2006; Lagouge et al., 2006; Beher et al., 2009; Pacholec et al., 2010), spermidine has been shown to inhibit acetylases (Erwin et al., 1984; Eisenberg et al., 2009). Based on this consideration, it appears paradoxical that neither of these two agents was able to provoke a general deacetylation state and that both of them actually stimulated a similar shift in the acetylation pattern, in which hundreds of proteins were deacetylated (more in the cytosol than in the nucleus), whereas several others were acetylated (more in the nucleus than in the cytosol). Cells harbor multiple deacetylases and acetylases (Hassig and Schreiber, 1997; Katan-Khaykovich and Struhl, 2002; Nakamura et al., 2010), and it appears plausible, yet remains to be proven, that inhibition of one (or a few) acetylase will activate compensatory reactions by other acetylases and/or impact the action of deacetylases so that the global cellular level of protein acetylation remains near to constant. As a significant trend, however, we observed that both resveratrol and spermidine stimulated the deacetylation of cytosolic proteins, such as ATG5 and LC3, and the acetylation of nuclear proteins, including multiple histones.

It has been recently reported that lifespan extension by spermidine treatment (during conditions of chronological aging) is linked to deacetylation of nuclear histones and to an increase in the transcription of different autophagy-related genes (Eisenberg et al., 2009). Interestingly, autophagy was rapidly induced by both spermidine and resveratrol in cytoplasts prepared from proliferating human cells, and an extranuclear variant of SIRT1 was as efficient in inducing autophagy as the predominantly nuclear WT SIRT1. Collectively, these data suggest that protein deacetylation first stimulates autophagy predominantly through a cytosolic mechanism. These results not only illustrate the differences between quiescent and proliferating cells in terms of autophagy modulation but also suggest that after a fast and nuclear-independent autophagic

Figure 5. Convergent acetylproteome modification after resveratrol or spermidine treatment. (A–C) Colon carcinoma HCT 116 cells were cultured for 2 wk in three different SILAC media containing different arginine and lysine isotopes. Cells were treated with 100-µM resveratrol (Resv) or spermidine (Spd) for 2 h, fractioned into cytoplasmic, nuclear, and mitochondrial extracts, processed for acetyl lysine peptide enrichment, and analyzed by MS. (A) Hierarchical clustering of drug-specific organellar distributions of all acetylated sites quantified in at least one fraction. Fold changes are calculated relative to untreated cells. Only sites regulated >1.5-fold were included in statistical analyses. (B) Graphical representation of peptides whose acetylation status was affected in a convergent or divergent way. n = 560.
Figure 6. **Significant motifs among sites undergoing acetylation.** (A) Hierarchical clustering of the organellar distributions of sites whose acetylation was increased by >1.5-fold in response to resveratrol or spermidine, at least in one organellar fraction. (B) Consensus acetylation motifs identified upon resveratrol (left) or spermidine (right) treatment are depicted using the MotifX algorithm (Schwartz and Gygi, 2005). Among sites that were hyperacetylated in response to both agents, the K(F/Y) motif is significantly enriched when tested against the whole proteome (P < 0.00001). (C) When testing against the largest acetylation site dataset from Choudhary et al. (2009) (acetylation background dataset [ABD]), the SxK motif is significant (P < 0.0001) for sites whose acetylation increased upon spermidine treatment. No general consensus motifs were found for sites whose acetylation increased in response to both agents.
Figure 7. Significant motifs among sites undergoing deacetylation. (A) Hierarchical clustering of the organellar distributions of sites whose deacetylation was increased by >1.5-fold in response to resveratrol or spermidine, at least in one organellar fraction. (B) Among sites that were hypoacetylated in response to both agents, the KP motif is significantly enriched when tested against ABD (P < 0.001). (C and D) When tested against the ABD (C) or the whole proteome (D), the KP motif is significant for sites undergoing hypoacetylation upon spermidine treatment (P < 0.0001). No general consensus motifs were found for sites whose acetylation decreased in response to both agents.
Resveratrol is a natural polyphenol contained in red wine and vegetables, whereas spermidine is a polyamine found in other healthy food, such as citrus fruit and soybean. When analyzed as individual compounds, neither polyphenols nor polyamines consumed with the normal diet may reach concentrations high enough to mediate pharmacological effects. Nonetheless, it is tempting to speculate that combinations of these agents—and perhaps that of other proautophagic dietary components—may affect the autophagic rheostat, as based on their distinct yet convergent mode of action.

**Materials and methods**

**Chemical, cell line, and culture conditions**

Unless otherwise specified, chemicals were purchased from Sigma-Aldrich, culture media and supplements for cell culture were obtained from Invitrogen, and plasticware was purchased from Corning. Human colon carcinoma HCT 116 cells (gift from B. Vogelstein, Howard Hughes Medical Institute, Baltimore, MD) were used as recipients to cotransfect the plasmids encoding wild-type SIRT1 and mSIRT1, respectively.
Figure 9. Low doses of resveratrol and spermidine synergistically induce autophagy in vitro and in vivo. (A) Human colorectal carcinoma HCT 116 cells were transfected with a GFP-LC3-encoding plasmid, cultured in complete medium for 24 h, and then treated with either vehicle (Co, control) or the indicated dose of resveratrol (Resv) or spermidine (Spd), alone or in combination, for 2 h. (top) Quantitative data. Bars depict the percentages (means ± SD; n = 3; **, P < 0.05) of cells showing the accumulation of GFP-LC3 in puncta (GFP-LC3vac). (bottom) Representative immunoblots showing endogenous LC3 lipidation. (B) Representative immunoblots showing endogenous LC3 lipidation in the presence of bafilomycin A1 (BafA1). (C–E) Transgenic C57BL/6 mice expressing a GFP-LC3 fusion protein were injected with resveratrol and spermidine at the indicated concentrations. 3 h later, mice were killed, and tissues were processed for immunofluorescence microscopy determinations of GFP-LC3vac. (C) Representative images. (D) Quantitative results. Bars represent the percentages (means ± SEM; n = 3; *, P < 0.05; and **, P < 0.01 as compared with the same tissue from untreated animals) of cells exhibiting GFP-LC3vac. (E) Representative immunoblots showing endogenous LC3 lipidation and p62 protein content in WT C57BL/6 mouse tissues. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
Plasmids, transfection, and RNA interference in human cell cultures

Cells were cultured in 12-well plates and transfected at 50% confluence with siRNAs targeting human SIRT1 (Ford et al., 2005), ATG5, or ATG7 (Thermo Fisher Scientific) or with an unrelated control siRNA by means of a preformed Profer siRNA kit (R&D Systems) according to the manufacturer’s instructions. After 24 h, cells were transfected with a plasmid coding for a GFP-LC3 fusion protein (Kabeya et al., 2000). Transient transfections were performed with the Attractene reagent (QIAGEN) as suggested by the manufacturer, and unless otherwise indicated, cells were analyzed 24 h after transfection. Cells were transfected with a plasmid coding for RFP fused to LC3 (RFP-LC3; obtained from Invitrogen) in the presence of an empty vector (pcDNA3) or of different constructs for the overexpression of GFP-tagged WT SIRT1 or a SIRT1 variant mutated in the nuclear localization signal, which mostly localizes in the nucleus (Tanno et al., 2007). For fluorescence microscopy determinations, cells cultured on coverslips were fixed in paraformaldehyde (4% vol/vol) for 15 min at RT, washed three times in PBS, and mounted with mounting medium ( Vectashield; Vector Laboratories).

Fluorescence microscopy

Confocal fluorescent images were captured using a confocal fluorescence microscope (TCS SP2; Leica). For experiments with HCT 116 cells, an ApoChromat 63× 1.3 NA immersion objective was used, whereas for the analysis of GFP-LC3 mice tissue sections, an ApoChromat 40× 1.25 NA immersion objective was used. All the acquisitions were made at RT with fixed cells/tissue slides. Images were acquired with a camera (DFC 350 FX 1.8; Leica) using LAS AF software (Leica) and processed with Photoshop (CS2; Adobe) software. Specifically, picture processing involved cropping of each picture to the area of interest, which mostly consisted of linear adjustments of contrast and brightness and was performed using Photoshop (with equal adjustment parameters for all pictures); no explicit correction was used. Nonconfocal microscopy of yeast strains carrying the EGF-P-tagged Atg8 protein was performed with a microscope (Axioskop; Carl Zeiss, Inc.) using a Plan Neofluar objective lens (Carl Zeiss, Inc.) with a 63× magnification and 1.25 NA in oil at RT. Images were taken with a camera (SPOT 9.0 Monochrome; Diagnostic Instruments, Inc.), acquired using the Metamorph software (A 6.2r4; Universal Imaging Corp.), and processed with IrfanView (version 3.97) and Photoshop (CS2) software. Specifically, picture processing involved coloring and cropping of representative areas and was performed with IrfanView. In addition, linear adjustments of contrast and brightness were applied with Photoshop (using equal adjustment parameters for all pictures); no explicit correction was used. Nonconfocal microscopy of C. elegans was performed with a microscope (Axioskop; Carl Zeiss, Inc.) using a Plan Neofluar 40× objective with a 0.75 NA and a 63× Plan Neofluar objective with an NA of 1.25 in oil at RT. Images were taken with a camera (AxioCam MRC5; Carl Zeiss, Inc.) with Axiosview software (Carl Zeiss, Inc.) without further processing. The different fluorophores used in this work were GFP and RFP for HCT 116 cells, EGFP for yeast experiments, DsRed and GFP-tagged WT SIRT1 or a SIRT1 variant mutated in the nuclear localization signal, which mostly localizes in the nucleus (Tanno et al., 2007). For yeast aging experiments, DsRed was added to stationary cultures at day 1 of the aging experiments (Eisenberg et al., 2009). Dihydroethidium staining was performed as previously described (Büttner et al., 2007), and the superoxide-driven conversion to ethidium was quantified by measuring the conversion of a-naphthyl phosphate to naphthalene using a GeniosPro fluorescence plate reader with excitation and emission wavelengths at 340 nm and 485 nm, respectively (Noda et al., 1995). To correct for intrinsic AP activity, WT or Δsir2 yeast cells lacking the pTN9 HindIII fragment were simultaneously assayed, and these values were used for background subtraction, giving the vacuolar (autophagic) AP activity.

C. elegans strains, genetics, and pharmacology

We followed standard procedures for C. elegans strain maintenance. Nematode-feeding temperature was kept at 20°C. The following strains were used in this study: N2, WT Bristol isolate, and VC199, sin2(1(ok4344))/IV. The VC199 strain was provided by the C. elegans Gene Knockout Project at the Oklahoma Medical Research Foundation, which is part of the International C. elegans Gene Knockout Consortium and the Cae- norhabditis Genetics Center and is funded by the National Institutes of Health National Center for Research Resources. The construction of the pIgG::DsRed-LGG-1 reporter plasmid has been described previously (Samara et al., 2008). Spermidine was dissolved in sterilized water to a stock solution concentration of 100 mM. Escherichia coli (OP50) bacteria on seeded nematode growth medium (NGM) plates were killed by UV irradiation for 10 min (0.3 J) using a UV cross-linker (Bio-Link BLX-E265; Vilber Lourmat). A range of spermidine concentrations was prepared by dilutions in 100 µl of sterilized water and applied to the top of the agar medium (7-ml NGM plates). Plates were then gently swirled to allow the drug to spread to the entire NGM surface. Identical drug-free water solutions were used for the control plates. Plates were then allowed to dry overnight. The procedure was repeated each time worms were transferred to fresh plates (every 2–3 d during the first 2 wk and every week thereafter). Worms were incubated at 20°C.

C. elegans autophagy measurements

Images from transgenic embryos expressing a DsRed::LGG-1 fusion protein were acquired using a 540 ± 15-nm band-pass excitation filter and a 575-nm long-pass emission filter. Experiments were performed at 20°C, with photography exposure time kept identical for each embryo.
Emission intensity was measured on grayscale images with a pixel depth of 8 bits (256 shades of gray). We calculated the mean and maximum pixel intensity for each embryo in these images using the ImageJ software (National Institutes of Health). For each transgenic line, we processed ≥25 images over at least three independent trials.

C. elegans lifespan analysis
Lifespan assays were performed at 20°C. Synchronous animal populations were generated by hypochlorite treatment of gravid adults to obtain tightly synchronized embryos that were allowed to develop into adulthood under appropriate conditions. Progeny were grown at 20°C through the L4 larval stage and then transferred to fresh plates in groups of 10–20 worms per plate for a total of 100–150 individuals per experiment. The day of egg harvest was set as d = 0. Animals were transferred to fresh plates every 2–4 d and were examined every day for touch-provoked movement and pharyngeal pumping until death. Worms that died (because of internally hatched eggs, extruded gonads, or desiccation upon crawling on the plate edge) were censored and incorporated as such into the dataset. Each survival assay was repeated at least three times.

**SILAC cell culture, sample processing, and analysis**
HCT 116 cells were cultured for 2 wk in three different SILAC media (Invitrogen) containing either (1) light isotypes of l-arginine and l-lysine (Arg6/Lys6), (2) -arginine-14C, l-lysine 2HCl 4.4, l-lysine 2HCl 4.4, -lysine-1C3H3, l-lysine 2HCl 4.4, l-lysine 2HCl 4.4, l-lysine 2HCl 4.4, (Euroisotop), or (3) -arginine-13C6, l-lysine 15N4 HCl (Arg10/Lys8; Invitrogen) and complemented as previously described (Blagoja and Mann, 2006). Cells were treated for 2 h with 100 µM spermidine (Arg10/Lys8) or 100 µM resveratrol (Arg6/Lys4) and then lysed and subdivided in nuclear, mitochondrial, and cytotoxic fractions as previously described (Gurbuxani et al., 2003). The cytosolic fraction was precipitated using ice-cold (–20°C) acetone (4 vol of the sample extract), vortexed, and placed for 2 h at –20°C. The resulting solution was centrifuged for 10 min at 16,000 g, and the supernatant was removed. The pellet was subsequently washed twice with ice-cold 4:1 acetone/water. The final pellet was dried using a concentrator (SpeedVac; Thermo Fisher Scientific) for 10–15 min. All fractions were dissolved in denaturant 6:2:4 urea/thiourea (both obtained from Merck), and Benzamide (Merck) was added as the nuclear fraction. All steps were performed at RT to avoid carbamylation of amines. Reduction of cysteines was performed with 5 mM DTT (Sigma-Aldrich) for 30 min followed by alkylation with 11-mM iodoacetamide for 20 min in the dark. The proteins were digested with 1:100 protease LysC (Wako Chemicals USA, Inc.) for 3.5 h, digested four times with 50-mM ammonium bicarbonate, and then digested with 1:100 trypsin (Promega) overnight. The nuclear fraction mixture was centrifuged at 10,000 g for 10 min, and the supernatant was filtered through a 0.45-µm Millex-HV filter (Millipore). From each fraction, ~100 µg of digested protein was collected for isoelectric focusing, a step required for subsequent normalization. The acetyl lysine peptide enrichment was performed as previously described (Choudhary et al., 2009). After peptide enrichment and isoelectric focusing, samples were subjected to MS analysis, and data were processed as described in the next paragraph.

**Preparation of cytoplasts**
Trypsinized cells previously transfected with GFP-LC3 cDNA were incubated in 3 ml of complete medium supplemented with 7.5 mg/ml cytochalasin B for 45 min at 37°C. This cell suspension was layered onto a discontinuous Ficol density gradient (3 ml of 55%, 1 ml of 90%, and 3 ml of 100% Ficol; GE Healthcare) in complete medium containing 7.5 mg/ml cytochalasin B. Gradients were prepared in ultracentrifuge tubes and pre-equilibrated at 37°C in a CO2 incubator overnight. Gradients containing cell suspensions were centrifuged in a prewarmed rotor (SW41; Beckman Coulter) at 100,000 g for 20 min at 32°C. The cytoplasm-enriched fraction was collected from the interface between 55% and 90% Ficol layers, washed in complete RPMI 1640 medium, and incubated for 4 h at 37°C before resveratrol, spermidine, or rapamycin treatment.

**Mouse experiments and tissue processing**
C57BL/6 mice obtained from Charles River Laboratory were bred and maintained at the Federation for Laboratory Animal Science Associations and the Animal Experimental Ethics Committee guidelines. They were housed in a temperature-controlled environment with 12 h light/dark cycles and received food and water ad libitum. Mice were injected intraperitoneally with 5 or 20 mg/kg spermidine and with 2.5 or 25 mg/kg 3 h before anesthesia and killing. Mice tissues were immediately frozen in liquid nitrogen after extraction and homogenized in a 20-mm Tris buffer, pH 7.4, containing 150-mM NaCl, 1% Triton X-100, 10-mM EDTA, and protease inhibitor cocktail (Complete; Roche). Tissue extracts were then centrifuged at 12,000 g at 4°C, and supernatants were collected. Protein concentration in the supernatants was evaluated by the bicinchoninic acid technique (BCA protein assay kit; Thermo Fisher Scientific).

**Quantitative analysis of GFP-LC3 dots in mouse tissue sections**
To avoid postmortem autophagy induction, dead mice were immediately perfused with 4% paraformaldehyde [wt/vol in PBS, pH 7.4]. Tissues were then harvested and further fixed with the same solution for ≥4 h followed by treatment with 15% sucrose [wt/vol in PBS] for 4 h and with 30% sucrose [wt/vol in PBS] overnight. Tissue samples were embedded in TissueTek OCT compound (Sakura) and stored at 70°C. 5-µm-thick tissue sections were generated with a cryostat (CM3050 S; Leica) and postfixed for 1 h, washed in PBS for 5 min, dried at RT for 30 min, and mounted with Vectashield antifading medium. In each organ, the number of GFP-LC3 dots was counted in five independent visual fields from at least five mice using a confocal fluorescence microscope (TCS SP2).

**SILAC sample preparation and analysis**
Upon digestion, peptides were concentrated and desalted on SepPak C18 (Waters) purification cartridges and eluted using a highly organic buffer (80% acetonitrile and 0.5% acetic acid). Eluates were lyophilized in a vacuum centrifuge, dissolved in immunoprecipitation buffer (50-mM MOPS, pH 7.2, 10-mM sodium phosphate, and 50-mM sodium chloride), and mixed with antibodies conjugated to cyanogen bromide–conjugated–agarose beads. The mixture was left for 12 h at 4°C on a rotation wheel. The flow-through (containing nonbound peptides) was removed, and beads were washed four times with the immunoprecipitation buffer and twice with deionized water. Bead-bound peptides were eluted with 0.1% trifluoroacetic acid. The same enrichment procedure was performed again on the flow-through containing the unbound peptides from the first enrichment. Six eluates and three separately collected digested protein samples were desalted using SepPak C18 cartridges, and 5% of each acetyl lysine–enriched eluate was used for MS analysis. The rest of the samples were subsequently separated into 12 fractions by isoelectric focusing using the 3100 OFFGEL Fractionator (Agilent Technologies) as previously described (Hübner et al., 2008). Gel strips and amphotafy buffer were purchased from GE Healthcare. Focusing was performed for 20 kV/h, the maximum current of 50 µA and the maximum power of 200 mW. 10 µl of 10% trifluoroacetic acid was added to each fraction and STAGE (stop and go extraction) tipped as previously described (Rappaport et al., 2007) before MS analysis, which was performed on either an LTQ Orbitrap XL spectrometer (Thermo Fisher Scientific) connected to a 1200 Series Nanoflow HPLC system (Agilent Technologies) or an LTQ Orbitrap Velos spectrometer (Thermo Fisher Scientific; Olsen et al., 2009) connected to an 1100 Series Nanoflow HPLC system (Agilent Technologies) using a nanoelectrospray ion source (Thermo Fisher Scientific). Peptides were separated by reverse phase chromatography using an in-house–made fused silica emitter (75-µm internal diameter) packed with 3-µm reversed phase material (Reprosil-Pur C18-AQ; Dr. Maisch GmbH). Peptides were loaded on a 5% solvent A (0.1% acetic acid) followed by a 100-min linear gradient to 50% solvent B (80% acetonitrile and 0.5% acetic acid). Survey full-scan MS spectra (mass to charge [m/z] range of 300–200, with a resolution of 60,000, at m/z of 400) were acquired followed by fragmentation of the 10 (in the case of using the LTQ Orbitrap XL) or the 20 (in the case of using the LTQ Orbitrap Velos) most intense multiply charged ions. Ions selected for MS/MS were placed on a dynamic exclusion list for 45 s. Real-time internal lock mass recalibration was used during data acquisition (Olsen et al., 2005). For unfractonated acetyl lysine–enriched eluates, an additional MS analysis was performed on the LTQ Orbitrap Velos using higher energy collision dissociation fragmentation (normalized collision energy of 40) of the 10 most intense ions from each MS spectrum creating MS/MS spectra at a resolution of 7,500. All samples used for protein normalization were analyzed on a mass spectrometer (LTQ FT ULTRA; Thermo Finnigan) in which the five most intense ions from each precursor scan were selected for fragmentation in the LTQ. In this case, no real-time lock mass recalibration was used. Reverse-phase chromatography settings were the same as described for the analysis performed on the Orbitrap spectrometers.

**SILAC data processing**
Raw files were processed with MaxQuant v1.0.13.13 (Cox and Mann, 2008) into centroided data and submitted to database searching with Mascot v2.2 (Matrix-Science). Preprocessing by MaxQuant was performed to determine charge states, mass tolerances, and SILAC states and to filter the MS/MS spectra, keeping the six most intense peaks within a 100-Da bin. Cysteine carbamidomethylation was chosen as a fixed modification, whereas...
N-terminal acetylation and methionine oxidation were chosen as variable modifications. Furthermore, acetylation of light, medium, and heavy isoforms of lysine (Lys0/Lys4/Lys8) was chosen as a variable modification. Processed MS/MS spectra were searched against a concatenated target decoy database of forward and reversed sequences from the International Protein Index database (152,616 sequences; FASTA file created 5/6/2008). For the search, trypsin/P+ DFP was chosen for the in silico protein digestion allowing four missed cleavages. The mass tolerance for the MS spectra acquired in the Orbitrap was set to 7 ppm, whereas the MS/MS tolerance was set to 0.6 D for the collision-induced dissociation MS/MS spectra from the ITQ and to 0.04 D for the higher energy collision dissociation MS/MS spectra. Upon peptide search, protein and peptide identification was performed given an estimated maximal false discovery rate of 1% at both the protein and peptide level. For false discovery rate calculation, posterior error probabilities were calculated based on peptides of at least six amino acids having a Mascot score of ≥10. For protein quantification, only unmodified peptides, peptides modified by N-terminal acetylation, and methionine oxidation were calculated. If a counterpart to a given lysine-acetylated peptide was identified, this counterpart was also excluded by protein quantitation. According to the protein group assignment performed by MaxQuant, both razor and unique peptides are used for protein quantification. A minimum of two ratio counts was required for protein quantification. For quantification of lysine-acetylated sites, the least modified peptides were used. The ratios for the sites were normalized by the corresponding protein ratios to account for eventual changes in protein abundance. In case a protein ratio was not determined, normalization was based on a logarithm transformation algorithm as previously described (Cox and Mann, 2008).

Cell respiration and mitochondrial substrate oxidations

Cell respiration and mitochondrial substrate oxidation were polarographically measured at 37°C in 250 µl of a buffer containing 0.3 M mannitol, 10 mM KCl, 5 mM MgCl2, 1 mg/ml BSA, and 10 mM KH2PO4, pH 7.4 (Rustin et al., 1994). Respiration was measured on intact cells (final concentration of 10^4/ml), which were subsequently permeabilized by 0.01% digitonin to study mitochondrial substrate oxidation. 10 mM malate plus 10 mM glutamate oxidation was measured in the presence of 200 µM ADP. 10 mM succinate oxidation by digitonin-permeabilized cells was measured in the presence of 2 µM rotenone and 200 µM ADP. Sequential addition of 2 µM oligomycin, a specific inhibitor of the mitochondrial ATPase, and 2 µM carbonyl cyanide m-chlorophenyl hydrazone, a potent mitochondrial uncoupler, allowed for the determination of the respiratory control value associated with succinate oxidation.

Functional analysis of proteins regulated by deacetylation or acetylation

To decipher the functional context of the proteins associated with the drug-specific regulation of proteins by deacetylation and acetylation, GO term (Ashburner et al., 2000) enrichment was performed using the Cytoscape (Shannon et al., 2003) plugin BiNGO (Biological Networks Gene Ontology tool; Maere et al., 2005) and PANTHER (Protein Analysis Through Evolutionary Relationships) classification system. For the enrichment analysis, proteins regulated by ≥1.5-fold were included, and p-values were calculated by Fisher’s exact test after the Benjamini–Hochberg adjustment for multiple testing (Benjamini and Hochberg, 1995). A significance level of 0.05 (corresponding to the maximal false discovery rate) and a minimum of five proteins in at least one of the subsets of each given significant GO term were set as thresholds.

Statistical analysis

Statistical analyses were performed using the Prism software package (GraphPad Software, Inc.), the Office 2003 Excel software package (Microsoft), and the statistical environment R (R Development Core Team). In cell culture experiments, values were compared using unpaired Student’s t tests. For multiple comparisons, we used the one-factor analysis of variance corrected by posthoc Bonferroni test. C. elegans survival curves were created using the product-limit method of Kaplan and Meier. The log-rank (Mantel–Cox) test was used to evaluate differences between survival and determine p-values.

Online supplemental material

Fig. S1 describes the kinetics of autophagy induction after 100-µM resveratrol and spermidine treatment and shows measurements of cell respiration and mitochondrial substrate oxidation in the absence (control) or presence of 100-µM resveratrol or spermidine. Fig. S2 shows the measurement of SILAC fraction purity and analysis of the acetylation status of autophagy essential proteins after spermidine and/or resveratrol treatment.
Supplemental material

Figure S1. The kinetics of autophagy induction after 100-µM resveratrol and spermidine treatment and measurements of cell respiration and mitochondrial substrate oxidation in the absence (control) or presence of 100-µM resveratrol or spermidine. (A and B) Representative immunoblots showing endogenous LC3B lipidation in colon carcinoma HCT 116 cells treated with 100-µM spermidine (Spd) or resveratrol (Resv) for the indicated time. (C–G) HCT 116 cells were left untreated (Co, control) or treated with 100-µM resveratrol or spermidine for 4 h and then processed for the polarographic measurement of oxidative phosphorylation. Bars depict nanomoles of O2 consumed per minute and milligrams of protein (prot; means ± SD; n = 3). CCCP, carbonyl cyanide m-chlorophenyl hydrazone. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
Figure S2. Measurement of SILAC fraction purity and analysis of the acetylation status of autophagy essential proteins after spermidine and/or resveratrol treatment. (A–C) Determination of the purity and quality of the organelle fractionation. (A) Fraction purity is measured by an enrichment score calculated from Wilcoxon testing for distribution location shift in compartment-specific peptides classified by GO cellular component terms compared with all identified peptides within the corresponding organelle fractions. The dashed line indicates a high significant score corresponding to $P < 0.001$ (Wilcoxon test). (B) Using a Western blot–like approach, 33 organelle-specific markers were selected and used to determine the purity of organelle fractions. The color key is a visualization of the fraction-specific number of identified peptides associated with the protein whose gene name is indicated as the row name. The fraction-wise relative number is indicated in the heat map trace histogram (black line). (C) Control immunoblots on the subcellular fractions used for the proteomic experiments. Cytoplasmic, mitochondrial, and nuclear fractions were blotted with antibodies specific for β-tubulin, voltage-dependent anion channel (VDAC), and histone H3. PARP, poly(ADP-ribose) polymerase. (D–E) Human colon carcinoma HCT 116 cells were treated with resveratrol (Resv) or spermidine (Spd) at the indicated concentrations for up to 2 h, processed for immunoprecipitation (IP) with an anti–acetyl lysine (Ac-Lys) antibody, and then subjected to immunoblotting (IB) with the depicted antibodies. Asterisks represent nonspecific bands detected by the antibody.
Figure S3. The biological processes associated with the differentially acetylated proteins in response to resveratrol and spermidine treatment by means of GO enrichment. (A) Summary of the GO biological processes found to be associated with the different protein groups subjected to convergent (de)acetylation in the different subcellular fractions after spermidine or resveratrol treatment. n = 375. (B) Fisher’s exact test was applied to identify significant (P < 0.05) biological processes associated with proteins regulated by >1.5-fold acetylation and deacetylation during treatment with resveratrol or spermidine. The enrichment score calculated based on the adjusted p-value is indicated in the color key, in which a score >3 corresponds to P < 0.001.
Table S1 is provided as an Excel file and shows a list of detected acetyl lysine-containing motifs in colon carcinoma HCT 116 cells upon SILAC and spermidine or resveratrol treatment.

Table S2 is provided as an Excel file and shows a list of proteins belonging to the human autophagy network interacting with the proteins shown in Table S1.