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

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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 cardio protection in the context of ischemic preconditioning; Moreau et al., 2010) and delay the pathogenic manifestations of aging (Levine and Kroemer, 2009). Given the

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utophagy 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.

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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.
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 rilmenidine 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 β (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 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 Δsir2 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 Δsir2 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 Δsir2 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.
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 proteins containing acetylated lysine residues and identified them by quantitative mass spectrometry (MS). Resveratrol or spermidine induced changes in the acetylation of 560 lysine-containing motifs corresponding to 375 different proteins (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 S2). Interestingly, 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 S2).

Both resveratrol and spermidine tended to induce the (de)acetylation of similar proteins, including that of autophagy-relevant substrates, such as ATG5 and LC3 (Fig. 5, A and B;...
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 dihydroethidium (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.
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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 and Fig. S2 E). 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...
Synergistic induction of autophagy by low doses of resveratrol and spermidine

Resveratrol (but not spermidine) induces autophagy through the activation of the deacetylase SIRT1 (Morselli et al., 2010), whereas spermidine is thought to act as an inhibitor of acetylases (Eisenberg et al., 2009). We reasoned that low doses of resveratrol and spermidine might synergistically induce autophagy by affecting the equilibrium state of (de)acetylation. To assess this possibility, we treated HCT 116 cells with different concentrations of resveratrol or spermidine, alone or in combination, and analyzed the effects of the different pharmacological combinations in terms of autophagy induction. As expected, both spermidine and resveratrol used at high doses (100 µM) induced GFP-LC3 punctation and LC3 lipidation (Fig. 9 A) in cultured cells. Interestingly, although none of the 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, A and B).

Convergent alterations in the phosphoproteome status after resveratrol and/or spermidine treatment. (A–C) Human colon carcinoma HCT 116 cells were treated for 2 h with vehicle (Co, control), 100-µM resveratrol (Resv), and 100-µM spermidine (Spd), alone or in combination (Resv + Spd). (A) Representative phosphoprotein arrays are shown. (B) Clustering analysis for the effects on protein kinase phosphorylation. (C) Representative immunoblots of selected kinases whose phosphorylation status was unaffected (PRKAA1, RPS6KB1, and acetyl-CoA carboxylase α [ACACA]) or affected by resveratrol or/and spermidine treatment (PTKB, AKT1, MAPK8, and CDKN1B), validating phosphoprotein array data. (D) 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 or the indicated dose of resveratrol or spermidine, alone or in combination, for 2 h. Quantitative data. Bars depict the percentages (means ± SD; n = 3; *, P < 0.05) of cells showing the accumulation of GFP-LC3 in puncta (GFP-LC3 vac). GAPDH, glyceraldehyde 3-phosphate dehydrogenase.
Discussion

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 100µM 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).
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.
Institute, Johns Hopkins University, Baltimore, MD; Bunz et al., 1999) were cultured in McCoy’s 5A medium containing 10% fetal bovine serum, 100 mg/liter sodium pyruvate, 10-nM Hepes buffer, 100 U/ml penicillin G sodium, and 100 µg/ml streptomycin sulfate ([5% CO2 at 37°C]). Cells were seeded in 6- and 12-well plates or in 10- and 15-cm dishes and grown for 24 h before treatment with 10µM EK527 (Tocris Bioscience), 10- or 100µM resveratrol, 10- or 100µM spermidine, and 1-µM rapamycin or 1-µM bafilomycin A1 (Tocris Bioscience) for the time indicated in each experimental figure legend.

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 an Oligofectamine transfection reagent (Oligofectamine; Invitrogen) following the manufacturer’s instructions. After 24 h, cells were transfected with a plasmid coding for a GFP-LC3 fusion (Kabeya et al., 2007). Transient plasmid 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 GFP fused to LC3 (RFPC-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% wt/vol) for 15 min at RT, washed three times in PBS, and mounted with mounting medium (Vectorshield; Vector Laboratories).

Fluorescence microscopy

Confocal fluorescent images were captured using a confocal fluorescence microscope (TCS SP2; Leica). For experiments with HCT 116 cells, an Apomorchron 40× 1.15 NA immersion objective was used, whereas for the analysis of GFP-LC3 mice tissue sections, an Apomorchron 40× 1.5 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 1.8.0; Leica) using LAS AF software (Leica) and processed with Photoshop (CS2; Adobe) software. Specifically, picture processing involved cropping and adjusting 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 EGFPTagged 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; Diagnostics Instruments, Inc.), acquired using the Metamorph software (A 2.4; 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 (AxioImager Z1; 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 for C. elegans analyses, and GFP for mice tissue sections. Nuclei were counterstained with Hoechst (Invitrogen). Immersion oil (Immerso; Carl Zeiss, Inc.) was used for all microscopy analyses.

Immunoblotting, immunoprecipitation, and phosphokinase array

For immunoblotting, cells were collected, washed with cold PBS, and lysed as previously described (Criollo et al., 2007). 25 µg of protein was then separated on 4–12% Bis-Tris acrylamide (Invitrogen) or 12% Tris-glycine SDS-PAGE precast gels (Bio-Rad) and electrotransferred to Immobilon membranes (Millipore) according to standard procedures. Unspecific binding sites were saturated by incubating membranes for 1 h in 0.05% Tween 20 (vol/vol in TBS) supplemented with 5% nonfat powdered milk (wt/vol in TBS) followed by overnight incubation with primary antibodies specific for acetylated lysine, phosphoacetyl-CoA carboxylase α (Ser79), acetyl-CoA carboxylase α, phospho-AKT1 (Thr308), AKT1, LC3, phosphomimetic-activated protein kinase ε (Thr183/Thr185), phospho-PRKAA1 (Thr172), PRKAA1, cyclin-dependent kinase inhibitor 1B, phosphoprotein tyrosine kinase 2 δ (Thr403), phosphoribosomal protein S6 kinase (Thr235/Ser242), ribosomal protein S6 kinase, stress-induced phosphoprotein 1 (Cell Signaling Technology), ATG7 (Sigma-Aldrich), phosphocysclin-dependent kinase inhibitor 1B (Abcam), TP53 (DO7), p62, SIRT1 (Santa Cruz Biotechnology), and peptidylprolyl isomerase A (cytoplasm A; Enzo Life Sciences, Inc.). Revelation was performed with appropriate HRP-labeled secondary antibodies (SouthernBiotech) plus a chemiluminescence substrate (SuperSignal West Pico; Thermo Fisher Scientific). An antiglyceroldehyde-3-phosphate dehydrogenase antibody (Millipore) was used to control the equal loading of lane. For immunoprecipitation, extracts from 8 × 106 cells were lysed, and 500 µg of protein was precleared for 1 h with 15 µl protein G–Sepharose 4 Fast Flow (GE Healthcare) followed by incubation for 2 h in the presence of an anticyctated lysine (Cell Signalling Technology) antibody. Subsequent immunoblotting was performed by means of TrueBlue-HRP (eBioScience) secondary antibodies. For phosphokinase array analyses, the Proteome Profiler kit (R&D Systems) was used according to the manufacturer’s instructions.

Yeast aging and autophagy measurements

For chronological aging experiments, WT BY4741, MATa his3 Δ1 leu2Δ0 met15Δ0 ura3Δ0 S. cerevisiae or the respective sir2 deletion mutant (Jα2R) from the European Saccharomyces Cerevisiae Archive for Functional Analysis were inoculated from fresh overnight cultures to an absorbance of 0.1 (1-106 cells/ml) and grown at 28°C on Synthetic Complete 2% glucose medium. Aliquots were taken to perform survival plating at the indicated time points (Fig. 2 D, line graph) as previously described (Herker et al., 2004). Representative aging experiments are shown with at least three independent samples. Spermidine 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 either on a fluorescence plate reader (GeniosPro; Tecan) or on a cytofluorometer (FACSaria; BD) followed by first-line statistical analysis by means of the FACSDiva software (BD). Autophagy was monitored by vascular localization of Atg8p using fluorescence microscopy or by immunoblotting of cells ectopically expressing an EGFPTagged chimera (Eisenberg et al., 2009) with anti-GFP (Sigma-Aldrich) and antiglyceroldehyde-3-phosphate dehydrogenase antibodies. For biochemical quantifications of the autophagic flux, AP activity was assayed according to published methods (Noda et al., 1995). In brief, WT or Jα2R BY4741 cells were transformed and selected for stable insertion of a pTN9 HindIII fragment encoding for the cytosolic Pho8BpN60 protein. AP activity was then assayed in 1.5 µg of crude protein extracts by measuring the conversion of α-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 Jα2R yeast cells lacking the pTN9 HindIII fragment were simultaneously assayed, and these values were used for background subtraction, giving the vacuolar (autoplastic) AP activity.

C. elegans strains, genetics, and pharmacology

We followed standard procedures for C. elegans strain maintenance. Nematode-rearing temperature was kept at 20°C. The following strains were used in this study: N2, WT Bristol isolate, and VC199, sin2.1(ok434)/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 Caenorhabditis Genetics Center and is funded by the National Institutes of Health National Center for Research Resources. The construction of the pIpa::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 (0.5 J) using a UV cross-linker (Bio-Link BLX-365; 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.
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 t = 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.

SIAC cell culture, sample processing, and analysis
HCT 116 cells were cultured for 2 wk in three different SIAC media (Invitrogen) containing either (1) light isotopes of l-arginine and l-lysine (Arg10/Lys0), (2) l-arginine–13C6, l-lysine 15N4 HCl (Eurotropic) and l-lysine 2HCl (Eurotropic) and l-lysine 15N4 HCl (Eurotropic) or (3) heavy isotopes of l-arginine–13C6, l-lysine 15N4 HCl (Eurotropic) and l-lysine 15N4 HCl (Eurotropic) and l-lysine 15N4 HCl (Eurotropic) or (3) heavy isotopes of l-arginine–13C6, l-lysine 15N4 HCl (Eurotropic) and l-lysine 15N4 HCl (Eurotropic) and l-lysine 15N4 HCl (Eurotropic). These media were preplated with a 150-mM NaCl, 1% Triton X-100, 10-mM EDTA, and 3% HCT 116 cells were cultured for 2 wk in three different SIAC media (Invitrogen) containing either (1) light isotopes of l-arginine and l-lysine (Arg10/Lys0), (2) l-arginine–13C6, l-lysine 2HCl (Eurotropic) and l-lysine 15N4 HCl (Eurotropic) or (3) heavy isotopes of l-arginine–13C6, l-lysine 15N4 HCl (Eurotropic) and l-lysine 15N4 HCl (Eurotropic) and l-lysine 15N4 HCl (Eurotropic) or (3) heavy isotopes of l-arginine–13C6, l-lysine 15N4 HCl (Eurotropic) and l-lysine 15N4 HCl (Eurotropic) and l-lysine 15N4 HCl (Eurotropic) or (3) heavy isotopes of l-arginine–13C6, l-lysine 15N4 HCl (Eurotropic) and l-lysine 15N4 HCl (Eurotropic) and l-lysine 15N4 HCl (Eurotropic). These media were preplated with a 150-mM NaCl, 1% Triton X-100, 10-mM EDTA, and 3% HCT 116 cells were cultured for 2 wk in three different SIAC media (Invitrogen) containing either (1) light isotopes of l-arginine and l-lysine (Arg10/Lys0), (2) l-arginine–13C6, l-lysine 2HCl (Eurotropic) and l-lysine 15N4 HCl (Eurotropic) or (3) heavy isotopes of l-arginine–13C6, l-lysine 15N4 HCl (Eurotropic) and l-lysine 15N4 HCl (Eurotropic) and l-lysine 15N4 HCl (Eurotropic) or (3) heavy isotopes of l-arginine–13C6, l-lysine 15N4 HCl (Eurotropic) and l-lysine 15N4 HCl (Eurotropic) and l-lysine 15N4 HCl (Eurotropic) or (3) heavy isotopes of l-arginine–13C6, l-lysine 15N4 HCl (Eurotropic) and l-lysine 15N4 HCl (Eurotropic) and l-lysine 15N4 HCl (Eurotropic). These media were preplated with a 150-mM NaCl, 1% Triton X-100, 10-mM EDTA, and 3% HCT 116 cells were cultured for 2 wk in three different SIAC media (Invitrogen) containing either (1) light isotopes of l-arginine and l-lysine (Arg10/Lys0), (2) l-arginine–13C6, l-lysine 2HCl (Eurotropic) and l-lysine 15N4 HCl (Eurotropic) or (3) heavy isotopes of l-arginine–13C6, l-lysine 15N4 HCl (Eurotropic) and l-lysine 15N4 HCl (Eurotropic) and l-lysine 15N4 HCl (Eurotropic) or (3) heavy isotopes of l-arginine–13C6, l-lysine 15N4 HCl (Eurotropic) and l-lysine 15N4 HCl (Eurotropic) and l-lysine 15N4 HCl (Eurotropic) or (3) heavy isotopes of l-arginine–13C6, l-lysine 15N4 HCl (Eurotropic) and l-lysine 15N4 HCl (Eurotropic) and l-lysine 15N4 HCl (Eurotropic). These media were preplated with a 150-mM NaCl, 1% Triton X-100, 10-mM EDTA, and 3% HCT 116 cells were cultured for 2 wk in three different SIAC media (Invitrogen) containing either (1) light isotopes of l-arginine and l-lysine (Arg10/Lys0), (2) l-arginine–13C6, l-lysine 2HCl (Eurotropic) and l-lysine 15N4 HCl (Eurotropic) or (3) heavy isotopes of l-arginine–13C6, l-lysine 15N4 HCl (Eurotropic) and l-lysine 15N4 HCl (Eurotropic) and l-lysine 15N4 HCl (Eurotropic) or (3) heavy isotopes of l-arginine–13C6, l-lysine 15N4 HCl (Eurotropic) and l-lysine 15N4 HCl (Eurotropic) and l-lysine 15N4 HCl (Eurotropic) or (3) heavy isotopes of l-arginine–13C6, l-lysine 15N4 HCl (Eurotropic) and l-lysine 15N4 HCl (Eurotropic) and l-lysine 15N4 HCl (Eurotropic). These media were preplated with a 150-mM NaCl, 1% Triton X-100, 10-mM EDTA, and 3% HCT 116 cells were cultured for 2 wk in three different SIAC media (Invitrogen) containing either (1) light isotopes of l-arginine and l-lysine (Arg10/Lys0), (2) l-arginine–13C6, l-lysine 2HCl (Eurotropic) and l-lysine 15N4 HCl (Eurotropic) or (3) heavy isotopes of l-arginine–13C6, l-lysine 15N4 HCl (Eurotropic) and l-lysine 15N4 HCl (Eurotropic) and l-lysine 15N4 HCl (Eurotropic) or (3) heavy isotopes of l-arginine–13C6, l-lysine 15N4 HCl (Eurotropic) and l-lysine 15N4 HCl (Eurotropic) and l-lysine 15N4 HCl (Eurotropic) or (3) heavy isotopes of l-arginine–13C6, l-lysine 15N4 HCl (Eurotropic) and l-lysine 15N4 HCl (Eurotropic) and l-lysine 15N4 HCl (Eurotropic). These media were preplated with a 150-mM NaCl, 1% Triton X-100, 10-mM EDTA, and 3% HCT 116 cells were cultured for 2 wk in three different SIAC media (Invitrogen) containing either (1) light isotopes of l-arginine and l-lysine (Arg10/Lys0), (2) l-arginine–13C6, l-lysine 2HCl (Eurotropic) and l-lysine 15N4 HCl (Eurotropi
N-terminal acetylation and methionine oxidation were chosen as variable modifications. Furthermore, acetylation of light, medium, and heavy iso- 
tope lysines (Lys0/Lys4/Lys8) was chosen as a variable modification. Pro- 
cessed MS/MS spectra were searched against a concatenated target 
decoy database of forward and reversed sequences from the Interna- 
tional Protein Index database (152,616 sequences; FASTA file created 
5/6/2008). For the search, trypsin/PI + DF was chosen for the in silico protease digestion allowing four miscleavages. 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 dissocia- 
tion MS/MS spectra. Upon peptide search, protein and peptide identifica- 
tion 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 counter peak to a given 
lysine-acetylated peptide was identified, this counterfeit 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 pro- 
tein 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 logarithmic transformation algorithm as previously described [Cox 
and Mann, 2008].

Cell respiration and mitochondrial substrate oxidations

Cell respiration and mitochondrial substrate oxidation were polarographi- 
cally 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 con- 
centration of ~10^-6/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-mM ADP. 
10-mM succinate oxidation by digitonin-permeabilized cells was measured 
in the presence of 2-µM oligomycin, a specific inhibitor of the mitochondrial ATPase, and 2-µM 
carbonyl cyanide m-chlorophenol hydrazone, a potent mitochondrial uncoupler, 
allowing 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 Ontol- 
ye tool; Maere et al., 2005) and PANTHER (Protein Analysis Through 
Evolutionary Relationships) classification system. For the enrichment analy- 
sis, proteins regulated by ≥1.5-fold were included, and p-values were cal- 
culated 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 vari- 
ability tool; Maere et al., 2005) and PANTHER (Protein Analysis Through 
Evolutionary Relationships) classification system. and co-regulates major cellular functions.

References


nrd2060

1038/nature09294

Benjamin, Y., and Y. Hochberg. 1995. Controlling the false discovery rate: a 
practical and powerful approach to multiple testing. J. Roy. Stat. Soc. B. 
57:289–300.

Blagovev, B., and M. Mann. 2006. Quantitative proteomics to study mitogen-activated 

Bunz, F., P.M. Hwang, C. Torrance, T. Waldman, Y. Zhang, L. Dillehay, J. 
Williams, C. Lengauer, K.W. Kinzler, and B. Vogelstein. 1999. Disruption of 
p53 in human cancer cells alters the responses to therapeutic agents. J. 
Clin. Invest. 104:263–269. doi:10.1172/JCI9866

Büttner, S., T. Eisenberg, D. Carmona-Gutierrez, D. Ruli, H. Knauer, C. 

Choudhary, C., C. Kumar, F. Gnad, M.L. Nielsen, M. Rehman, T.C. Walther, J.V. 
Olsen, and M. Mann. 2009. Lysine acetylation targets protein complexes 
doi:10.1126/science.1173571

Cox, J., and M. Mann. 2008. MassQuant enables high peptide identification 
rates, individualized p.p.b.-range mass accuracies and proteome-wide 
nt.1511

Crielio, A., M.C. Mairui, E. Tasdemir, I. Vitale, A.A. Fiebig, D. Andrews, 
J. Molgo, J. Diaz, S. Lavandero, F. Harper, et al. 2007. Regulation of 
atophagy by the inositol triphosphate receptor. Cell Death Differ. 
14:1029–1039.

extensive apoptosis by depolarizing mitochondrial membranes and acti- 
vating caspase-9 in acute lymphoblastic leukemia cells. Cancer Res. 
61:4731–4739.