

Longevity-relevant regulation of autophagy at the level of the acetylproteome

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The acetylase inhibitor, spermidine and the deacetylase activator, resveratrol, both induce autophagy and prolong life span of the model organism *Caenorhabditis elegans* in an autophagy-dependent fashion. Based on these premises, we investigated the differences and similarities in spermidine and resveratrol-induced autophagy. The deacetylase sirtuin 1 (SIRT1) and its orthologs are required for the autophagy induction by resveratrol but dispensable for autophagy stimulation by spermidine in human cells, *Saccharomyces cerevisiae* and *C. elegans*. SIRT1 is also dispensable for life-span extension by spermidine. Mass spectrometry analysis of the human acetylproteome revealed that resveratrol and/or spermidine induce changes in the acetylation of 560 peptides corresponding to 375 different proteins. Among these, 170 proteins are part of the recently elucidated human autophagy protein network. Importantly, spermidine and resveratrol frequently affect the acetylation pattern in a similar fashion. In the cytoplasm, spermidine and resveratrol induce convergent protein de-acetylation more frequently than convergent acetylation, while in the nucleus, acetylation is dominantly triggered by both agents. We surmise that subtle and concerted alterations in the acetylproteome regulate autophagy at multiple levels.

Macroautophagy (which we refer to as “autophagy”) constitutes an essential mechanism of adaptation to external or internal stress that increases the fitness of individual cells and even entire organisms. Autophagy plays a cardinal role in cellular homeostasis, facilitates the mobilization of energy reserves when external resources are scarce, and is indispensable for the removal of toxic protein aggregates and damaged organelles. At the organismal level, autophagy can mediate cardioprotection and neuroprotection (for instance in the context of ischemic preconditioning), delay the pathogenic manifestations of aging and prolong life span.

A myriad of distinct stimuli and noxious agents can induce a rapid homeostatic autophagy that becomes apparent within minutes or hours. Accordingly, there is increasing evidence that a first wave of autophagy induction does not require transcriptional reprogramming and changes in the expression of pro-autophagic gene products, but rather relies on post-translational modifications of proteins, which include but are not limited to changes in protein phosphorylation, acetylation and ubiquitination, resulting in modifications

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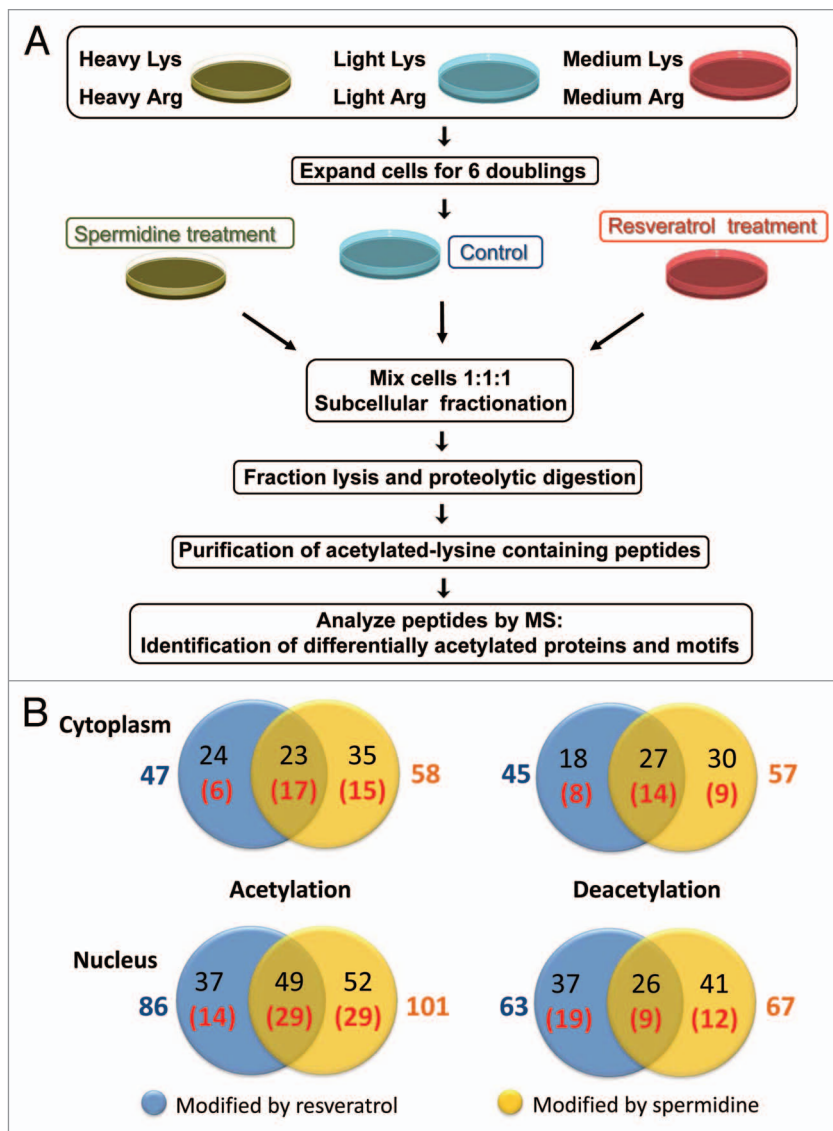


Figure 1. Modifications of the autophagy-relevant acetylproteome by resveratrol and spermidine. (A) Schematic overview of the experiment. Three cultures of HCT116 colon cancer cells were cultured for several weeks in the presence of three distinct stable isotope-labeled amino acids: standard light Lys and Arg ($^{12}\text{C}_6$ L-lysine and $^{12}\text{C}_6$ L-arginine, respectively), medium-weight Lys and Arg (2HCl 4,4,5,5- D_4 L-lysine and $^{13}\text{C}_6$ L-arginine, respectively) and heavy Lys and Arg ($^{13}\text{C}_6^{15}\text{N}_4$ L-lysine and $^{13}\text{C}_6^{15}\text{N}_4$ HCl L-arginine, respectively) resulting in labeling of cellular proteins. Then, the cultures were either left untreated or treated with spermidine or resveratrol. All the cellular populations were mixed and then processed by cellular fractionation, purification of the lysine-acetylated proteins and quantitative mass spectrometry, placing special emphasis on the identification of acetylated peptides whose quantity is altered (by a factor >1.2) by resveratrol or spermidine. (B) Effects of resveratrol and spermidine treatment in the acetylation of nuclear and cytosolic proteins. Venn diagrams show the distribution of the proteins whose acetylation status is convergently (overlapping areas) or divergently (non-overlapping areas) modified by resveratrol or spermidine (by a factor >1.2) in cytosolic and nuclear fractions. Red numbers in parentheses indicate the number of proteins belonging to the known autophagy-relevant protein network (ARPN) whose acetylation status is significantly modified by resveratrol and/or spermidine.

of enzymatic activities, the composition of multiprotein complexes and their subcellular localization/organization.

Two structurally unrelated pharmacological agents, resveratrol (a polyphenol)

and spermidine (a polyamine), induce autophagy and increase longevity and these two effects are indeed linked by a cause-effect relationship. Thus, autophagy-incompetent strains of yeast (*S. cerevisiae*)

or *Drosophila melanogaster* fail to manifest an increase in life span when they are treated with spermidine. Moreover, the knockdown of several essential autophagy genes abolishes the life-span-extending effect of both spermidine and resveratrol in *C. elegans*.

Resveratrol is an activator of the NAD^+ -dependent deacetylase, sirtuin 1 (SIRT1). We found that the induction of autophagy and longevity by resveratrol (or caloric restriction, which is the physiological activator of SIRT1) indeed depends on the expression of the *C. elegans* ortholog of SIRT1. Moreover, the genetic or pharmacological inhibition of SIRT1 prevents the induction of autophagy by resveratrol in human cells. In contrast, spermidine, which is an inhibitor of histone acetylases, does not require SIRT1 to induce autophagy in any of the three systems that we investigated: human cells, *C. elegans* and *S. cerevisiae*. In these model organisms, SIRT1 is also dispensable for the life-span-prolonging effect of spermidine.

The aforementioned results clearly indicate that the initial signaling events that are triggered by resveratrol and spermidine are distinct. Nonetheless, we wondered whether the deacetylase activator, resveratrol and the acetylase inhibitor, spermidine, would induce autophagy by similar modifications of the autophagy-relevant acetylproteome. To address this question, we performed stable isotope labeling with amino acids in cell culture (SILAC), thus marking the proteins of control cells, resveratrol-treated cells and spermidine-treated cells with three distinct stable isotopes. Then, the three cultures were mixed and lysed simultaneously, followed by subcellular fractionation (to separate the cytoplasm and nuclei), purification of the proteins/peptides containing acetylated lysine residues and their identification by quantitative mass spectrometry (Fig. 1A). This experimental design allowed for the comparative quantification of the acetylproteome and its modifications by resveratrol and spermidine. We found that resveratrol or spermidine provoke alterations (>1.2 fold) in the acetylation of 560 lysine-containing motifs from 375 distinct proteins. Importantly, 170 proteins whose acetylation status is modified in response to resveratrol or spermidine treatment

overlap with proteins from the recently elucidated entire human autophagy protein network consisting of 2,540 distinct intracellular proteins (Fig. 1B).

Both resveratrol and spermidine tended to induce the convergent (de)acetylation of the same proteins. No major differences were detected in the consensus (de)acetylation sites that were modified in response to resveratrol as compared to those affected by spermidine. In the nucleus, resveratrol and spermidine mostly trigger convergent acetylation reactions. In the cytosol, by contrast, both agents provoke both convergent acetylation and convergent deacetylation of multiple proteins to a similar extent, although the number of convergently deacetylated proteins is higher. We have recently reported that essential autophagy genes are required for life-span extension due to spermidine during conditions of chronological aging. This observed longevity increase is linked to changes in the acetylation of nuclear histones and to a transcriptional increase of different autophagy-related genes. Thus, the fact that resveratrol and spermidine

are able to induce autophagy in cytoplasts (enucleated cells) suggests that nuclear acetylation reactions are dispensable for spermidine and/or resveratrol-dependent autophagy induction and that (de)acetylation of critical cytoplasmic proteins mediates the pro-autophagic activity of spermidine and resveratrol. Collectively, 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 response, transcriptional reprogramming is required to maintain an increased basal autophagic activity, thus contributing to the previously reported life-span extension.

Taken together, all these observations suggest that multiple post-translational modifications of the acetylproteome can likely regulate autophagy. Considering that energy metabolism might influence the acetylproteome both via an increase in SIRT1 activity and by a reduction in the overall abundance of acetyl donors, it will be interesting to study the detailed (and perhaps circadian) changes in the

acetylproteome, as they are likewise modulated by the nutritional status, as putative biomarkers of autophagy and longevity in humans.

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