Priority Research paper

# Inhibition of mammalian S6 kinase by resveratrol suppresses autophagy

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***Abbreviations:*** *p70 S6 kinase (S6K1); microtubule-associated protein 1 light chain 3 (LC3); monodansylcadaverine (MDC); mammalian target of rapamycin (mTOR); resveratrol (res); mouse embryonic fibroblasts (MEFs)*
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**Abstract: Resveratrol is a plant-derived polyphenol that promotes health and disease resistance in rodent models, and extends lifespan in lower organisms. A major challenge is to understand the biological processes and molecular pathways by which resveratrol induces these beneficial effects. Autophagy is a critical process by which cells turn over damaged components and maintain bioenergetic requirements. Disruption of the normal balance between pro- and anti-autophagic signals is linked to cancer, liver disease, and neurodegenerative disorders. Here we show that resveratrol attenuates autophagy in response to nutrient limitation or rapamycin in multiple cell lines through a pathway independent of a known target, SIRT1. In a large-scale *in vitro* kinase screen we identified p70 S6 kinase (S6K1) as a target of resveratrol. Blocking S6K1 activity by expression of a dominant-negative mutant or RNA interference is sufficient to disrupt autophagy to a similar extent as resveratrol. Furthermore, co-administration of resveratrol with S6K1 knockdown does not produce an additive effect. These data indicate that S6K1 is important for the full induction of autophagy in mammals and raise the possibility that some of the beneficial effects of resveratrol are due to modulation of S6K1 activity.**

## INTRODUCTION

Autophagy is an essential process by which eukaryotic cells turn over long-lived cytosolic components, clear damaged proteins and organelles, and maintain bio-energetic requirements during conditions of nutrient and growth factor withdrawal [1]. Degradation and recycling of cellular components can involve the uptake of small amounts of cytoplasm at the vacuole or lysosome surface (microautophagy) or, in response to a strong stimulus such as starvation, the formation of specialized double membraned-organelles termed autophagosomes, which engulf larger portions of cytoplasm or organelles before fusing with a vacuole or lysosome (macro-auto-

phagy), hereafter referred to simply as autophagy [1-3]. While this process is an important part of the normal balance between anabolic and catabolic processes and can prolong survival during nutrient limitation, autophagy is also an alternate death pathway that facilitates type II programmed cell death [4-6]. For this reason, imbalances in this pathway can contribute to seemingly diverse pathologies.

Resveratrol is a small polyphenol that extends the lifespan of simple model organisms, ostensibly by mimicking caloric restriction [7, 8]. In rodents, resveratrol protects from a variety of age-related diseases including cancer, cardiovascular disease, neurodegeneration, obesity and diabetes [9-14]. Although there is evidence that some of resveratrol's actions are mediated by activation of the SIRT1 deacetylase, the mechanisms underlying the numerous beneficial effects of resveratrol remain to be elucidated.

It has previously been reported that 24-48 hours of resveratrol treatment induces autophagy in cancer cells grown in rich media, suggesting a mechanism by which resveratrol might enhance cell death and suppress tumor growth [15-18]. Here we report the effect of resveratrol treatment on the normal induction of autophagy over 4-6 hours following nutrient withdrawal in tumor and non-tumor cell lines. In contrast to the activation of the autophagic pathway observed in tumor cells in complete media, we find that resveratrol markedly inhibits the starvation-induced autophagic response. We show that this effect does not require SIRT1, and identify p70 S6 kinase (S6K1) as a target of resveratrol that is responsible for the inhibition of starvation-induced autophagy.

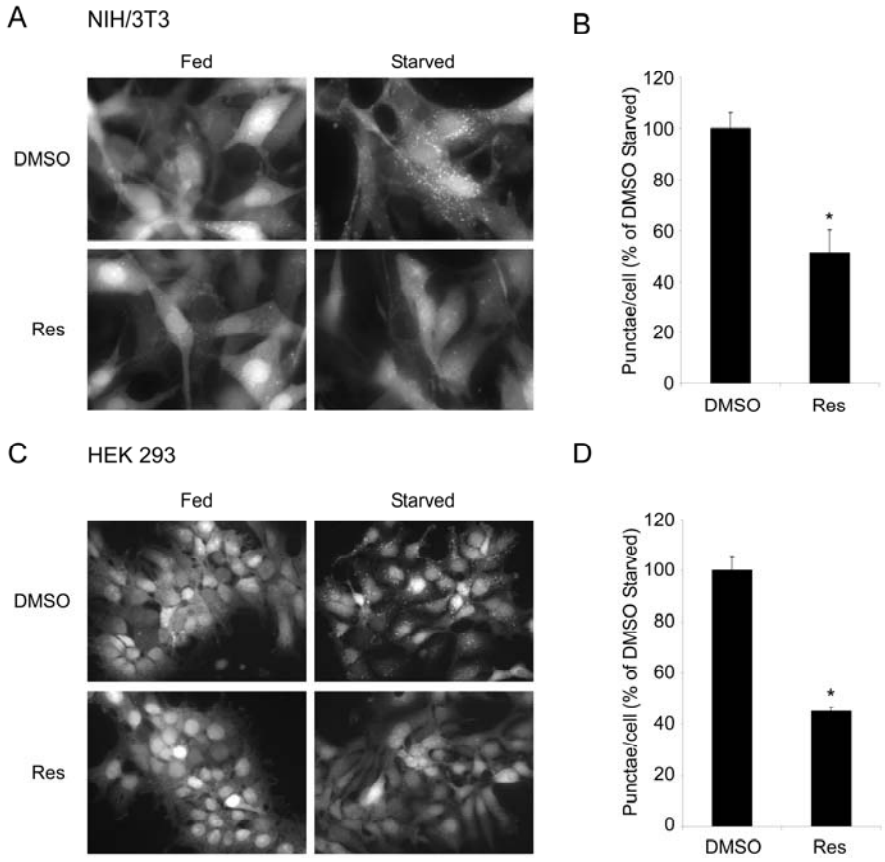


**Figure 1. Resveratrol inhibits autophagy in mammalian cells.** (**A**) NIH/3T3 cells stably expressing the GFP-LC3 fusion protein were subjected to nutrient withdrawal by replacing growth media (Fed) with Earle's buffered saline solution (Starved) and treated with either DMSO or 50 μM resveratrol (Res) for 2 hours. Representative fields at 63X (oil immersion) magnification are shown. (**B**) Quantification of punctae/cell in (A) of at least 4 fields per treatment are represented as a percentage of the starved DMSO treated cells. (**C**) HEK293 cells stably expressing the GFP-LC3 fusion protein were subjected to starvation and either DMSO or 50 μM Res for 6 hours. Representative fields at 40X magnification are shown. (**D**) Quantification was performed on HEK293 cells as in (B). Error bars represent s.e.m. * ($p < 0.0022$).

## RESULTS

### Suppression of nutrient starvation- and rapamycin-induced autophagy by resveratrol

Autophagy is an important component of the cellular response to nutrient stress and growth factor withdrawal. We therefore tested whether resveratrol treatment would influence regulation of autophagy under these conditions. Autophagy was assessed by monitoring the relocalization of a component of the autophagy machinery, LC3, from the cytoplasm to the forming autophagosome [19]. NIH/3T3 cells or HEK293 cells stably expressing a GFP-LC3 fusion protein were generated and were induced to undergo autophagy in the presence or absence of resveratrol. Treatment with resveratrol resulted in a dramatic reduction in the number of starvation-induced GFP-LC3 punctae (Figure 1). Similar inhibitory effects on autophagy were observed in other cell lines, including human tumor cell lines (HeLa and U2OS), as well as mouse embryonic fibroblasts (MEFs) using mono-dansylcadaverine (MDC), a fluorescent compound that stains late autophagosomes (Supplementary Figures S1 and S3) [20]. Since previous studies have described an increase in autophagy following 24 hours of resveratrol treatment in nutrient rich media, we also tested the effects of resveratrol under these conditions. Consistent with these results, we observed an induction of auto-phagosome formation in cells treated with resveratrol for 24 hours in complete media containing serum (Supplementary Figure S2). Thus, the influence of resveratrol on autophagy is context dependent, and in the case of autophagy induced by nutrient limitation, resveratrol is inhibitory.

Rapamycin is an inhibitor of the nutrient sensing mTOR-Raptor complex and has been shown to induce autophagy [21, 22]. In yeast, it had been shown that resveratrol can reverse some markers of autophagy induced by rapamycin [23]. Consistent with these results and similar to the results seen under nutrient limitation, rapamycin-induced autophagy is almost completely abrogated by resveratrol treatment (Figure 2A and B). Decreased GFP-LC3 punctae could be due either to increased flux or a block in autophagy. To distinguish between these two possibilities, we examined LC3-II accumulation with and without Bafilomycin A1, an inhibitor of lysosome degradation. Under both conditions, resveratrol was able to block the accumulation of LC3-II, indicating a suppression of autophagy rather than an enhancement of lysosomal clearance (Figure 2C).

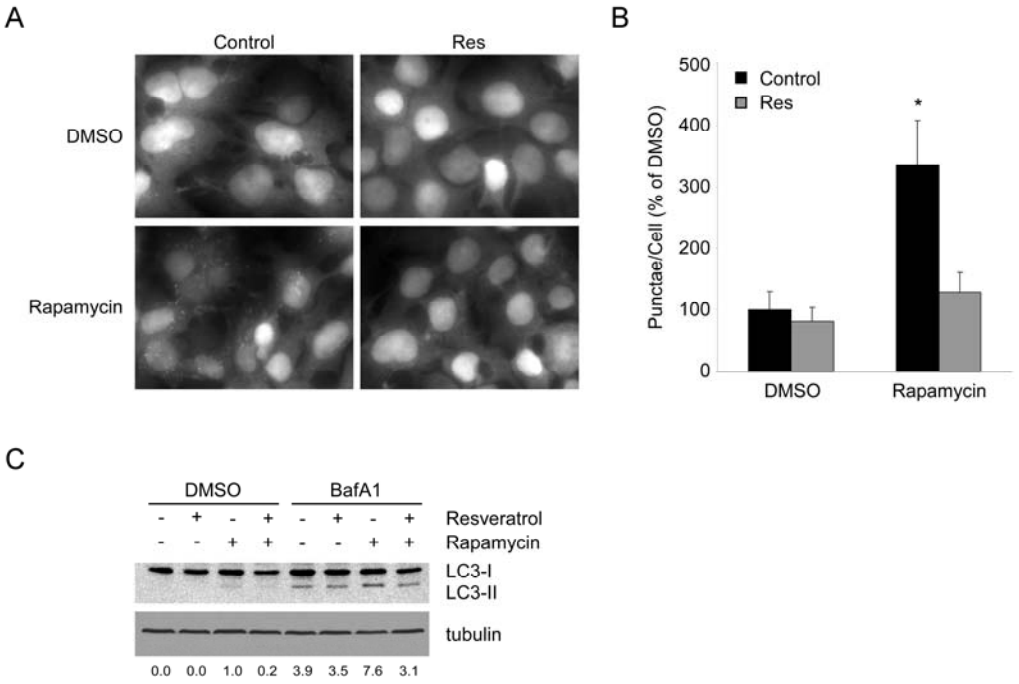


**Figure 2. Resveratrol suppresses autophagy under TOR inhibition.** (**A**) HEK293 cells stably expressing GFP-LC3 growing in complete media were pretreated with DMSO or 50 μM resveratrol (Res) for 1 hour, prior to addition of DMSO or 200 nM rapamycin for 4 hours. 40X magnification fields have been cropped and zoomed for ease of punctae visualization. (**B**) Quantification of punctae/cell from (**A**) of 10 fields per treatment are represented as a percentage of DMSO treated cells. Error bars represent s.d.m. * ($p < 0.0001$) (**C**) HEK293 GFP-LC3 cells were pretreated for 1 hour with DMSO or resveratrol and subsequently treated with DMSO or 1 mM rapamycin in the presence or absence of 100 nM Bafilomycin A1 for 4 hours. A representative western blot of endogenous LC3 and tubulin are shown. Numbers represent the ratio of LC3-II to tubulin for each condition normalized to Rapamycin in the absence of BafA1.

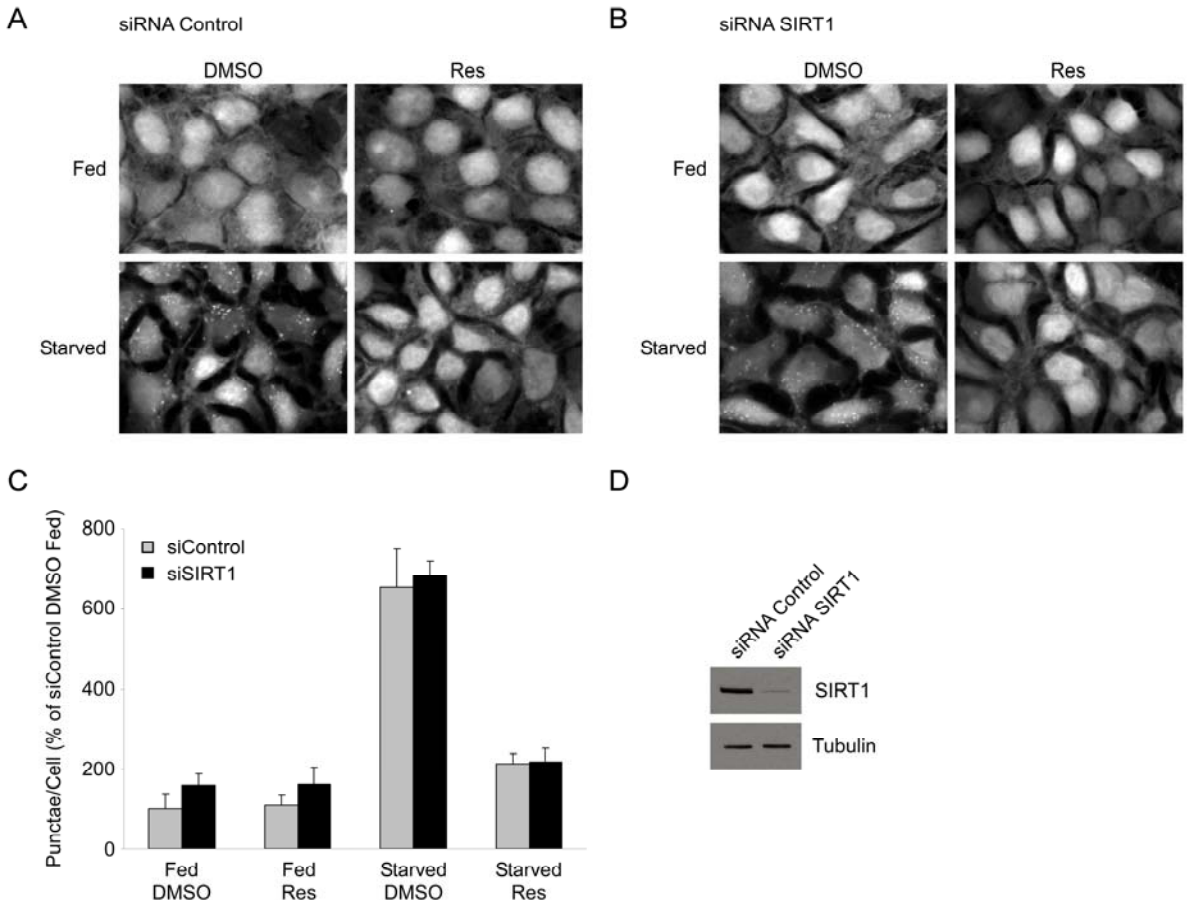


**Figure 3. Resveratrol suppresses autophagy independently of SIRT1.** HEK293 cells stably expressing GFP-LC3 were transfected with either a control siRNA (**A**) or an siRNA directed against SIRT1 (**B**) for 72 hours. Subsequently, cells were subjected to nutrient starvation with or without 50 μM resveratrol (Res) treatment for 4 hours. 40X magnification fields have been cropped and zoomed for ease of punctae visualization. (**C**) Quantification of punctae/cell from (A) and (B) of 4 fields are represented as a percentage of fed DMSO treated control siRNA cells. Error bars represent s.d.m. (**D**) Representative western blot showing typical knockdown of SIRT1 by siRNA transfection in HEK293 GFP-LC3 cells.

We next tested whether SIRT1, an $NAD^+$-dependent deacetylase that is activated by resveratrol [8], was required for this effect. To test this hypothesis, HEK 293 GFP-LC3 cells were transfected with either control siRNA or an siRNA directed against the SIRT1 deacetylase and subsequently nutrient starved in the presence or absence of resveratrol. We found that both control and SIRT1 knockdown cells displayed a similar level of induction of GFP-LC3 punctae and that resveratrol still produced an equivalent suppression of autophagy (Figure 3). Consistent with these results, SIRT1+/+ and SIRT1-/- MEFs induced autophagy in response to nutrient withdrawal, and in both cell lines the inhibitory effect of resveratrol on autophagy was comparable (Supplementary Figure S3). These data indicate that inhibition of starvation-induced autophagy by resveratrol is molecularly distinct from the induction seen in previous studies and is not mediated by the SIRT1-dependent pathway that has previously been described [24]. It will be interesting to explore the differences in these systems that engage or disengage SIRT1 during autophagic induction.

**Kinase profiling of resveratrol *in vitro***

Resveratrol has previously been shown to inhibit several kinases including PKC and Src [25] and is structurally similar to the flavanoid quercetin (Figure 4A), which is an inhibitor of PI 3-kinase [26]. Therefore, we hypothesized that the effect of resveratrol

on autophagy could be related to inhibition of one or more upstream kinases. To test this, we performed an *in vitro* kinase screen and determined an inhibition profile for resveratrol. Out of 100 kinases tested, Jak2, NLK, p70 S6 kinase (S6K1), Pim-1, and Pim-2 emerged as potential targets of resveratrol (Figure 4B, Supplementary Table 1). With the exception of S6K1, these kinases play primary roles in the hematopoietic system, and were thus viewed as unlikely to have been responsible for the effect we were studying. Furthermore, although JNK had previously been shown to play a positive role in autophagy, its activity was not significantly inhibited by resveratrol at this dose [27]. On the other hand, S6K1 is known to play a requisite role in the regulation of autophagy in *Drosophila* [28], making it a promising candidate for the *in vivo* target of resveratrol that is responsible for inhibition of autophagy.

To confirm the inhibition of S6 kinase by resveratrol *in vitro*, we determined the effect of the compound on immunoprecipitated HA-tagged S6K1 from HEK293 cells using purified GST-tagged full-length recombinant S6 ribosomal protein (S6) as a substrate. In agreement with the primary screen, we found that resveratrol inhibited the activity of S6 kinase in a dose-dependent manner, exhibiting an IC50 of ~25 μM (Figure 4C and D, and Supplementary Figure S4).

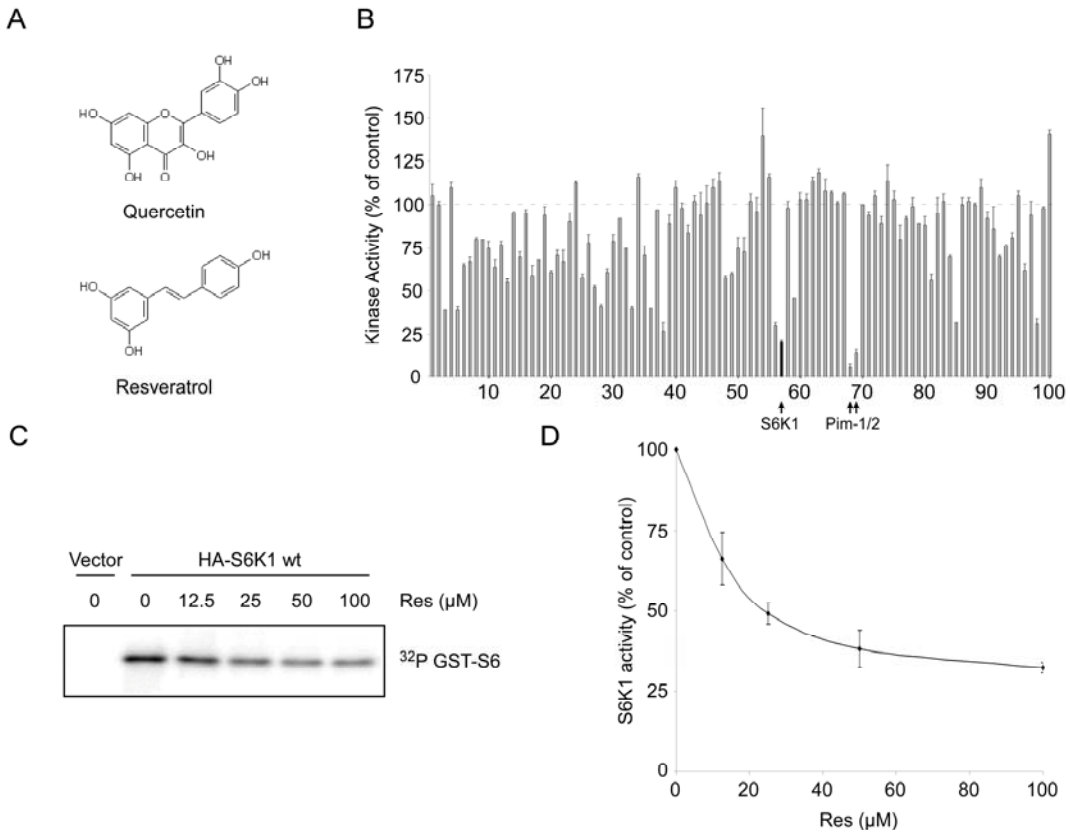


**Figure 4. Resveratrol inhibits S6K1 *in vitro*.** (**A**) Structural similarity between resveratrol and quercetin, a known kinase inhibitor. (**B**) Kinase inhibition profile for resveratrol at 20 μM obtained using KinaseProfiler™ (Upstate). Dashed line represents 100% activity as compared to control. Black filled-in bar on the graph indicates S6K1. Complete data set is provided in Supplementary Table 1. Error bars represent s.d.m. (**C**) Phosphorylation of recombinant GST-tagged S6 by immunoprecipitated HA-S6K1 under increasing concentrations of resveratrol (Res). Autoradiograph depicts S6K1 phosphorylation of GST-S6. (**D**) Average of three separate kinase assay experiments as performed in (**C**). Densitometry was performed using NIH ImageJ. Error bars represent s.e.m.