

Small molecule activators of sirtuins extend *Saccharomyces cerevisiae* lifespan

Konrad T. Howitz¹, Kevin J. Bitterman², Haim Y. Cohen², Dudley W. Lamming², Siva Lavu², Jason G. Wood², Robert E. Zipkin¹, Phuong Chung¹, Anne Kisielewski¹, Li-Li Zhang¹, Brandy Scherer¹ & David A. Sinclair²

¹BIOMOL Research Laboratories, Inc., 5120 Butler Pike, Plymouth Meeting, Pennsylvania 19462, USA

²Department of Pathology, Harvard Medical School, 200 Longwood Avenue, Boston, Massachusetts 02115, USA

In diverse organisms, calorie restriction slows the pace of ageing and increases maximum lifespan. In the budding yeast *Saccharomyces cerevisiae*, calorie restriction extends lifespan by increasing the activity of Sir2 (ref. 1), a member of the conserved sirtuin family of NAD⁺-dependent protein deacetylases^{2–6}. Included in this family are SIR-2.1, a *Caenorhabditis elegans* enzyme that regulates lifespan⁷, and SIRT1, a human deacetylase that promotes cell survival by negatively regulating the p53 tumour suppressor^{8–10}. Here we report the discovery of three classes of small molecules that activate sirtuins. We show that the potent activator resveratrol, a polyphenol found in red wine, lowers the Michaelis constant of SIRT1 for both the acetylated substrate and NAD⁺, and increases cell survival by stimulating SIRT1-dependent deacetylation of p53. In yeast, resveratrol mimics calorie restriction by stimulating Sir2, increasing DNA stability and extending lifespan by 70%. We discuss possible evolutionary origins of this phenomenon and suggest new lines of research into the therapeutic use of sirtuin activators.

Longevity regulatory genes have been identified in many eukaryotes, including rodents, flies, nematode worms and even single-celled organisms such as baker's yeast (reviewed in ref. 11). These genes seem to be part of an evolutionarily conserved longevity pathway that evolved to promote survival in response to deteriorating environmental conditions. We recently reported that the pace of ageing in yeast is governed by *PNC1*, a calorie restriction-responsive and stress-responsive gene that depletes nicotinamide¹², a physiological inhibitor of Sir2¹³. On the basis of these results we proposed that calorie restriction might confer health benefits because it is a mild stress that provokes an organismal defence response¹².

On the basis of the positive health effects of calorie restriction in mammals¹⁴, we sought to identify small molecules that could modulate sirtuin activity. To this end, we screened a number of small molecule libraries, which included analogues of ϵ -acetyl lysine, NAD⁺, NAD⁺ precursors, nucleotides and purinergic ligands. Fluorescent deacetylation assays were performed on human SIRT1 using a synthetic peptide substrate that encompassed lysine 382 (K382) of p53, a known target of SIRT1 *in vivo*^{8–10} and a preferred target *in vitro* (see Supplementary Fig. 1).

The initial compound screen identified several inhibitors of SIRT1 (Supplementary Table 7). However, the most notable outcome was the identification of two structurally similar compounds, quercetin and piceatannol, that stimulated SIRT1 activity five- and eightfold, respectively (Table 1). Both quercetin and piceatannol have previously been identified as protein kinase inhibitors^{15,16}. Comparison of the two compounds suggested a possible structure-activity relationship. The *trans*-stilbene ring structures of piceatannol are superimposable on the flavonoid A and B rings of quercetin, with the ether oxygen and carbon-2 of the C ring aligning with the ethylene carbons in piceatannol (Table 1). Furthermore, the 5, 7, 3'

and 4' hydroxyl group positions in quercetin can be aligned, respectively, with the 3, 5, 3' and 4' hydroxyls of piceatannol.

Both quercetin and piceatannol are polyphenols, members of a large and diverse group of plant secondary metabolites that include flavones, stilbenes, flavanones, isoflavones, catechins (flavan-3-ols), chalcones, tannins and anthocyanidins^{17,18}. A secondary screen of these compound families identified 15 additional SIRT1 activators that provided more than twofold stimulation (Table 1; see also Supplementary Tables 1, 2 and 4). We refer to the sirtuin-activating compounds as STACs.

Many, but not all of the most active STACs included hydroxyls in the two meta-positions of the A ring, *trans* to the B ring with a 4' or 3',4' hydroxyl pattern (Table 1; see also Supplementary Tables 1 and 2). A potentially coplanar orientation and *trans*-positioning of the

Table 1 Stimulation of SIRT1 catalytic rate by plant polyphenols

Compound (100 μ M)	Ratio to control (mean \pm s.e.)	Structure
Resveratrol (3,5,4'-trihydroxy- <i>trans</i> -stilbene)	13.4 \pm 1.0	
Butein (3,4,2',4'-tetrahydroxychalcone)	8.53 \pm 0.89	
Piceatannol (3,5,3',4'-tetrahydroxy- <i>trans</i> -stilbene)	7.90 \pm 0.50	
Isoliquiritigenin (4,2',4'-trihydroxychalcone)	7.57 \pm 0.84	
Fisetin (3,7,3',4'-tetrahydroxyflavone)	6.58 \pm 0.69	
Quercetin (3,5,7,3',4'-pentahydroxyflavone)	4.59 \pm 0.47	

Rate measurements with 25 μ M NAD⁺ and 25 μ M p53-382 acetylated peptide substrate were performed as described in Methods. All ratio data were calculated from experiments in which the total deacetylation in the control reaction was 0.25–1.25 μ M peptide or 1–5% of the initial concentration of acetylated peptide. s.e., standard error.

hydroxylated rings also appear to be important for activity. The most potent activator was resveratrol (3,5,4'-trihydroxystilbene), a polyphenol found in red wine that is associated with a surprising number of health benefits, most notably the mitigation of age-related diseases, including neurodegeneration, carcinogenesis and atherosclerosis¹⁷⁻¹⁹.

The biological effects of polyphenols are frequently attributed to

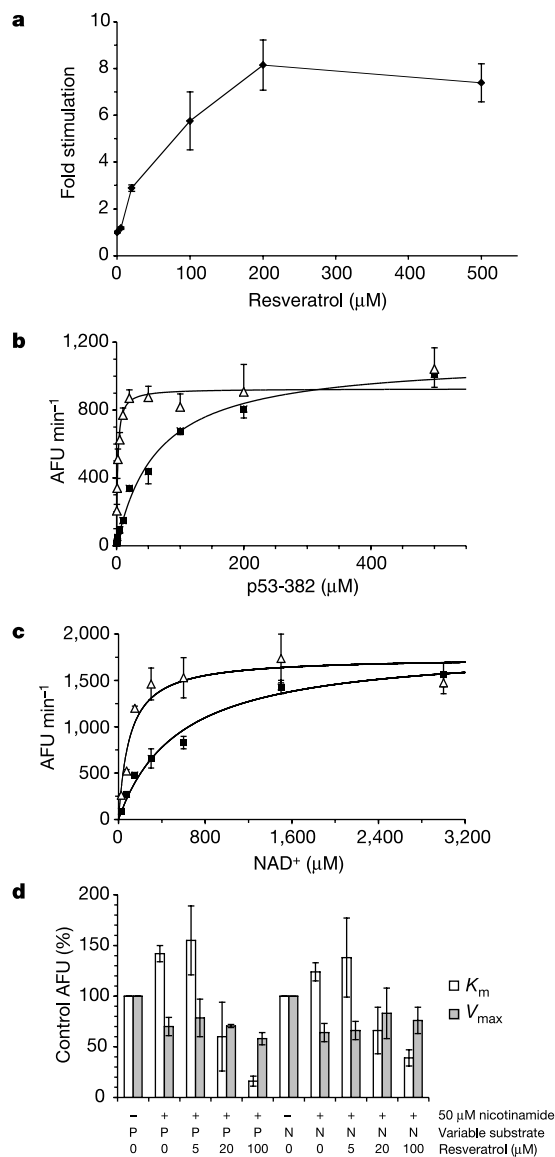


Figure 1 Effects of resveratrol on the kinetics of recombinant SIRT1. **a**, Resveratrol dose-response of SIRT1 catalytic rate. Fold stimulation values are derived from the means of six to nine determinations. **b**, SIRT1 initial rate at 3 mM NAD⁺, as a function of p53-382 acetylated peptide concentration in the presence (triangles) or absence (squares) of 100 μM resveratrol. Lines represent nonlinear least-square fits to the Michaelis-Menten equation. K_m (control, squares) = 64 μM, K_m (plus resveratrol, triangles) = 1.8 μM; V_{max} (control, squares) = 1,107 AFU min⁻¹, V_{max} (plus resveratrol, triangles) = 926 AFU min⁻¹. **c**, SIRT1 initial rate at 1 mM p53-382 acetylated peptide, as a function of NAD⁺ concentration, in the presence (triangles) or absence (squares) of 100 μM resveratrol as in **b**. K_m (control, squares) = 558 μM, K_m (plus resveratrol, triangles) = 101 μM; V_{max} (control, squares) = 1,863 AFU min⁻¹, V_{max} (plus resveratrol, triangles) = 1,749 AFU min⁻¹. **d**, Kinetic constants are relative to untreated control and represent the mean of two determinations. AFU, arbitrary fluorescence unit; N, NAD⁺; P, p53 acetylated peptide.

antioxidant, metal-ion-chelating and/or free-radical-scavenging activity¹⁷. We considered the possibility that the stimulation of SIRT1 might simply represent the repair of oxidative- or metal-ion-induced damage to the recombinant protein. We discounted this explanation based on three observations. First, a variety of free-radical protective compounds, including antioxidants, chelators and radical scavengers failed to stimulate SIRT1 (see Supplementary Table 6). Second, among various polyphenols of equivalent antioxidant capacity²⁰ we observed diverse SIRT1-stimulating activity. Third, as described below, we showed that resveratrol could stimulate the activity of native sirtuins *in vivo*.

Dose-response experiments showed that resveratrol doubled the rate of deacetylation by SIRT1 at about 11 μM and was saturated at 100–200 μM (Fig. 1a). Although resveratrol had no significant effect on the two V_{max} determinations when either substrate or NAD⁺ was varied (Fig. 1b, c), it had pronounced effects on the two apparent Michaelis constants (K_m). Its effect on the acetylated

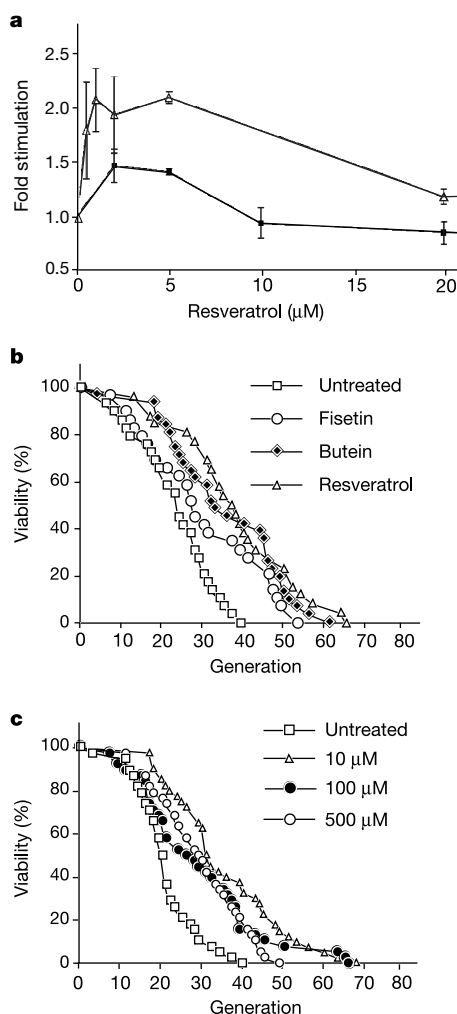


Figure 2 Effects of polyphenols on Sir2 and *S. cerevisiae* lifespan. **a**, Rates were determined using either FdL acetylated lysine substrate (squares) or an acetylated fluorogenic peptide (triangles) based on the sequence encompassing histone H4 Lys 16, a target of Sir2 *in vivo* (Supplementary Fig. 1). Fold stimulations represent the mean of at least three determinations. **b**, Replicative lifespan is the number of daughter cells an individual mother cell produces before dying. Lifespan analyses were determined by micro-manipulating individual yeast cells²³ on complete 2% glucose medium with 10 μM compound, unless otherwise stated. Average lifespan for wild type, 22.9 generations; fisetin, 30.0; butein, 35.5; resveratrol, 36.8. **c**, Average lifespan for wild type untreated, 21.0 generations; grown on resveratrol 10 μM, 35.7; 100 μM, 29.4; 500 μM, 29.3.

peptide K_m was particularly striking, amounting to a 35-fold decrease (Fig. 1b). Resveratrol also lowered the K_m for NAD^+ over fivefold (Fig. 1c). As resveratrol acts only on K_m , it could be classified as an allosteric effector of the 'K system' type²¹, which may indicate that only the substrate-binding affinity of the enzyme has been altered. In the presence of the negative regulator nicotinamide, resveratrol stimulated SIRT1, but there also appeared to be complex, concentration-dependent effects (Fig. 1d). Protein structural studies are underway to determine how resveratrol activates SIRT1 and to elucidate the interaction between the effects of nicotinamide and polyphenols.

To investigate the effects of sirtuin activators *in vivo*, we turned to *S. cerevisiae*. In this organism, a single extra copy of *SIR2* is sufficient to mimic calorie restriction¹. We determined that the optimal resveratrol concentration for activation of recombinant Sir2 was 2–5 μ M and that, in contrast to recombinant SIRT1, the rate stimulation was no more than about twofold (Fig. 2a). Resveratrol and other STACs in the stilbene, flavone and chalcone families were then tested for their effect on yeast replicative lifespan²². Three compounds—butein, fisetin and resveratrol—increased average lifespan by 31%, 55% and 70%, respectively, and all three significantly increased maximum lifespan (Fig. 2b). Higher concentrations of resveratrol provided no added lifespan benefit (Fig. 2c), and there was no lasting effect of the compound on the lifespan of pre-treated young cells (data not shown). Quercetin and piceatannol had no significant effect on lifespan (data not shown), possibly due to oxidation of the compounds in the medium or insufficient uptake into cells.

For subsequent experiments we focused on resveratrol. Glucose restriction, a form of calorie restriction in yeast, resulted in no significant extension of long-lived, resveratrol-treated cells (Fig. 3a), indicating that resveratrol probably acts through the same pathway as calorie restriction. Consistent with this, resveratrol had no effect on the lifespan of a *sir2* null mutant (Fig. 3b). Given that resveratrol

is reported to have fungicidal properties at high concentrations and that mild stress can extend yeast lifespan by activating *PNC1* (ref. 12), it was plausible that resveratrol was extending lifespan by inducing *PNC1*, rather than acting on Sir2 directly. However, resveratrol extended the lifespan of a *pnc1* null mutant nearly as well as it did wild-type cells (Fig. 3b). Together these data show that resveratrol acts downstream of *PNC1* and requires *SIR2* for its effect. Although we do not discount the possibility that resveratrol might act on Sir2 indirectly, the simplest explanation of our observations is that resveratrol increases lifespan by directly stimulating Sir2 activity *in vivo*.

The ability of Sir2 to extend yeast lifespan is thought to stem from its role in stabilizing repetitive DNA^{1,22–24}. Homologous recombination between ribosomal DNA (rDNA) repeats can generate an extrachromosomal circular DNA molecule that is replicated until it reaches toxic levels in old cells²³. Consistent with this, resveratrol reduced the frequency of rDNA recombination by about 60% in a *SIR2*-dependent manner (Fig. 3c, d). In the presence of nicotinamide, recombination was also decreased by resveratrol, in agreement with the kinetic data (Fig. 3c). Interestingly, each of the STACs we tested had only minor effects on rDNA silencing compared with the 2 \times *SIR2* positive control (Fig. 3e, f). Together, these findings add to the body of evidence that yeast ageing is caused by genomic instability^{1,22–25}, not gene dysregulation²⁶.

Another measure of lifespan in *S. cerevisiae* is the length of time that cells can survive in a metabolically active but nutrient-deprived state. Ageing under these conditions (that is, chronological ageing) is primarily due to oxidative damage. Resveratrol (10 μ M or 100 μ M) failed to extend chronological lifespan (not shown), indicating that the effect of resveratrol on sirtuins may be more relevant than its antioxidant activity^{17,18}.

To test whether STACs could stimulate human sirtuins *in vivo*, we developed a cell-based fluorescence deacetylase assay (Supplementary Figs 2 and 3a–c). A selection of sirtuin-stimulatory and non-

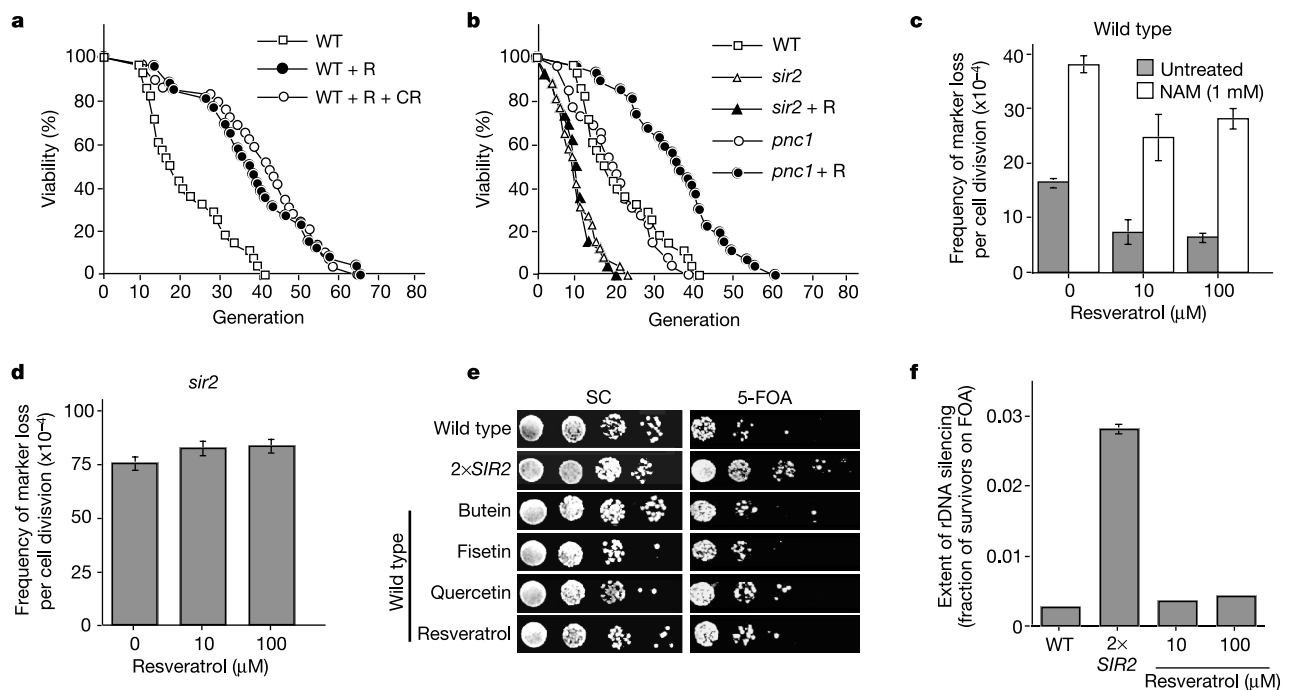


Figure 3 Resveratrol extends lifespan by mimicking calorie restriction and suppressing rDNA recombination. **a**, Average lifespan for wild type (WT) untreated, 19.0 generations; wild type plus resveratrol (WT + R), 37.8; glucose (calorie)-restricted plus resveratrol (WT + CR + R), 39.9. **b**, Average lifespan for wild type, 19.0 generations; *sir2*, 9.9; *sir2* plus resveratrol, 10.0; *pnc1*, 19.2; *pnc1* plus resveratrol, 33.1. **c, d**, Recombination

frequencies for wild type and *sir2* strains were determined by monitoring loss of the rDNA::*ADE2* marker gene. **e**, Pre-treated *RDN1::URA3* cells were spotted as tenfold serial dilutions on SC medium with or without 5-fluoro-orotic acid (5-FOA). Increased silencing results in increased survival on 5-FOA medium. **f**, Level of rDNA silencing represents numbers of surviving cells on 5-FOA/total plated.

stimulatory polyphenols were analysed using this *in vivo* assay and values were plotted against their fold stimulation of SIRT1 *in vitro* (Fig. 4a). Compounds with little or no activity *in vitro* clustered around the negative control (group A). Another grouping of strong *in vitro* activators is clearly distanced from the low-activity cluster in both dimensions (group B). These data indicate that certain polyphenols can activate human sirtuins *in vivo*.

Next, we assessed whether resveratrol could activate SIRT1 *in vivo*. One known target of SIRT1 is lysine 382 of p53 (K382). Deacetylation of this residue by SIRT1 decreases the activity and half-life of p53, and increases cell survival under a variety of DNA-damaging

conditions^{8–10}. Treatment of cells with a low concentration (0.5 μM) of resveratrol increased cell survival after ionizing irradiation (Fig. 4b). To test whether this was due to modulation of SIRT1 activity, we generated an antibody that specifically recognizes the acetylated form of p53-K382 (Ac-K382) (Fig. 4c and data not shown). U2OS cells treated with 0.5 μM resveratrol showed a marked decrease (about 75%) in the level of Ac-K382 (Fig. 4d; see also Supplementary Fig. 4a). At higher concentrations of resveratrol (>50 μM) the effect was reversed (Fig. 4d and data not shown), which may explain the dichotomy in the literature regarding the effects of resveratrol on cell viability^{17,27,28}. Treatment of HEK 293 cells with resveratrol led to a dose-dependent decrease in Ac-K382 levels that was diminished by overexpression of a dominant-negative SIRT1 allele (see Fig. 4e and Supplementary Fig. 4b). These data strongly indicate that resveratrol can promote cell survival by stimulating SIRT1 *in vivo*.

We have discovered small molecule sirtuin activators (STACs), and have shown that they can promote the survival of eukaryotic cells. Sirtuins have been found in diverse eukaryotes including fungi, protozoans, metazoans and plants²⁹, and probably evolved early in life's history¹¹. Plants produce a variety of polyphenols such as resveratrol in response to stresses including dehydration, nutrient deprivation, ultraviolet radiation and pathogens³⁰. Therefore it is plausible that plants synthesize these molecules in order to regulate a sirtuin-mediated stress response. This would be consistent with the recently discovered relationship between environmental stress and Sir2 activity in yeast¹². Perhaps these compounds have stimulatory activity on sirtuins from fungi and animals because they mimic an endogenous activator. Alternatively, animal and fungal sirtuins may have retained or developed an ability to respond to plant stress metabolites because they are a useful indicator of a deteriorating environment and/or food supply. Consistent with these ideas, preliminary experiments indicate that resveratrol can also extend the lifespan of multicellular animals, including the nematode *C. elegans* and the fruitfly *Drosophila melanogaster* (J.G.W., D.A.S. and M. Tatar, unpublished results).

A broad range of human health benefits have been reported for plant polyphenols including cardioprotection, neuroprotection and cancer suppression^{17–19}. Interestingly, similar beneficial effects are observed for calorie-restricted rodents¹⁴. We postulate that many of the effects of polyphenols might be the result of a calorie-restriction-mimetic defence response mediated by sirtuins. Part of this response may involve suppressing p53 and delaying apoptosis to give cells additional time to repair damage and to prevent unnecessary cell death. Our results also suggest that survival and longevity at the cellular and organismal levels are intimately linked. The newly appreciated ability of polyphenols to promote survival and longevity by activating sirtuins indicates a new line of investigation into the effects of these and related molecules on age-related human diseases. □

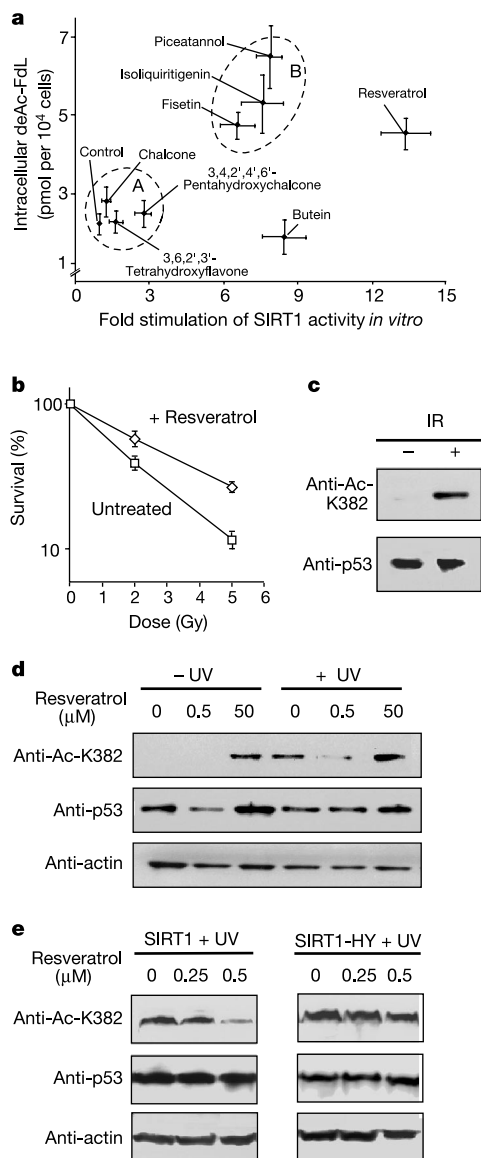


Figure 4 STACs stimulate sirtuin activity in human cells. **a**, STACs can stimulate TSA-insensitive FdL deacetylation (Supplementary Figs 2 and 3). Intracellular deacetylated FdL (deAc-FdL) levels are plotted against fold stimulation in the *in vitro* SIRT1 assay. **b**, Survival of HEK 293 cells in the presence of 0 (squares) or 0.5 μM (diamonds) resveratrol after exposure to 0, 2 or 5 Gy of ionizing radiation (IR). **c**, U2OS osteosarcoma cells were exposed to 0 or 10 Gy of ionizing radiation (IR). Cell lysates were prepared 4 h after irradiation and probed with indicated antibodies. **d**, U2OS cells were pre-treated with resveratrol for 4 h after which cells were exposed to 0 or 50 J cm^{-2} of ultraviolet (UV) radiation. **e**, HEK 293 cells expressing wild-type SIRT1 or dominant-negative SIRT1 H363Y (SIRT1-HY) were pre-treated with resveratrol and exposed to ultraviolet radiation as above.

Methods

Compound libraries and deacetylation assays

His₆-tagged recombinant SIRT1 and recombinant Sir2 were prepared as described¹³. A total of 0.1–1.0 μg of SIRT1 and 1.5 μg of Sir2 were used per deacetylation assay (50 μl total reaction) using ‘Fluor de Lys’ (FdL) as described¹³. SIRT1 screening assays used the p53-382 acetylated substrate (FdL-SIRT1, BIOMOL). Themed compound libraries (BIOMOL) were used for primary and secondary screening. Most polyphenol compounds were dissolved at 10 mM in dimethylsulphoxide (DMSO) on the day of the assay. For water-soluble compounds and negative controls, 1% v/v DMSO was added to the assay. *In vitro* fluorescence assays were read in white, half-volume 96-well microplates (Corning) with a CytoFluor II fluorescence plate reader (PerSeptive Biosystems, excitation 360 nm, emission 460 nm, gain = 85). HeLa cells were grown and the cellular deacetylation assays performed and read, as above, but in full-volume 96-well microplates (Corning). Unless otherwise indicated all initial rate measurements were means of three or more replicates, obtained with single incubation times, at which point 5% or less of the substrate initially present had been deacetylated.

For the SIRT1 resveratrol dose-response experiments, values are derived from the slopes of fluorescence (arbitrary fluorescence units, AFU) against time plots with data obtained at 0, 5, 10 and 20 min of deacetylation using 25 μM each of NAD⁺ and p53-382

acetylated peptide. Sir2 assays were performed similarly using either 100 μM FdL or 25 μM acetylated H4 peptide (FdL-H4-Ac-K16). Calculation of net fluorescence included the subtraction of a blank value by adding an inhibitor (200 μM suramin or 1 mM nicotinamide) to the reaction in the case of SIRT1, and in the case of Sir2 by omitting the enzyme from the reaction. For polyphenols that partially quenched the fluorescence produced in the assay, correction factors were obtained by determining the fluorescence increase due to a 3 μM spike of an FdL deacetylated standard (BIOMOL).

Medium and strains

All yeast strains were grown at 30 °C in complete yeast extract/bactopeptone, 2.0% (w/v) glucose (YPD) medium unless stated otherwise. Calorie restriction was induced in 0.5% glucose. Synthetic complete (SC) medium consisted of 1.67% yeast nitrogen base, 2% glucose and 40 mg l⁻¹ each of auxotrophic markers. SIRT2 was integrated in extra copy and disrupted as described¹. Other strains are described elsewhere¹³. For cellular deacetylation assays, HeLa S3 cells were used. U2OS osteosarcoma and human embryonic kidney (HEK 293) cells were cultured adherently in DMEM containing 10% fetal calf serum with 1.0% glutamine and 1.0% penicillin/streptomycin. HEK 293 overexpressing dominant-negative SIRT1 H363Y was a gift of R. Frye.

Lifespan determinations

Lifespan measurements were performed using PSY316AT MAT α as previously described¹³. All compounds for lifespan analyses were dissolved in 95% ethanol. Plates were dried and used within 24 h. Cells were pre-incubated on their respective medium for at least 15 h and equilibrated on the medium for a minimum of 4 h before micro-manipulating them. At least 30 cells were examined per experiment and each experiment was performed at least twice. Statistical significance of lifespan differences was determined using the Wilcoxon rank sum test. Differences are stated to be significant when the confidence is higher than 95%.

Silencing and recombination assays

Ribosomal DNA silencing assays were performed as previously described¹³. Ribosomal DNA recombination frequencies were determined by plating W303AR cells²³ on YPD medium with low adenine/histidine and counting the fraction of half-red sector colonies using Bio-Rad Quantity One software. At least 6,000 cells were analysed per experiment and all experiments were performed in triplicate. All strains were pre-grown for 15 h with the relevant compound before plating.

Cellular survival assay

HEK 293 cells treated with either 0.5 μM resveratrol or 0.5% DMSO for 8 h were trypsinized, collected and exposed to 0, 2 or 5 Gy of ionizing radiation. A total of 300 cells per sample were then plated in duplicate in 10 cm dishes. Medium was changed the following day and after 10 days, colonies were stained with crystal violet and counted. Error bars represent the standard error of the mean.

Proteins and western analyses

Recombinant Sir2–glutathione S-transferase was expressed and purified from *Escherichia coli* as previously described except that lysates were prepared using sonication¹³. Recombinant SIRT1 from *E. coli* was prepared as described¹³. Polyclonal antiserum against p53-Ac-K382 was generated using an acetylated peptide antigen as previously described⁹, with the following modifications. Anti-Ac-K382 antibody was affinity purified using non-acetylated p53-K382 peptides and stored in PBS at –70 °C. Western hybridizations using anti-acetylated K382 or anti-actin (Chemicon) antibody were performed at 1:1,000 dilution of antibody. Hybridizations with polyclonal p53 antibody (Santa Cruz) used a 1:500 dilution. Cell extracts were prepared 4 h after irradiation from cells pre-treated with resveratrol for 4 h. Lysis buffer contained 150 mM NaCl, 1 mM MgCl₂, 10% glycerol, 1% NP40, 1 mM dithiothreitol and anti-protease cocktail (Roche).

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Competing interests statement The authors declare competing financial interests: details accompany the paper on www.nature.com/nature.

Correspondence and requests for materials should be addressed to D.S. (david_sinclair@hms.harvard.edu).