

Sirtuin activators mimic caloric restriction and delay ageing in metazoans

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Caloric restriction extends lifespan in numerous species. In the budding yeast *Saccharomyces cerevisiae* this effect requires Sir2 (ref. 1), a member of the sirtuin family of NAD⁺-dependent deacetylases^{2,3}. Sirtuin activating compounds (STACs) can promote the survival of human cells and extend the replicative lifespan of yeast⁴. Here we show that resveratrol and other STACs activate sirtuins from *Caenorhabditis elegans* and *Drosophila melanogaster*, and extend the lifespan of these animals without reducing fecundity. Lifespan extension is dependent on functional Sir2, and is not observed when nutrients are restricted. Together these data indicate that STACs slow metazoan ageing by mechanisms that may be related to caloric restriction.

Sir2-like proteins (sirtuins) are a family of NAD⁺-dependent deacetylases conserved from *Escherichia coli* to humans^{5–9} (Fig. 1a) that play important roles in gene silencing, DNA repair, rDNA recombination and ageing in model organisms^{2,10–12}. When diet is restricted (caloric restriction), lifespan is extended in diverse species, suggesting that there is a conserved mechanism for nutrient regulation of ageing^{13–17}. In budding yeast, extra copies of SIR2 extend lifespan by 30%, apparently by mimicking caloric restriction^{1,18}. We recently described a group of compounds (STACs) that stimulate the catalytic activity of yeast and human sirtuins, and extend the replicative lifespan of yeast cells by up to 60% (ref. 4).

To establish whether STACs could activate sirtuins from multicellular animals, we developed a cell-based deacetylation assay for *D. melanogaster* S2 cells. Unlike other classes of deacetylases, the sirtuins are insensitive to the inhibitor trichostatin A (TSA). Several classes of polyphenolic STACs, including chalcones, flavones and stilbenes, increased the rate of TSA-insensitive deacetylation (Fig. 1b). To determine whether this activity was due to direct stimulation of a Sir2 homologue, we purified recombinant SIR-2.1 of *C. elegans* and Sir2 of *D. melanogaster* and determined the effect of various STACs on enzymatic activity *in vitro* (Fig. 1c, d). In a dose-dependent manner, resveratrol stimulated deacetylation up to 2.5-fold for SIR-2.1 (Fig. 1e) and 2.4-fold for Sir2 (Fig. 1f). As previously observed with the yeast and human Sir2 enzymes, resveratrol lowered the K_m of SIR-2.1 for the co-substrate NAD⁺ (Fig. 1g).

Because resveratrol can significantly extend replicative lifespan in yeast⁴, we asked whether STACs could also extend lifespan in the metazoans *C. elegans* and *D. melanogaster*. Wild-type worms were transferred to plates containing 0 or 100 μ M of resveratrol shortly after reaching adulthood. Lifespan was extended up to 14%, using either heat-killed or live *E. coli* as food supply (Fig. 2a, c, respectively; Supplementary Table ST1; averaged across trials at 100 μ M resveratrol, lifespan was extended 10% on live and dead *E. coli*), and

mortality was decreased across all adult ages (Supplementary Fig. S1). To test whether the lifespan extension depends on functional SIR-2.1, we constructed a *sir-2.1* null mutant. The lifespan of this strain was not appreciably shorter than the wild-type N2 control, and adults treated with resveratrol did not exhibit a significant lifespan extension relative to untreated worms (Fig. 2b, d; Supplementary Table ST1). There was no decrease in fecundity associated with resveratrol treatment (Fig. 2e). To rule out the possibility that resveratrol was causing the animals to eat less,

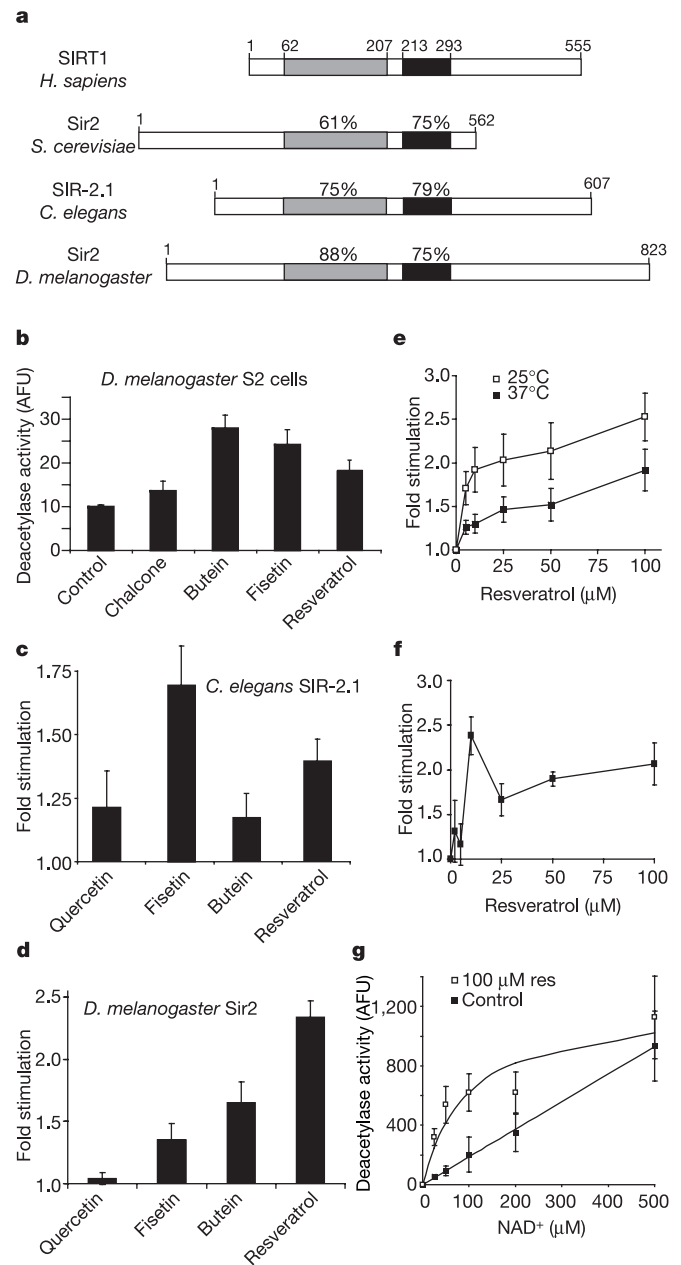


Figure 1 Effect of polyphenolic STACs on sirtuins. **a**, Sir2 polypeptides from various species. The NAD⁺-binding pocket (grey), substrate-binding groove (black) and per cent homology to SIRT1 are shown. **b**, Effect of polyphenolic STACs (500 μ M) on TSA-insensitive deacetylase activity in *Drosophila* S2 cells. **c**, Fold stimulation of SIR-2.1 by STACs (10 μ M). **d**, Fold stimulation of *Drosophila* Sir2 by STACs (10 μ M). Values are the mean of at least three determinations (\pm s.e.). **e**, **f**, Activation of *C. elegans* SIR-2.1 (**e**) and *Drosophila* Sir2 (**f**) by resveratrol (\pm s.e.). **g**, SIR-2.1 initial rate as a function of NAD⁺ concentration (\pm s.e.). AFU, arbitrary fluorescence units.

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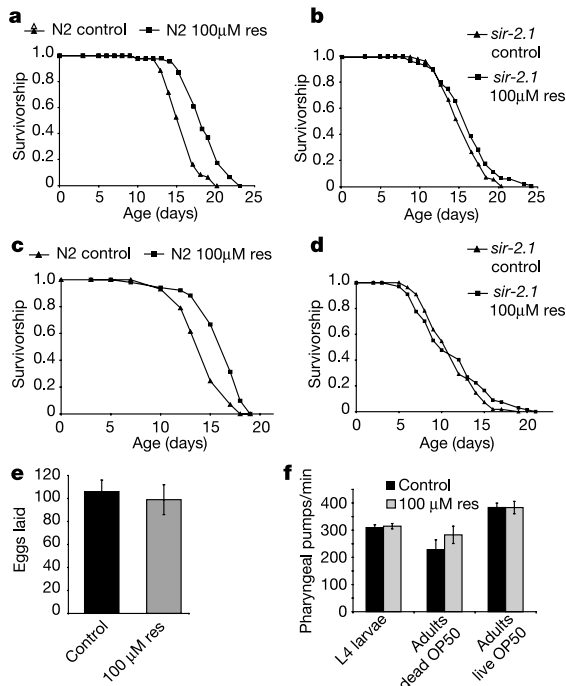


Figure 2 *C. elegans* survival on resveratrol. **a**, Survivorship of adult wild-type N2 *C. elegans* treated with resveratrol fed with heat-killed OP50 *E. coli*. **b**, Survivorship of *sir-2.1* mutants treated with resveratrol fed with heat-killed OP50. Adult lifespan of *sir-2.1* animals does not differ significantly from N2 controls. **c**, Survivorship of wild-type N2 *C. elegans* on 100 μM resveratrol fed with live OP50. **d**, Survivorship of *sir-2.1* mutants on 100 μM resveratrol fed with live OP50. **e**, Fecundity of adult hermaphrodites on 100 μM resveratrol (±s.d.). **f**, Feeding rates of L4 larval and adult hermaphrodites treated with 100 μM resveratrol (±s.d.).

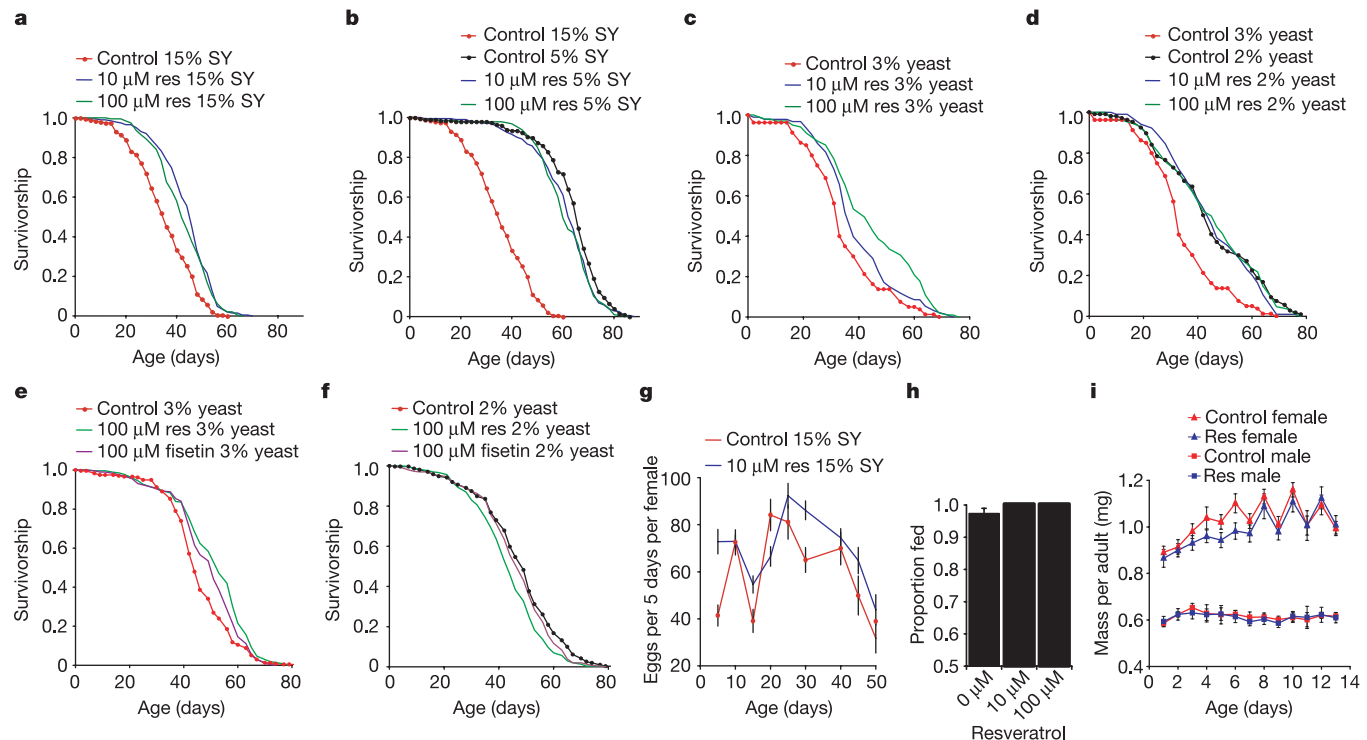


Figure 3 Survival of wild-type female *D. melanogaster* adults fed resveratrol or fisetin. **a**, Canton-S on 15% SY media. **b**, Canton-S on 5% SY media. **c**, yw on 3% CSY media. **d**, yw on 2% CSY media. **e**, yw on 3% CSY media. **f**, yw on 2% CSY media. **g**, Mean 5 day

thereby inducing a caloric restriction effect indirectly, we measured feeding rates of both L4 larval and adult worms with or without resveratrol and found no differences (Fig. 2f). Throughout the lifespan trials, we used the reproductive suppressant FUDR to prevent accumulation of progeny on the treatment plates.

We also tested whether STACs could extend lifespan in *D. melanogaster* using the standard laboratory wild-type strain Canton-S and normal fly culturing conditions (vials), and a yw marked wild-type strain and demographic culturing conditions (cages) (Supplementary Table ST2). Across independent tests in males and females on abundant diet, lifespan was extended up to 23% with fisetin and up to 29% with resveratrol (Fig. 3a, c, e; Supplementary Table ST2; averaged across these trials with 100 μM resveratrol, lifespan was extended 20% in females and 16% in males). Increased longevity was associated with reduced mortality before day 40 (Supplementary Fig. S1). A restricted diet increased lifespan by 40% in females and by 14% in males (averaged across trials), and under these conditions neither resveratrol nor fisetin further increased longevity (Fig. 3b, d, f). The lack of an additive effect of resveratrol and a low calorie diet suggests that resveratrol extends lifespan through a mechanism related to caloric restriction.

Surprisingly, while diet manipulations that extend *D. melanogaster* longevity typically reduce fecundity^{19,20}, longevity-extending doses of resveratrol modestly increased egg production (10 μM resveratrol: 69.8 eggs/5 days, s.e. = 2.2; control: 59.9 eggs/5 days, s.e. = 2.2; $t = 3.17$, $P = 0.0017$), particularly in the earliest days of adult life (Fig. 3g). The increase in egg production suggests that the lifespan extending effect of resveratrol in *D. melanogaster* was not due to caloric restriction induced by food aversion or lack of appetite. Consistent with this, no decrease in food uptake was seen with resveratrol-fed flies (Fig. 3h). Furthermore, resveratrol-fed flies maintained normal weight (Fig. 3i), except during days 3–6 when resveratrol fed females were laying significantly more eggs than control fed females.

fecundity per female of Canton-S on 15% SY media ± resveratrol. **h**, Proportion of yw females feeding on diet ± resveratrol in crop-filling assay. **i**, Mean body mass of Canton-S flies on diet ± resveratrol (10 μM). Errors represent s.e.

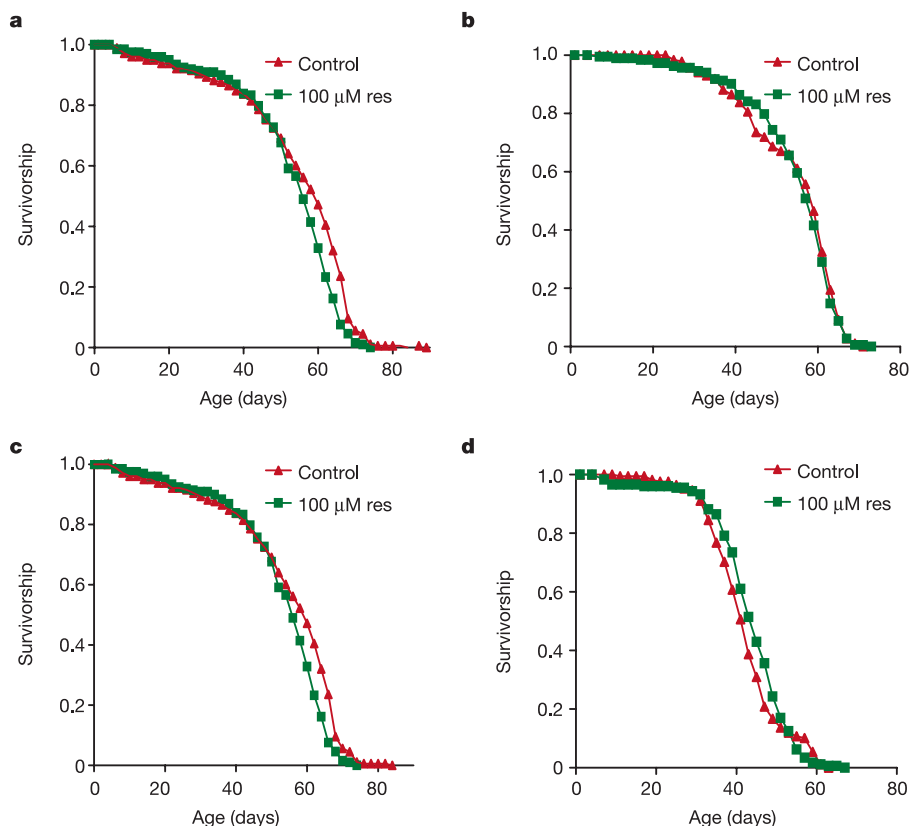


Figure 4 Survival of *D. melanogaster* adults with mutant alleles of *Sir2* fed resveratrol (100 μM). Females (**a**) and males (**b**) with loss-of-function genotype *Sir2*^{4.5}/*Sir2*^{5.26}. Females (**c**) and males (**d**) with strong hypomorphic genotype *Sir2*¹⁷/*Sir2*^{KG00871}.

To determine whether resveratrol extends fly lifespan in a *Sir2*-dependent manner, we analysed a *Sir2* allelic series with increasing amounts of *Sir2*. Adult offspring from crosses between independently derived alleles of *Sir2* were tested. Resveratrol failed to extend lifespan in flies completely lacking functional *Sir2* (*Sir2*^{4.5}/*Sir2*^{5.26}) (Fig. 4a, b) or in flies in which *Sir2* is severely decreased (*Sir2*¹⁷/*Sir2*^{KG00871}) (Fig. 4c, d). Resveratrol increased longevity a small but statistically significant amount in flies homozygous for a hypomorphic *Sir2* allele (*Sir2*^{KG0087}/*Sir2*^{KG0087}) (Supplementary Table ST2, trial 6) and increased lifespan up to 17% in flies with one copy of the hypomorphic allele and one copy of a wild-type *Sir2* (Canton-S/*Sir2*^{KG0087}) (Supplementary Table ST2, trial 7). These data suggest that the ability of resveratrol to extend fly lifespan requires functional *Sir2*.

We previously reported that STACs extend the replicative lifespan of yeast cells by mimicking caloric restriction⁴. Here we show that STACs can extend lifespan in *C. elegans* and *D. melanogaster*, both of which are composed of primarily non-dividing (post-mitotic) cells as adults, and whose somatic and reproductive ageing are independent measures of senescence. In both species, resveratrol increases lifespan in a *Sir2*-dependent manner and, at least for the fly, this action appears to function through a pathway related to caloric restriction.

Our observation that resveratrol can increase longevity without an apparent cost of reproduction is counter to prevalent concepts of senescence evolution. However, STACs may still entail trade-offs that involve other traits, or that occur only under some environmental conditions^{21,22}. Plants synthesize STACs such as resveratrol in response to stress and nutrient limitation²³, possibly to activate their own sirtuin pathways⁴. These molecules may activate animal

sirtuins because they serve as plant defence mechanisms against consumers or because they are ancestrally orthologous to endogenous activators within metazoans. Alternatively, animals may use plant stress molecules as a cue to prepare for the decline in their environment or food supply⁴. Understanding the adaptive significance, endogenous function and evolutionary origin of sirtuin activators will lead to further insights into the underlying mechanisms of longevity regulation, and could aid in the development of interventions that provide the health benefits of caloric restriction. □

Methods

C. elegans media, strains, lifespan and feeding assays

Bristol N2 (*Caenorhabditis* Genetics Center) was used as the wild-type strain. The *sir-2.1* mutant strain was generated by backcrossing VC199 (*sir-2.1(ok434)*) to N2 four times. For the lifespan assays, synchronized animals were transferred to treatment plates as young adults (2 days after hatching, day 0 of assay), and were transferred to fresh treatment plates every 2 days for the first 6 to 8 days of the assay. Treatment plates were standard NGM media with the reproductive suppressant FUDR (Sigma; 100 mg l^{-1}) containing resveratrol or solvent (DMSO, which does not affect lifespan) added either directly into the agar before pouring (for live OP50 trials) or diluted into PBS and added to the surface of a dry plate to the indicated final concentration (for dead OP50 trials). For heat-killed *E. coli* trials, OP50 cultures were heated to 65 °C for 30 min, then pelleted and resuspended in 1/10 volume in S Basal supplemented with 10 mM MgSO_4 . For *E. coli* killed by ampicillin and kanamycin, overnight cultures of OP50 were treated with 500 $\mu\text{g ml}^{-1}$ ampicillin and 250 $\mu\text{g ml}^{-1}$ kanamycin overnight at 37 °C. In all assays, worms were monitored daily for mortality by gently probing with a platinum pick. Assays were performed at 24 °C.

To assay worm feeding rates, worms at the indicated stages were placed on treatment plates (no FUDR) for 4–5 h, then videoed for 1 min using a Pixelink PL-662 camera. The frame rate was slowed and the pumping rate of the pharynx was counted. To assay fecundity, gravid hermaphrodites (5 per plate, raised from synchronized L1s on normal or treatment plates) were allowed to lay eggs on their respective media for 5 h, and the total number of eggs was counted.

D. melanogaster media, strains, feeding assay and lifespan assays

Survival assays were conducted independently with adult *D. melanogaster* in two laboratories. At Brown University, all trials used an *yw* marked wild-type strain. Larvae were reared on standard cornmeal-sugar-yeast (CSY) agar diet (cornmeal 5%, sucrose 10.5%, SAF yeast 2% and agar 0.7%). Newly eclosed adults were placed in 1-litre demography cages with approximately 75 males and 75 females. Three to four replicate 1-litre demography cages were used for each treatment group in each trial. Every two days, dead flies were removed and scored, and food vials were replenished. Food vials contained CSY diet with SAF yeast as either 2% or 3% by weight. Test compounds in 100 μ l of EtOH (or blank EtOH in controls) were mixed into melted aliquots of the adult food media to make a final concentration of 0, 10 or 100 μ M. Fresh stock solutions and adult media were prepared weekly. At the University of Connecticut Health Center, lifespan trials were conducted with the wild type strain Canton-S, *Sir2*^{4,5} and *Sir2*^{5,26} (S. Smolik), *Sir2*¹⁷ (S. Astrom) and *Sir2*^{KG00871} (*Drosophila* Stock Center). Larvae for all tests were reared on standard CSY diet. Newly eclosed adults were incubated in plastic shell vials containing 5 ml of 15% sugar-yeast diet (15% SY) or 5% sugar-yeast (5% SY) diet (15% SY: 15% yeast, 15% sucrose, 2% agar; 5% SY: 5% yeast, 5% sucrose, 2% agar as per ref. 20). In all trials, ~20 males with ~20 females were placed into each of 10 vials per treatment group. Every two days, flies were passed into new vials and dead flies were counted. Resveratrol in EtOH (or EtOH alone in controls) was added to the media during its preparation after it had cooled to 65 °C and mixed vigorously. Final compound concentrations were 0, 10, 100 or 200 μ M. Fresh stock solution and adult media was prepared weekly.

Feeding rate was measured in *yw* females with the crop-filling assay²⁴. Females were held overnight with water and placed on 2% CSY diet containing food colour (FDA Blue 1) and 0, 10 or 100 μ M resveratrol with EtOH. The presence of dye-marked food in the crop was scored in sets of 20 females across five 5-min intervals. For body mass measurements, 10 vials with 20 males and 20 females each of wild type CS-5 flies were kept on 15% SY diet with EtOH or with resveratrol in EtOH (10 μ M). Males and females were weighed daily.

Sirtuin purification and deacetylation assays

These are described in Supplementary Methods.

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- Lin, S. J., Defossez, P. A. & Guarente, L. Requirement of NAD and SIR2 for life-span extension by caloric restriction in *Saccharomyces cerevisiae*. *Science* **289**, 2126–2128 (2000).
- Gasser, S. C. M. The molecular biology of the SIR proteins. *Gene* **279**, 1–16 (2001).
- Hekimi, S. & Guarente, L. Genetics and the specificity of the aging process. *Science* **299**, 1351–1354 (2003).
- Howitz, K. T. *et al.* Small molecule activators of sirtuins extend *Saccharomyces cerevisiae* lifespan. *Nature* **425**, 191–196 (2003).
- Landry, J. *et al.* The silencing protein SIR2 and its homologs are NAD-dependent protein deacetylases. *Proc. Natl Acad. Sci. USA* **97**, 5807–5811 (2000).
- Imai, S., Armstrong, C. M., Kaerberlein, M. & Guarente, L. Transcriptional silencing and longevity protein Sir2 is an NAD-dependent histone deacetylase. *Nature* **403**, 795–800 (2000).
- Smith, J. S. *et al.* A phylogenetically conserved NAD⁺-dependent protein deacetylase activity in the Sir2 protein family. *Proc. Natl Acad. Sci. USA* **97**, 6658–6663 (2000).
- Tanner, K. G., Landry, J., Sternglanz, R. & Denu, J. M. Silent information regulator 2 family of NAD-dependent histone/protein deacetylases generates a unique product, 1-O-acetyl-ADP-ribose. *Proc. Natl Acad. Sci. USA* **97**, 14178–14182 (2000).

- Tanny, J. C., Dowd, G. J., Huang, J., Hiltz, H. & Moazed, D. An enzymatic activity in the yeast Sir2 protein that is essential for gene silencing. *Cell* **99**, 735–745 (1999).
- Guarente, L. Sir2 links chromatin silencing, metabolism, and aging. *Genes Dev.* **14**, 1021–1026 (2000).
- Tissenbaum, H. A. & Guarente, L. Increased dosage of a sir-2 gene extends lifespan in *Caenorhabditis elegans*. *Nature* **410**, 227–230 (2001).
- Rogina, B., Helfand, S. L. & Frankel, S. Longevity regulation by *Drosophila* Rpd3 deacetylase and caloric restriction. *Science* **298**, 1745 (2002).
- Jiang, J. C., Jaruga, E., Repnevskaya, M. V. & Jazwinski, S. M. An intervention resembling caloric restriction prolongs life span and retards aging in yeast. *FASEB J.* **14**, 2135–2137 (2000).
- Kenyon, C. A conserved regulatory mechanism for aging. *Cell* **105**, 165–168 (2001).
- Masoro, E. J. Caloric restriction and aging: an update. *Exp. Gerontol.* **35**, 299–305 (2000).
- Koubova, J. & Guarente, L. How does calorie restriction work? *Genes Dev.* **17**, 313–321 (2003).
- Sinclair, D. A. Paradigms and pitfalls of yeast longevity research. *Mech. Ageing Dev.* **123**, 857–867 (2002).
- Kaerberlein, M., McVey, M. & Guarente, L. The SIR2/3/4 complex and SIR2 alone promote longevity in *Saccharomyces cerevisiae* by two different mechanisms. *Genes Dev.* **13**, 2570–2580 (1999).
- Chippindale, A. K., Leroi, A. M., Kim, S. B. & Rose, M. R. Phenotypic plasticity and selection in *Drosophila* life-history evolution. *J. Evol. Biol.* **6**, 171–193 (1993).
- Chapman, T. & Partridge, L. Female fitness in *Drosophila melanogaster*: an interaction between the effect of nutrition and of encounter rate with males. *Proc. R. Soc. Lond. B* **263**, 755–759 (1996).
- Walker, D. W., McColl, G., Jenkins, N. L., Harris, J. & Lithgow, G. J. Evolution of lifespan in *C. elegans*. *Nature* **405**, 296–297 (2000).
- Marden, J. H., Rogina, B., Montooth, K. L. & Helfand, S. L. Conditional tradeoffs between aging and organismal performance of Indy long-lived mutant flies. *Proc. Natl Acad. Sci. USA* **100**, 3369–3373 (2003).
- Soleas, G. J., Diamandis, E. P. & Goldberg, D. M. Resveratrol: a molecule whose time has come? And gone? *Clin. Biochem.* **30**, 91–113 (1997).
- Edgecomb, R. S., Harth, C. E. & Schneiderman, A. M. Regulation of feeding behavior in adult *Drosophila melanogaster* varies with feeding regime and nutritional state. *J. Exp. Biol.* **197**, 215–235 (1994).

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