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MicroReview

Small molecules that regulate lifespan: evidence for xenohormesis

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Summary

Barring genetic manipulation, the diet known as calorie restriction (CR) is currently the only way to slow down ageing in mammals. The fact that CR works on most species, even microorganisms, implies a conserved underlying mechanism. Recent findings in the yeast *Saccharomyces cerevisiae* indicate that CR extends lifespan because it is a mild biological stressor that activates Sir2, a key component of yeast longevity and the founding member of the sirtuin family of deacetylases. The sirtuin family appears to have first arisen in primordial eukaryotes, possibly to help them cope with adverse conditions. Today they are found in plants, yeast, and animals and may underlie the remarkable health benefits of CR. Interestingly, a class of polyphenolic molecules produced by plants in response to stress can activate the sirtuins from yeast and metazoans. At least in the case of yeast, these molecules greatly extend lifespan by mimicking CR. One explanation for this surprising observation is the 'xenohormesis hypothesis', the idea that organisms have evolved to respond to stress signalling molecules produced by other species in their environment. In this way, organisms can prepare in advance for a deteriorating environment and/or loss of food supply.

Calorie restriction

Over 70 years ago, McCay and colleagues observed that rats fed 40% fewer calories tend to live longer and look younger and healthier than well-fed animals. In numerous subsequent studies it has been documented that calorie restriction (CR) delays most diseases of ageing including cancer, atherosclerosis, type II diabetes and even neurodegeneration. As a result, there is intense interest in how CR works at the molecular level and in finding small molecules that can mimic its effects.

A number of theories have been advanced to explain the effect of CR. It was initially thought that CR works by retarding development, but two observations argue against this theory: CR can be started in adult animals and still extend lifespan, and animals placed on a restricted diet every other day grow normally but receive similar health benefits (reviewed in Masoro, 2000). Another theory is that the CR effect is simply an artefact of comparing animals to overfed controls. It is true that wild mice are rarely exposed to abundant food sources, and it seems reasonable to suppose that the effects of CR arise simply from not overfeeding the animals. However, feeding rodents what is considered a healthy diet is not sufficient to extend lifespan, and the applicability of CR to so many different types of organisms argues against this hypothesis. Moreover, the benefits of CR are seen even when mice are fed controlled amounts of food rather than *ad libitum* (Koubova and Guarente, 2003). Finally, even if some of the effects of CR are a result of excessive food consumption in controls, very few would argue that this research is not applicable to people in today's society.

Another theory is that CR works by slowing down metabolic activity and reducing oxidative damage (reviewed in Masoro, 2000). This is an appealing theory because free-radical damage is viewed as one of the major causes of ageing in mammals. Indeed, studies in yeast, worms and flies have shown that protecting against oxidative damage can extend lifespan (Parkes *et al.*, 1998; Longo *et al.*, 1999; Longo and Finch, 2003). However, this theory has a major flaw: although CR mice experience an initial drop in metabolic rate, their metabolic rate returns to normal several weeks after initiation of the diet (Masoro, 2000).

A more recent and controversial theory proposes that CR is a form of hormesis (Masoro, 2000; Anderson *et al.*, 2003a; Strauss, 2003). The term 'hormesis' refers to the process by which a mild stress provides health benefits

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by causing the organism to mount a defence response. The hormesis hypothesis of CR, therefore, is based on the idea that low calorie intake is in itself a mild stress, which invokes a general stress response that promotes better health and longer life. This theory explains why CR animals are more resistant to a broad array of stresses and fits with prominent theories about the allocation of resources to survival and somatic maintenance during times of stress (Kirkwood *et al*., 2000).

The age-defying effects of CR have now been observed in most laboratory species, including dogs, nematode worms, fruit flies, and the budding yeast *Saccharomyces cerevisiae* (Masoro, 2000). Such a high degree of conservation typically implies the existence of a universal underlying mechanism. If the mechanism of CR is truly conserved, even in a general sense, then understanding how a simple organism responds to CR could provide valuable clues as to how CR works in mammals. The most rapid progress in the understanding basic mechanisms of CR has come from studying baker's yeast, *S. cerevisiae.*

Genomic instability is a cause of yeast ageing

Determining the lifespan of individual yeast cells is possible because *S. cerevisiae* undergoes asymmetric cell division. Yeast 'replicative lifespan' is defined as the number of daughters produced by a cell before it senesces, and ageing is associated with a number of phenotypic changes, such as a larger size, slower cell cycle and sterility resulting from the loss of gene silencing at mating-type loci (Bitterman *et al*., 2003). In young cells, the nucleolus (which encloses the ribosomal DNA) is a crescent-shaped structure; in old cells it becomes enlarged and fragments into multiple, rounded structures (Sinclair *et al*., 1997). These changes can be slowed down by reducing the amount of glucose or amino acids in the medium, a treatment akin to calorie restriction in higher organisms (Jiang *et al*., 2000; Lin *et al*., 2000).

If ageing is brought about by wear and tear, then not all biological systems will wear and tear at the same rate. Some systems such as basic metabolic pathways are robust because they are inherently self-regulating and contain inbuilt redundancy. Other systems, such as the genome, require considerable energy for maintenance and damage is often irreversible (Sinclair, 2002). Such systems are most likely to fail as an organism ages. Consistent with this idea, a major cause of yeast ageing stems from the instability of repetitive DNA (Sinclair and Guarente, 1997). The yeast ribosomal DNA (rDNA) locus consists of 100–200 tandemly arrayed 9 kb repeats encoding the ribosomal RNAs. Homologous recombination between repeats can result in the excision of extrachromosomal circular forms of rDNA known as ERCs, which can replicate during S-phase but are inefficiently segregated to daughter cells. As a result, the abundance of ERCs increases exponentially in mother cells at a rate determined by cell division, until they reach more than 1000 copies. The mechanism by which ERCs cause death is not known, but given that their total DNA content can be greater than the yeast genome, it is likely that they cause ageing by titrating away vital transcription and/or replication factors.

As proof of this model, the introduction of either an ERC or a yeast plasmid into a young cell causes premature ageing (Sinclair and Guarente, 1997; Falcon and Aris, 2003), and the rate at which these circular DNAs accumulate correlates with the yeast lifespan (Falcon and Aris, 2003). Moreover, over 15 genetic manipulations or small molecules that suppress rDNA recombination have been found to extend lifespan (Defossez *et al*., 1999; Kaeberlein *et al*., 1999; Bitterman *et al*., 2003), some of which have no effect on rDNA silencing (Sinclair, 2002; Howitz *et al*., 2003).

Another determinant of yeast lifespan is the retrograde signalling pathway that ensures that nuclear genes are regulated in synchrony with mitochondrial activity (Liao and Butow, 1993). Although there has been debate about the relative contribution of the retrograde response and ERCs to yeast ageing, a recent paper has shown that these two phenomena are in fact opposite sides of the same coin and are coordinated by the retrograde response factor Rtg2 (Borghouts *et al*., 2004). The involvement of mitochondria in yeast ageing is intriguing in light of studies implicating this organelle in *Caenorhabditis elegans* longevity (Hekimi and Guarente, 2003).

SIR2, the silent protector of DNA stability

Genetic manipulations that suppress rDNA recombination and extend lifespan fall into two categories. Some suppress recombination by preventing rDNA replication forks from stalling (e.g. *fob1Δ*), whereas others decrease recombination by promoting the formation of transcriptionally silent heterochromatin (e.g. *SIR2* overexpression) (Kaeberlein *et al*., 1999; Bitterman *et al*., 2003). Three types of loci are silenced in *S. cerevisiae*: the telomeres, the two mating-type loci, and the rDNA. Different complexes of these proteins form at each locus to promote silencing (Fig. 1), but Sir2 is a key component in all of them. An Sir1/2/3/4 complex functions to silence transcription at the mating-type loci whereas a Sir2/3/4 complex mediates silencing at telomeres (Kaeberlein *et al*., 1999). Silencing at the rDNA is achieved by the RENT (regulator of nucleolar silencing and telophase exit) complex, which contains Sir2, Cdc14, and Net1 (Huang and Moazed, 2003).

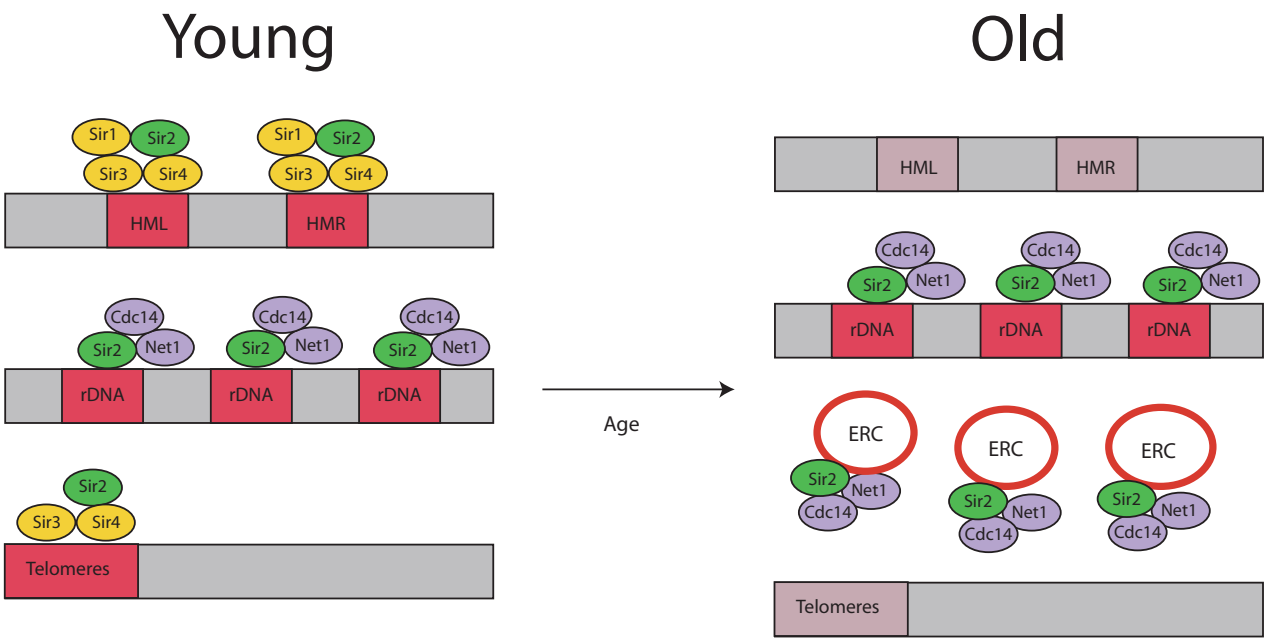

Fig. 1. Redistribution of Sir proteins in ageing yeast. Sir complexes localize to the mating loci, rDNA, and telomeres in young yeast. Sir2 suppresses recombination at the rDNA array in combination with Cdc14 and Net1. As yeast age, Sir complexes relocalize from the mating loci and telomeres to the nucleolus. This may be a result of extrachromosomal rDNA circle (ERC) formation in the nucleolus.

In 1999, yeast Sir2 was found to have ADP-ribosyltransferase activity that required NAD^+ as a cofactor (Tanny *et al.*, 1999). This may be a major activity of Sir2 homologues in other organisms, such as TbSIR2RP1 in *Trypanosoma brucei*, and this activity may have a role in DNA repair in yeast (Garcia-Salcedo *et al.*, 2003). However, about a year after this first study, three groups reported a more robust enzymatic activity for Sir2: NAD^+-dependent histone deacetylation (reviewed in Denu, 2003). It is now generally accepted that this activity allows Sir2 to form silent heterochromatin by removing acetyl groups from specific lysines in the N-terminal tails of histones H3 and H4, causing them to adopt a more compact conformation (Koubova and Guarente, 2003).

The regulation of Sir2 by CR

In yeast, Sir2 is essential for lifespan extension by CR and a variety of other stresses, including increased temperature, amino acid restriction, and osmotic shock (Swiecilo *et al.*, 2000; Anderson *et al.*, 2003a). However, Sir2 protein levels do not change in response to any of these treatments. Thus, Sir2 enzymatic activity must be regulated in response to these stimuli. An early theory was that levels of the co-substrate, NAD^+, regulated the enzyme (Lin *et al.*, 2002). An extension of this model was that the increase in respiration resulting from CR promotes the re-oxidation of NADH to NAD^+ in mitochondria, thus boosting NAD^+ levels and activating Sir2. In yeast, cellular levels of NAD are maintained by a variety of mechanisms, including *de novo* synthesis of NAD from tryptophan and the NAD salvage pathway that regenerates NAD from nicotinamide. Hst1, a homologue of Sir2, has recently been shown to regulate NAD levels by repressing the transcription of genes in the *de novo* pathway (Bedalov *et al.*, 2003). The problem with this model was that NAD levels in yeast are extremely stable: three studies have now shown that CR does not increase NAD^+ (Ashrafi *et al.*, 2000; Anderson *et al.*, 2003a; Lin *et al.*, 2004), and one study even indicated that NAD^+ levels decrease during CR (Anderson *et al.*, 2003a).

Another theory is that alterations in the NAD^+/NADH ratio regulate Sir2 activity (Lin *et al.*, 2002; 2004). Consistent with this model, high levels of NADH can inhibit Sir2 *in vitro*, and, based on an assay of cell extracts, the amount of NADH in yeast cells appears relatively high, with an NAD^+ : NADH ratio of ≈ 2 (Lin *et al.*, 2002). Moreover, levels of NADH in this assay seem to be lower in glucose-restricted cells, and overexpression of Nde1 and Nde2, two mitochondrial NADH dehydrogenases, results in an extension of lifespan similar to that seen under CR (Lin *et al.*, 2004). Despite this supporting evidence, some aspects of this model remain unresolved. For example, the model does not yet explain how a variety of other treatments extend lifespan, such as amino acid restriction, high salt, nitrogen restriction, and heat shock. Perhaps more importantly, a different study that used nuclear magnetic resonance to measure freely available NAD in living

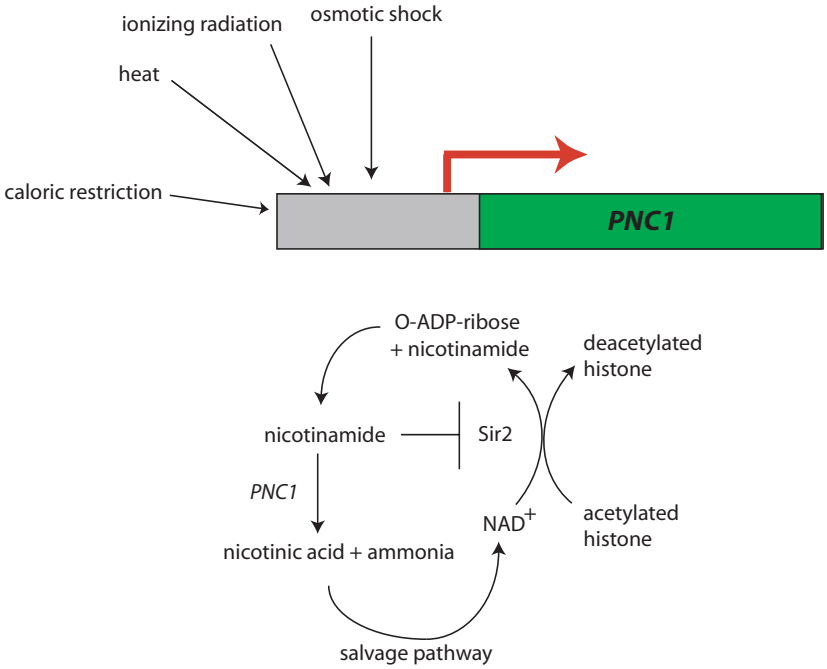

Fig. 2. Regulation of Sir2 activity in yeast. Top: Transcription of *PNC1* can be induced by a variety of stresses, indicating that calorie restriction (CR) works by eliciting a mild stress response within the organism. Bottom: Regulation of Sir2 by *PNC1* and nicotinamide.

cells determined that the ratio of NAD^+ : NADH is >20, a number that agrees with classical estimates (Anderson *et al*., 2003b), and at this ratio the activity of SIRT1 is not appreciably affected by NADH (Imai *et al*., 2000; Anderson *et al*., 2003a). Furthermore, addition of acetaldehyde to cells, which is known to decrease NADH levels in yeast, does not affect Sir2 activity *in vivo* (Anderson *et al*., 2003a).

A different but not mutually exclusive hypothesis is that Sir2 is regulated by a gene called *PNC1* (pyrazinamidase-nicotinamidase 1) (Anderson *et al*., 2003a; Gallo *et al*., 2004), which encodes an essential component of the NAD salvage pathway that converts nicotinamide to nicotinic acid (Fig. 2). Free nicotinamide is a product of the Sir2 reaction and an inhibitor of Sir2 *in vitro* and *in vivo* (Landry *et al*., 2000; Bitterman *et al*., 2002; Anderson *et al*., 2003b). When cells are subjected to CR or any of the other treatments known to extend yeast lifespan, *PNC1* is highly upregulated and this upregulation is necessary for lifespan extension (Anderson *et al*., 2003a; Gallo *et al*., 2004). Thus, *PNC1* seems to serve as an environmental sensor that translates CR and environmental stress signals into Sir2 activation. Whether *PNC1* or NAD^+/NADH plays the major role in regulating Sir2 during CR is an ongoing debate.

Despite the differences in complexity between yeast and higher organisms, Sir2 enzymes (also known as the 'sirtuins') appear to have a conserved role in promoting

Table 1. Summary of sirtuins that have been characterized

Organism	Gene	Effect	Localization
S. solfataricus	*ssSir2*	Deacetylation of Alba, an archaeal chromatin protein	
L. major	*LmSIR2*		Cytoplasmic
S. cerevisiae	*SIR2*	Histone deacetylase, silencing, increases lifespan	Nuclear
	HST1	HM silencing, sporulation	Nuclear
	HST2	Silencing	Cytoplasmic
	HST3	Telomeric silencing, radiation resistance	Nuclear
	HST4	Telomeric silencing, radiation resistance	Nuclear
C. elegans	*sir2.1*	In daf-16 pathway, increases lifespan	Nuclear and cytoplasmic
D. melanogaster	*αSIR2*		Nuclear and cytoplasmic
H. sapiens	*SirT1*	Deacetylates p53, FOXO3a Ku70	Nuclear, some cytoplasmic
	SirT2	Deacetylates tubulin, cell cycle	Cytoplasmic, possibly nuclear
	SirT3		Mitochondrial
	SirT4		Mitochondrial
	SirT5		Cytoplasmic
	SirT6		Nuclear
	SirT7		Nucleolar

cell survival and promoting longevity. In *C. elegans*, increased dosage of the closest Sir2 homologue, *sir-2.1*, extends lifespan by 50% (Tissenbaum and Guarente, 2001). This extension is DAF-16-dependent, suggesting in worms SIR-2.1 functions in the insulin/IGF-1 signalling pathway. Humans possess seven Sir2 homologues, SIRT1–7 (see Table 1). SIRT1, the closest homologue to yeast Sir2, has recently been shown to deacetylate and regulate FOXO3, a mammalian homologue of DAF-16, thus linking SIRT1 to the insulin/IGF-1 longevity pathway (Brunet *et al*., 2004, Motto *et al*., 2004). The next major challenge will be to determine whether sirtuins are involved in mediating the CR effect in metazoans and, if so, how they are regulated by caloric intake.

Small molecules that modulate sirtuin activity

Since the discovery that Sir2 is a regulator of yeast longevity, there has been a great deal of interest in finding small molecules that can alter the activity of this enzyme family. Nicotinamide and its analogues such as N-methyl nicotinamide are effective inhibitors of sirtuin activity and may be valuable in a clinical setting (Bitterman *et al*., 2002; Anderson *et al*., 2003a). Several synthetic compounds have also been identified that can inhibit sirtuins including splitomicin (Bedalov *et al*., 2001), and sirtuinol (Grozinger *et al*., 2001). These compounds contain aromatic structures, and the compounds identified by Grozinger *et al*. share a hydroxynapthaldehyde moiety that seems to be important for inhibition.

While sirtuin inhibitors may prove to be useful in treating some diseases, activators of sirtuins could be the key to extending lifespan in higher organisms. Recently, 18 small molecules that can increase human SIRT1 activity were identified, including resveratrol, butein, and piceatannol (Howitz *et al*., 2003). All of these molecules come from plants and are similar in structure. Analysis of the structure–activity relationship of these compounds suggests that the hydroxylated *trans*-stilbene ring structure is key to their ability to activate (Howitz *et al*., 2003). From a biochemical standpoint, the stimulatory effects of the molecules are notable because they are the first examples of small molecule agonists of a deacetylase.

The compound with the greatest stimulatory activity was resveratrol, a polyphenol that is found in numerous plant species including grapes, peanuts, and some Asian medicinal herbs. Relatively low concentrations of these polyphenols added to yeast medium (<10 μM) can extend yeast lifespan by up to ≈ 65%. Experiments in *C. elegans* and *Drosophila* so far seem promising (Wood *et al*., 2004). Interestingly, a number of health benefits in humans have already been attributed to resveratrol, including cardioprotection, neuroprotection, and cancer chemoprevention. It will be interesting to explore whether any of these effects are caused by sirtuin activation.

Sirtuin activators: molecular mimics or example of xenohormesis?

Sirtuin genes are found in all eukaryotes examined so far, including plants, fungi, and animals. It is therefore safe to assume that sirtuins are very ancient enzymes that existed in the common ancestor of today's eukaryotes, possibly more than a billion years ago. As far as we can tell from a few laboratory organisms, their function in promoting longevity and stress resistance is also conserved. Because plants possess multiple sirtuins, it is reasonable to imagine that the sirtuin-activating polyphenols are actually stress-signalling molecules that coordinate sirtuin-mediated defences in plants (Howitz *et al*., 2003). In fact, many of the polyphenols that activate the sirtuins, such as resveratrol and quercetin, are synthesized by plants during times of stress (e.g. infection, starvation, and dehydration). This hypothesis, that this class of polyphenols are signalling molecules, contrasts with the mainstream view of them as antioxidants or phytoalexins (i.e. plant antibiotics) (Stojanovic *et al*., 2001).

These findings raise a big question: why do plant stress molecules activate sirtuins from yeast and humans? It is highly unlikely that this is simply an inherent property of these enzymes because it would be lost rapidly in the absence of positive selection. So what selective force could have maintained this property of sirtuins since the major eukaryotes diverged? One possibility is that fungi and plants modulate their sirtuins with endogenous molecules that have yet to be discovered. These putative molecules must be quite different in structure from polyphenols, however, because *S. cerevisiae* and metazoans do not possess genes that resemble those used for plant polyphenol biosynthesis.

Another explanation is that animals and fungi have since lost their ability to synthesize polyphenols but have retained the ability to be activated by these plant molecules because they provide a highly useful advance warning of a deteriorating environment and/or food supply, allowing organisms to begin conserving resources and increasing cell defences (Howitz *et al*., 2003) (Fig. 3). This interspecies communication of stress signals has been termed 'xenohormesis' by Howitz and Sinclair (Howitz *et al*., 2003). The hypothesis makes a number of predictions: we should find a bounty of medicinal molecules in stressed plants; that xenohormetic molecules should interact with a variety of enzymes involved in regulating stress responses and survival; and the molecules should be relatively safe for

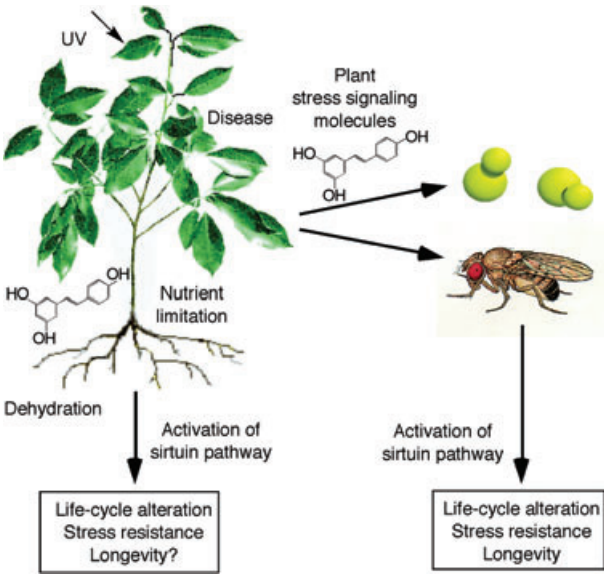

Fig. 3. The xenohormesis hypothesis. Sirtuin enzymes evolved early in life's history to increase somatic maintenance and survival during times of adversity. The xenohormesis hypothesis of Howitz and Sinclair proposes that primordial species synthesized polyphenolic molecules to stimulate sirtuins during times of stress. Plants have retained this ability. Survival pathways in fungi and animals have retained the ability to respond to plant stress signalling molecules because they provide useful prediction about the state of the environment and/or food supply. This ability would allow organisms to prepare for and survive adversity when they might otherwise perish (Howitz *et al*., 2003).

human consumption. An intriguing prediction of the hypothesis is that we might one day use xenohormetic molecules therapeutically, to provoke an artificial sirtuin response that could provide the same remarkable health benefits currently reserved for calorie-restricted animals.

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