# Aging and Survival: The Genetics of Life Span Extension by Dietary Restriction

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## Abstract

Reducing food intake to induce undernutrition but not malnutrition extends the life spans of multiple species, ranging from single-celled organisms to mammals. This increase in longevity by dietary restriction (DR) is coupled to profound beneficial effects on age-related pathology. Historically, much of the work on DR has been undertaken using rodent models, and 70 years of research has revealed much about the physiological changes DR induces. However, little is known about the genetic pathways that regulate the DR response and whether or not they are conserved between species. Elucidating these pathways may facilitate the design of targeted pharmaceutical treatments for a range of age-related pathologies. Here, we discuss how recent work in nonmammalian model organisms has revealed new insight into the genetics of DR and how the discovery of DR-specific transcription factors will advance our understanding of this phenomenon.

## Contents



## INTRODUCTION

It is now over 70 years since McCay et al. (1) first reported that restricting the food intake of laboratory rats dramatically increased their life span. Since then dietary restriction (DR) has become something of a gold standard in aging research and is still the only environmental intervention shown to extend the longevity of both invertebrates and vertebrates (2). As such, DR is regarded as a true public mechanism of life span extension. However, despite 70 years of work and a large body of publications reporting the effects of DR on physiology, senescence, and age-related disease, the genetic pathways that influence this remarkable phenomenon remain at worst elusive and at best controversial. The literature is dominated with correlative reports showing that age-related changes to a range of physiological traits can be attenuated or delayed by DR, but it is difficult to separate those that are causal from those that are casual. A lack of genetic data may be because, up until recently, most work on DR was performed on rodents, and classical genetic analysis was difficult to perform. Motivation to elucidate the underlying genetic mechanisms behind this life span increase remains because of the beneficial impact DR has on a wide range of age-related pathologies. DR in rodents has been shown to delay the onset and reduce the severity of many diseases, including cardiovascular disease, diabetes, autoimmune disease, cataracts, osteoporosis, neurodegenerative diseases, and, in particular, many forms of cancer (for a review see Chapter 5 of Reference 3). Far from simply prolonging the life span of an aged and frail individual, DR seems to maintain laboratory animals in a relatively youthful and healthy state beyond the point at which their gluttonous counterparts begin to fail.

Protocols for applying DR in mammals are designed to induce "undernutrition without malnutrition" (2), and the food intake this represents is usually 30% to 40% less than ad libitum levels. If life span extension via DR truly functions through evolutionarily conserved mechanisms, using model organisms to elucidate the genetic pathways involved may ultimately allow for the design of targeted pharmaceutical treatments with the potential for dramatic health benefits, without the need for strict dietary regimes and the associated detrimental physical and psychological side effects that they can impose (4). It is beyond the scope of a review of this size to summarize the vast effects of DR on physiology and life span, and this has been done comprehensively elsewhere (2, 3, 5). Here, we aim to summarize what the recent research on invertebrate models has taught us about the genetic pathways that regulate the response to DR, which factors seem to be evolutionarily conserved between species, and how newly identified DR-specific transcription factors may move the field further toward identifying the mechanisms by which DR enhances longevity.

**DR:** dietary restriction

**Public mechanism:** a mechanism conserved between evolutionarily diverse species

## DIETARY RESTRICTION: THE ULTIMATE PUBLIC MECHANISM OF LIFE SPAN EXTENSION?

DR has been shown to increase life span in a wide range of species (**Table 1**). These include commonly used model organisms, such as the budding yeast *Saccharomyces cerevisiae* (6, 7), the nematode *Caenorhabditis elegans* (8, 9), the fruit fly *Drosophila melanogaster* (10, 11), and rodents (1–3), along with many species less often used for laboratory research, such as spiders, water striders, rotifers, grasshoppers, water fleas, fish, hamsters, and dogs (**Table 1**). Three studies on the effects of DR on the life span of rhesus monkeys (12–14) and one on squirrel monkeys (13) are still ongoing. Although it is still too early to show conclusively if DR extends life span in these long-term studies, early signs are promising because biomarkers of age seem delayed in the DR cohorts (15). One report described an increase of seven years in the median life span of rhesus monkeys under DR (16), but this result should perhaps be treated as preliminary; out of the 117 monkeys in the study, only 8 were on a DR regime, and of those only 3 died, making the sample size too small to be conclusive. It has also been suggested that DR results in health benefits to humans (17), although again there is as yet little conclusive evidence. The effect of DR (25% reduction) in nonobese humans is currently under investigation in the Comprehensive Assessment of Long-term Effects of Reducing Intake of Energy project (17).

## DIETARY RESTRICTION AS AN ADAPTIVE TRAIT

The prevailing theory regarding the response of organisms to DR is that this mechanism evolved to provide animals protection during times of famine (18, 19). Because DR extends life span in nearly every species tested, the ability to increase longevity when food is scarce likely confers some fitness advantage that caused the trait to be selected for during evolution. One of the hallmarks of DR is that although restricted animals live longer than controls they show reduced yet prolonged reproduction. DR has been shown to reduce both daily and lifetime fecundity in *C. elegans* (9, 20) and *D. melanogaster* (11) and also to delay reproductive maturity in rats (21). Hence, the increase in life span seen during DR may represent an evolutionarily adaptive strategy for reallocating limited resources. During periods of food scarcity in the wild when reproduction might be costly and the chances of offspring surviving low, upregulating somatic maintenance at the cost of growth and reproduction would increase the evolutionary fitness of the organism (18, 19). The debate as to whether the beneficial effects of DR are due to reversing artificially high feeding levels in the laboratory is discussed in the accompanying sidebar, Is Dietary Restriction Rescuing the Gluttonous Effects of Life in the Laboratory? However, if increased life span in response to DR is an evolutionary adaptation to increase survival in the wild, it must be regulated genetically, and those pathways involved might be conserved between species. Yet, despite the finding that some form of food restriction increases longevity in such a diversity of species, the protocols by which DR is applied vary tremendously for different organisms (**Table 1**), and the mechanisms responsible for the life span increase are yet to be determined in any of them. Indeed, scrutiny of several experiments reported as examples of DR extending the life span of diverse species reveals that many of them involve dietary regimes that severely restrict growth along with extending life span (**Table 1**). Therefore, although the ability to display plasticity of life span in response to food shortage may increase individuals' fitness in divergent species, it remains possible that the mechanisms by which this plasticity is achieved are not conserved and represent examples of convergent evolution. Uncovering the genetic pathways that regulate the DR response in different species will allow us to determine if this is the case.

**Convergent evolution:** the independent evolution of similar adaptive traits in unrelated organisms, often in response to the same environmental pressure

**Table 1 The effect of dietary restriction (DR) on life span in different organisms. Life span is increased in a wide range of species, although the protocol used to apply DR varies greatly for different species/laboratories**

| Species | DR regime | Life span measure | Increase | Reference |
|---|---|---|---|---|
| *S. cerevisiae* | Glucose dilution | RLS | 75% | (6) |
| | SDC versus water | Mean CLS | 300% | (183) |
| | Asparagine/glutamate restriction | CLS | Not reported | (138) |
| *Tokophrya infusionum* | Fed reduced number of *Tetrahymena* | Maximum life span | Not reported | (184) |
| *C. elegans* | *eat-2 (ad1113)* mutation | Mean life span | 46% | (42) |
| | Axenic media | Mean life span | 85% | (185) |
| | Bacterial dilution in liquid | Mean life span | 52% | (9) |
| | Reduction of bactopeptone in plates | Mean life span | 30% | (186) |
| | Dietary deprivation during adulthood | Mean life span | 50% | (41, 81) |
| *D. melanogaster* | Reduction in yeast paste availability | Mean life span | 28% | (11) |
| | Dilution of media | Median life span | 66% | (10) |
| Medflies | Dilution of nutrients | Median life span | 22% | (187) |
| Grasshoppers | Reduction by 40% of ad libitum | Median life span | 62% | (188) |
| Spiders | Reduction in number of *D. melanogaster* fed | Median life span | 212% | (189) |
| Water striders | Reduction in number of *D. melanogaster* fed | Increase in life span on low food | 20 days | (190) |
| Water fleas | Dilution of manure infusion media with pond water | Mean life span | 69% | (191) |
| Rotifers | Algae deprivation | Mean life span | 60% | (192) |
| Guppies | Reduced sludge worm intake | Maximum life span | Not reported | (193) |
| Trout | Dried skim milk with liver supplement versus without | Survival | Not reported | (194) |
| Hamsters | Reduction of food by 50% | Median | 30% | (195) |
| Mice | Ad libitum versus 40 kcal/week from weaning | Mean | 65% | (25) |
| | From 1 year, 160 kcal versus 90 kcal/week | Mean | 20% | (44) |
| | Every other day feeding | Mean | 27% | (196) |
| | Methionine restriction | Maximal life span[b] | 10% | (140) |
| Rats | Reduced food intake to growth-restricting levels interspersed with periodic growth-promoting diets | Mean (males) | 85% | (1) |
| | Reduction of ad libitum by 60% | Median | 47% | (197) |
| | Methionine restriction | Mean life span | 42% | (141) |
| | Every other day feeding | Mean life span | 83% | (198) |
| Dogs | Reduced to 75% of control food intake | Median life span | 16% | (199) |
| Rhesus monkeys | Restricted chow to maintain lean target weight of 10–11 kg | Median life span | 28% | (16) |

[a]Abbreviations: CLS, chronological life span; RLS, replicative life span; SDC, standard yeast growth medium.
[b]Maximal represents the mean life span of the longest lived 10% within a cohort.

## DETERMINING INTERVENTIONS THAT MIMIC DIETARY RESTRICTION

The response of life span to reduced food intake can be represented by a bell-shaped curve (**Figure 1*a***) whereby the maximum life span is achieved at a narrow sweet spot of food consumption, below and above which animals show reduced life span. This characteristic response has been observed in yeast (21), *C. elegans* (8, 20, 22), and *Drosophila* (23). In mice, progressive reduction in food intake also results in incremental increases in life span extension (24), representing the right-hand side of the bell curve. Unlike life span, reproductive capacity correlates with food level and is maximized at ad libitum intake levels (9, 10, 20, 25).

Examining the effect any intervention, be it genetic or pharmacological, has on this bell curve response of life span to reduced nutrient intake can be used to assess the intervention's role in the DR pathway (**Figure 1**). Any intervention that upregulates the same longevity assurance processes as DR will have the greatest effect on life span in well-fed animals (**Figure 1*b***). This longevity increase will diminish as food intake is reduced until no effect is seen at the nutritional sweet spot that optimizes longevity of the controls (**Figure 1*b***) because at this nutritional level longevity assurance processes are already maximized. An intervention that limits nutrient intake/absorption or reduces cellular energy even when food availability is high will trigger a DR response. This type of DR mimetic will again show the maximum increase in longevity at high food intake levels but will also push organisms into the starvation range sooner than controls as food intake is decreased, resulting in a shifting of the curve to the right (**Figure 1*b***). Interventions that disable any downstream "master regulator" of the DR response will block all interaction between the dietary regime and life span, resulting in a flattening of the curve and a similar life span for animals irrespective of their nutritional status (**Figure 1*c***). These alterations to the bell curve response are indicative of interventions involved in the DR pathway and are quantifiably different from interventions that either increase or reduce the longevity of the animal in a DR-independent manner (**Figure 1*d***). That the response to DR is continuous rather than discrete may explain the

## IS DIETARY RESTRICTION RESCUING THE GLUTTONOUS EFFECTS OF LIFE IN THE LABORATORY?

It has been suggested that DR increases life span by rescuing the pathological effects of overfeeding (177–179), and interestingly, DR did not increase the life span of wild-caught mice (180). One interpretation of this result is that DR restores lab mice back to the adiposity levels of wild mice and as such removes the unnatural gluttony caused by years of selection in laboratory conditions. However, although DR rodents are leaner than ad libitum-fed controls, it is not the obesity of ad libitum-fed animals that causes the difference in life span. Leptin-deficient *ob/ob* obese mice (181) live as long under a DR regime as wild-type mice despite having 50% adipose tissue and weighing more than shorter-lived, ad libitum-fed wild-type mice (182). Furthermore, comparisons of food intake of laboratory-housed mice to wild counterparts show that mice eat more in the wild than under ad libitum feeding in captivity (182a). The lack of a DR effect on the wild-caught mice may be due to too severe a DR regime. Although the regime in this study was similar to those successfully used elsewhere to increase the life span of standard lab strains, no positive laboratory strain control was included (180). Furthermore, the DR wild-caught mice did show reduced levels of cancer incidence and hormonal changes commonly seen in diet-restricted lab mice, and the longest lived 8% of mice in the study came from the DR group, suggesting that although no positive effect on life span was seen, there was a DR response in these mice. Additionally, it is not clear that the wild-caught mice can live long in response to any perturbation that increases the life span of laboratory mice, such as reduced insulin/IGF-1 signaling. Further study may resolve this issue, and it is certainly of interest to speculate if westernized humans more closely resemble the wild-caught mice or their more gluttonous lab counterparts.

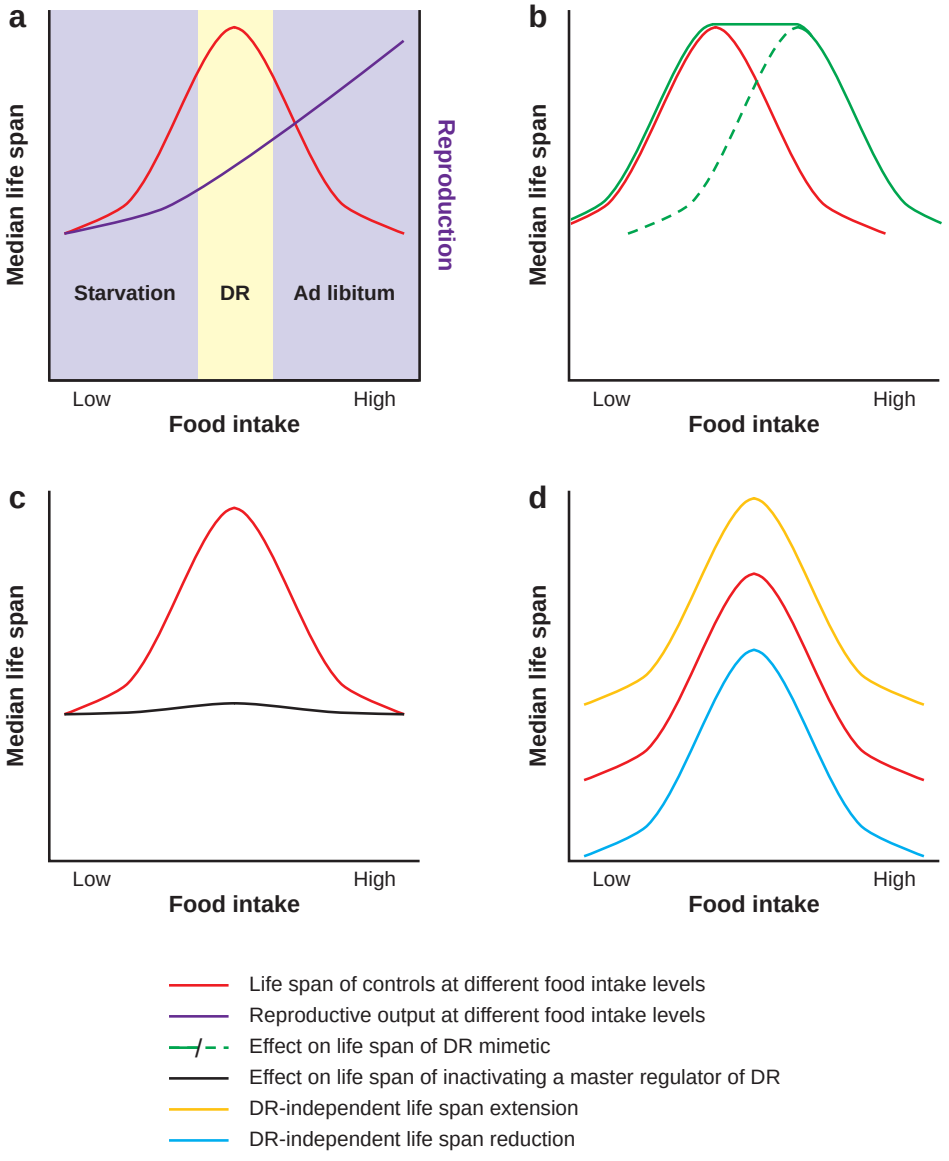


**Figure 1**

The effect of dietary restriction (DR) on life span. (*a*) Median life span increases as food intake is reduced from ad libitum levels (high food intake) until a nutrient regime that optimizes longevity is reached, past which further restriction results in reduced life span due to starvation (low food intake) (*red line*). Reproductive capacity correlates with dietary intake and optimal reproduction is seen at ad libitum food levels (*purple*). (*b*) Interventions that activate the same longevity assurance targets as DR will increase life span compared to controls at ad libitum food levels, but this effect will diminish as food intake is decreased (*solid green line*). Interventions that limit food uptake/cellular energy levels will increase life span at ad libitum levels but will push animals into starvation at a higher level of food intake than that which causes starvation in control animals (*dashed green line*). (*c*) Disabling any master regulator of DR will block the response of life span to changes in food intake, resulting in a flattening of the bell curve (*black line*). (*d*) Interventions that affect life span in a DR-independent manner will either increase (*yellow line*) or decrease (*blue line*) life span to the same extent at all levels of food consumption.

few published examples of DR failing to increase life span (26–29) because imposing too severe a regime will push the organisms past the optimal sweet spot to the point where no life span extension or reduced longevity could result.

## OVERVIEW

Uncovering the genetic pathways that regulate the response to DR in different species, and determining how upstream changes in nutrient sensing and signaling lead to downstream upregulation of longevity assurance genes, will allow researchers to accurately determine if the mechanisms behind the DR phenomenon are indeed conserved between species and therefore if research into DR in model organisms will prove to be a productive strategy for developing biomedical therapeutics for treatment of human disease. The remainder of this review summarizes the evidence for and against a role in the DR response for those pathways already known to affect longevity.

### Insulin Signaling

Mutations in genes that encode components of the insulin/insulin-like growth factor signaling (IIS) pathway result in a robust extension of life span that is conserved from *C. elegans* (30, 31) to *Drosophila* (32, 33) and mammals (34–36). IIS represents a key nutrient signaling pathway that coordinates traits such as development and growth with resource availability (37). As such, reduced IIS seemed a good candidate mechanism through which DR might be extending life span. Indeed, in the fruit fly, mutations to *chico*, which encodes the insulin receptor substrate, extended life span by 48% when nutrient intake was high, but when food was limited, *chico* flies were shorter lived than controls, as would be expected if these mutants were already partially dietarily restricted by their genotype (23) (**Figure 1*b***). However, this suggestion from early experiments in *Drosophila* that DR functions exclusively through reduced IIS has not been supported by subsequent work in *C. elegans*.

In worms, IIS causes phosphorylation of the forkhead transcription factor DAF-16 (38), resulting in its nuclear exclusion and deactivation through association with 14-3-3 proteins such as FTT-2 (39). During stress or starvation, DAF-16 is translocated to the nucleus where it upregulates a panel of longevity assurance genes (40). Functional DAF-16 is required for the extension of life span by reduced IIS (31) because the increased longevity that results from mutations to genes encoding either the insulin-like growth factor (IGF) receptor DAF-2 or downstream signaling kinases is completely suppressed by null mutations of *daf-16* (31). Therefore, if DR functions exclusively through the insulin pathway, it too should be blocked by removal of functional DAF-16. Several reports indicate that this is not the case. DR in *C. elegans* by reduction of its food source (bacteria), by complete removal of food in adulthood, or by the genetic surrogate of DR *eat-2* mutation (which reduces the animal's ability to pump food through its pharynx) all extend the life span of *daf-16* null mutants (8, 22, 41, 42). Although the response to DR is dampened when DAF-16 is impaired (8, 22), suggesting that part of this response may be mediated by IIS, it is clear that life span extension by DR is not exclusively the product of environmentally induced reduction of insulin signaling.

Until recently, evidence that life span extension by DR was not dependent upon insulin signaling had only been clearly demonstrated in the worm, but this has now been shown to also be the case for *Drosophila*. DR applied late in life yields robust extension of life span in nematodes (9), flies (43), and mammals (44). However, this is contrary to the temporal requirements of reduced insulin signaling. In the worm, RNA-mediated interference (RNAi) of *daf-2* late in life results in only marginal changes to life span (45), and recent work in *Drosophila* shows a similar decline in the longevity response when *dFoxo* (the fly orthologue of *daf-16*) is overexpressed late in

**IIS:** insulin/insulin-like growth factor signaling

**RNAi:** RNA-mediated interference

adulthood (46), suggesting that the modes of action of DR and IIS in the fly, as in the worm, are at least partially separate from one another. More conclusively, subjecting *dFoxo* null *Drosophila* to DR still results in robust increases to longevity (46a), although the dependence of life span extension by reduced IIS upon *dFoxo* in fruit flies has yet to be shown.

In mammals, the Forkhead box type O (FoxO) group of transcription factors (comprising FoxO1, FoxO3, FoxO4, and FoxO6) are homologous to DAF-16 and dFOXO (47). The regulation of FoxO1, FoxO3, and FoxO4 activity is controlled in a manner conserved from the invertebrate systems (48). Phosphorylation by AKT/protein kinase B (PKB) results in the nuclear exclusion of the FoxOs by their association with 14-3-3 proteins (49–51). The effect on the life span of reduced IIS components is also strikingly conserved from lower organisms to mammals. Robust life span extension is seen in growth hormone-deficient Ames dwarf mice (52); growth hormone receptor knockout (GHRKO) mice (53) (growth hormone positively regulates the production of IGF-1); IGF-1 receptor knockout heterozygous mice (35); fat-specific insulin receptor knockout mice (34); and recently, insulin receptor substrate 2 knockout mice (36).

The link between DR in mice and reduced IIS is now an area of active investigation (see Reference 54 for a review). Ames dwarves and GHRKO mice share some physiological characteristics with DR animals, such as reduced body size, core temperature, circulating insulin levels, and reproductive capacity (54). Ames mice also show the increased activity levels often seen in wild-type mice on a DR feeding regime (54). However, food consumption per unit mass of Ames and GHRKO mice is increased relative to controls, and these animals become obese with age (54). Furthermore, in contrast to DR, IGF-1 knockout heterozygous mice do not have reduced body size or compromised reproduction (35). Comparison of transcriptional profiles of wild-type and GHRKO mice on control and DR feeding regimes reveals only partial overlap in expression changes (55, 56), suggesting that the IIS and DR pathways are at least in part separable.

The effects of DR on IIS mutant mice has not been comprehensively studied, and the results seen in the different IIS mouse models tested to date have been conflicting. Long-lived Ames mice subjected to DR show further increases in longevity (57); in contrast, subjecting GHRKO mice to DR failed to increase their life span (58). It is not known if the life span extension seen in IGF-1 heterozygous mice is dependent on any or all of the FoxO proteins, and experiments testing the role these transcription factors play in life span extension either by reduced IIS or DR in mammalian systems should greatly advance our understanding of the extent to which these two pathways overlap.

## Sirtuins and Dietary Restriction

The $NAD^+$-dependent histone deacetylase Sir2p was the first reported regulator of DR-induced longevity and is considered specific to and inherently required for this pathway. Sir2p was found to mediate the response to DR because mutant strains of the budding yeast *S. cerevisiae* that were null for *SIR2* did not show the increase in replicative capacity seen in wild-type yeast cultured in media with reduced glucose concentration (59). The number of budding events that a mother cell can undergo is readily quantifiable and is used as a measure of decline with age known as replicative life span (RLS). This is in contrast to another assay of yeast longevity known as chronological life span (CLS). RLS is extended by overexpressing *SIR2* (60) and shortened when *SIR2* is deleted (61). One factor that limits the RLS of budding yeast is the buildup of extrachromosomal rDNA circles (ERCs) within the nucleus (62), and it is through changes to the copy number of ERCs that Sir2p is thought to effect RLS. Chromatin silencing by Sir2p suppresses recombination events in the tandemly repeated rDNA sequence and therefore reduces the buildup of ERCs (63). The short RLS of yeast *sir2* Δ

**Ames dwarf mice:** mice with loss-of-function *Prop-1* mutations and with deficiencies in growth hormone, thyroid-stimulating hormone, and prolactin

**RLS:** replicative life span

**Chronological life span (CLS):** the time yeast can maintain a nondividing state in liquid before losing replicative ability when replated onto growth medium

**ERCs:** extrachromosomal rDNA circles

mutants is suppressed by mutations in *FOB1* (60), a gene encoding a replication fork protein whose deletion reduces ERC formation and extends life span (64), and overexpression *of SIR2* does not extend the life span of *fob1*Δ mutants. The ability of Sir2p to silence chromatin requires $NAD^+$ as a substrate (65). It has therefore been proposed that DR in yeast extends life span by upregulation of Sir2p activity as a result of increased $NAD^+$/NADH ratios produced as the cells shift from fermentation to respiration when glucose becomes limited (7). An alternate model has also been proposed in which glucose restriction increases Sir2p activity through increased expression of *PNC-1*; the product of *PNC-1* converts nicotinamide into nicotinic acid (66). Nicotinamide inhibits sirtuin activity; therefore increased *PNC-1* would activate Sir2p indirectly via removal of this inhibition (66).

Although ERCs do not play a role in the life span of higher organisms, a role for Sir2p in the DR pathway is conserved, as overexpression of the *SIR2* homologue *sir-2.1*, in *C. elegans* (67), and *dSir2*, in *Drosophila* (68), also increases longevity; and life span extension by DR is suppressed in animals mutant for *sir-2.1* (69) or *dSir2* (68). Furthermore, DR of mice lacking functional SIRT1, the mammalian orthologue of Sir2p, does not result in the increased foraging behavior that is usually characteristic of DR in that species (70), although longevity studies have yet to be reported. Taken together these results indicate that Sir2p is essential for the response of RLS to glucose limitation in yeast and suggest an evolutionarily conserved function for Sir2p homologues in the modulation of survival of higher organisms.

Recent results have challenged the original findings that mutations to *SIR2* or its homologues (known collectively as the sirtuins) completely block the longevity effects of DR in yeast (71), and the role of the sirtuins in DR has become a highly contested area within the field of aging research. The exclusivity of Sir2p as a regulator of DR in budding yeast was questioned when its role was examined in strains other than PSY316 (on which the original experiments were performed) (72). Both BY4742 and W303R strains null for *SIR2* and *FOB1* show a normal increase in RLS in response to lowering glucose concentration in the food medium (61, 72). Furthermore, the only yeast strain in which RLS extension by glucose restriction is shown to require functional Sir2p does not show increased RLS when *SIR2* is overexpressed (72), whereas those in which overexpression of *SIR2* does extend RLS show Sir2p-independent DR responses (72) (**Table 2**).

There are five sirtuins in budding yeast (73), allowing the potential for redundancy between them for the role of mediating the response to DR in different strains. In support of this redundancy, the Sir2p-independent life span extension by DR seen in the W303AR5 strain (a nearly identical derivative of W303R) carrying *sir2*Δ and *fob1*Δ mutations (72) was blocked by the addition of nicotinamide, a general inhibitor of sirtuins in yeast (74). Interestingly, deletion of the homologue of *SIR2* 2 (*HST2*) in combination with *sir2*Δ and *fob1*Δ mutations in the W303AR5 background did block the response to DR (74),

**Table 2 The contrasting and strain-specific effects of Sir2 and Resveratrol on the replicative life span (RLS) of budding yeast**

| Strain | Sir2p-dependent DR? | Increased RLS from increased *SIR2* expression? | Increased RLS from Resveratrol? | References |
|---|---|---|---|---|
| PSY316 | Yes | No | Yes | (59, 72, 92, 98) |
| BY4742 | No | Yes | No | (72, 98) |
| W303R | No | Yes | No | (60, 74, 98) |

suggesting that, in this strain, life span extension by DR is mediated by a mechanism involving both of these sirtuins. However, Hst2p's role in the Sir2p-independent DR pathway has also been contested; glucose restriction has been reported to extend the RLS of a strain with deletions in four of the five sirtuin genes, including both *SIR2* and *HST2* (75). The reason for the disparity in the results on the role of Hst2p in DR remains unresolved and may be due to differences in the degree of glucose restriction applied. However, what is certain is that in at least some genetic backgrounds, reducing glucose concentrations in yeast null for *SIR2* can extend RLS, and as such Sir2p cannot be the single master regulator of DR in this species (74, 75).

The relevance of yeast RLS to aging in higher organisms also remains to be shown. The cells of adult *C. elegans* and *Drosophila* are postmitotic, and it is therefore unclear why the role of Sir2p in RLS should be relevant to aging in systems not undergoing cell division. Furthermore, buildup of ERCs during cell division, which limits the RLS of budding yeast, is not seen in mammalian cells (76). RLS may be more representative of reproductive capacity; although if this is the case, the response of budding yeast to DR is different from those of higher organisms because reproduction is reduced during DR in worms, flies, and rodents (9, 10, 20, 25). Instead, RLS may relate to the capacity for stem cell maintenance in higher organisms.

The CLS of yeast cells is also used as an assay of longevity and may provide a better system for identifying conserved pathways that affect aging of animals whose soma is composed of predominantly differentiated cells. Interestingly, the CLS of yeast can also be increased by DR, but this effect is independent of Sir2p (77). Indeed, recent results show that increased CLS via DR is not dependent on any one of the five sirtuins (78). In contrast to the effects on RLS, deletion of *SIR2* did not shorten the CLS of cells, and no extension of CLS was seen when *SIR2* was overexpressed (78). The lack of an effect on CLS when expression levels of *SIR2* are altered is similar to results seen for RLS in a *fob1*Δ mutant background, and interestingly, no accumulation of ERCs is seen during chronological aging (79), suggesting that life span extension that is independent of ERCs is also independent of Sir2p. However, the effect of multiple deletions to the sirtuins on CLS by DR has yet to be examined.

The initial results linking DR to Sir2p in yeast therefore seem specific both to particular strains and to one assay of senescence, RLS. However, a key role of *SIR2* homologues in DR has also been reported in flies and worms (67–69), and if this role is truly conserved to mammals also, perhaps fortunate choices made in the initial yeast experiments have indeed led to the discovery of a true DR-specific regulator of longevity. Although the mechanism by which Sir2p acts may not be conserved, the adaptive advantage of a modulator of life span that can respond to changes in the environment might well be. However, although the idea of an evolutionarily conserved regulator of DR is appealing, there are actually surprisingly few studies on the sirtuins in worms or flies, and caution should be applied before solid conclusions are drawn from these limited studies, especially given that only one sirtuin orthologue has been examined.

In *C. elegans*, mutations in *sir-2.1* significantly reduce the life span of *eat-2* worms (69). However, the critical test of the dependence of worm DR on *sir-2.1* requires that there be no statistically significant difference between the life span of *sir-2.1* mutant worms and the *eat-2 II*; *sir-2.1 IV* double mutants. This statistical comparison is not reported in the paper (69), although mean life spans of 15.4 days and 18.4 days for *sir-2.1 (ok434)* and *eat-2 (ad465) II; sir-2.1 (ok434) IV* worms, respectively (a 19.4% increase), suggest that the *eat-2 (ad465)* allele did extend life span in a *sir-2.1* mutant background in these experiments. Indeed, elsewhere in the literature, DR has been reported to extend the life span of *sir-2.1* mutants either by *eat-2* mutation (80) or

by reducing nutrient intake directly (41, 74, 81). There are four sirtuins in *C. elegans*, but as yet, there are no studies investigating the role of any of them in DR except SIR-2.1. Although overexpression of *sir-2.1* increases the life span in worms, it does so via a mechanism dependent on the FoxO transcription factor DAF-16 (67), which is not required for life span extension by DR in *C. elegans* (8, 22, 41, 42). Further work is needed to conclude that DR extends the life span of *C. elegans* in a sirtuin-dependent mechanism.

The *Drosophila* Sir2p orthologue dSir2 was reported to have a key role in life span extension by DR because feeding a nutrient-diluted media to flies that were trans-heterozygous for *dSir2* null mutations and that therefore had reduced *dSir2* expression did not extend life span (68). However, it is unclear if this level of food dilution consistently increased life span of control flies in this study, and because the food dilution that maximizes life span in *Drosophila* varies for different genotypes (23), the longevity of *dSir2* mutants should be tested across a range of nutrient levels to conclusively determine whether their life span is unresponsive to changes in diet and to rule out the possibility of a false-negative response to DR.

Recent results suggest a complex regulatory network involving the sirtuins and the mammalian FoxO transcription factors. SIRT1 has been shown to deacetylate FoxO1 (82), FoxO3a (the closest mammalian homologue of the worm DAF-16) (83–85), and FoxO4 (83–85), and SIRT2 also has FoxO1 and FoxO3a deacetylase ability (86, 87). Furthermore, increased SIRT1 expression in response to starvation is dependent on FoxO3a (88), and life span extension by overexpression of *sir-2.1* in *C. elegans* is blocked in a *daf-16* null background (67). Although removal of *daf-16* does not block the response of life span to DR, it does appear to dampen it (8, 22), and it may be that the role of the sirtuins in DR is through interaction with the insulin/IGF-1-like signaling pathway. Recent results suggest that yeast lacking *SIR2* also show a dampened, rather than blocked, response to DR with only a marginal RLS extension seen when glucose concentration was reduced from 2% to 0.5% as opposed to a 30% increase in RLS at a glucose concentration of 0.05% (89).

Together, the sirtuins play a role in DR, but it seems unlikely that any single one of them will prove to be a master regulator of DR conserved across different species. Indeed, an increase in hepatic expression of SIRT1 was induced by DR in GHRKO mice, yet these animals did not show increased life span compared to ad libitum-fed controls, implying that an increase in SIRT1 expression in the liver alone is insufficient for life span extension (54). However, knockdown of hepatic SIRT1 via injection of small hairpin RNA impacts fasting-mediated control of glucose and cholesterol metabolism (90), indicating that SIRT1 does play a key role in the control of hepatic glucose metabolism in response to fasting and potentially to DR. The suppression of typical DR-induced behavioral changes in mice lacking SIRT1 subjected to a DR regime (70) and the fact that mice overexpressing SIRT1 phenocopy some aspects of DR (91) are encouraging, and the life span of the animals in both of these experiments should prove informative.

**Resveratrol:** a polyphenol produced by plants in response to stress

**Resveratrol.** The work in yeast suggesting that life span extension via DR was the result of increased Sir2p activity was novel as it represented an example of a positive regulator of life span and as such opened the door for searches for chemical compounds that could activate sirtuins and potentially represent pharmacological DR mimetics. A screen for sirtuin-activating compounds revealed that Resveratrol could increase the activity of the human Sir2p homologue SIRT1 in vitro (92). Resveratrol increased the RLS of yeast by 70%, and DR produced no additive RLS effect in conjunction with Resveratrol administration (92). Moreover, no effect on RLS was seen when Resveratrol was administered to *sir2*Δ mutant strains (92). In contrast,

**AMPK:** AMP-activated protein kinase

**TOR:** target of rapamycin

Resveratrol does not increase the CLS of yeast (92). Recently developed synthetic compounds, structurally similar to Resveratrol, have both increased stability and greater effects on yeast RLS (93).

The longevity effects of Resveratrol appear to be conserved to multicellular organisms. Subsequent studies reported *sir2*-dependent life span extension by Resveratrol in both *C. elegans* and *Drosophila* (94). Moreover, experiments in the Killifish *Nothobranchius furzeri*, a short-lived fish with a life span of 13 weeks, suggest that these effects are conserved to vertebrates because life span was increased by up to 56% by Resveratrol administration in this species (95), although the role of sirtuins in this effect remains unknown. In mammals, Resveratrol has also been shown to have beneficial effects on the survival of mice fed a high-calorie diet from one year of age compared to equally gluttonous mice lacking Resveratrol administration (96) and to decrease weight gain on high-calorie diets (97). High-calorie-fed mice treated with Resveratrol also phenocopy some aspects of DR such as increased locomotor activity, show reduced organ pathology relative to controls, and exhibit transcriptional profiles that resemble those of mice fed the standard rather than the high-calorie diet (96, 97). However, it is still too early to say if this treatment will increase the median or maximum life span of the high-calorie-fed animals, whether the effect is dose dependent, or what effect, if any, Resveratrol treatment will have on mice fed a standard diet.

As with the work on sirtuins, controversy exists in the field both as to the ability of Resveratrol to extend life span per se (97a) and on its specificity as an activator of sirtuins. The original experiments, reporting extension of budding yeast RLS given Resveratrol (92), were in the same PSY316 strain in which overexpression of *SIR2* did not extend life span (72). Resveratrol's ability to increase Sir2p activity in vivo was implied by reduced ERCs in cells given the compound. Increased Sir2p activity also leads to an increase in transcriptional silencing at telomeres (65), which can be assayed using a modified PSY316 strain of yeast in which the *ADE2* gene (required for adenine biosynthesis) has been translocated near a telomeric region (98). Increased Sir2p activity suppresses transcription of subtelomeric *ADE2*, which can be visualized by changes to the color of streaked colonies caused by a buildup of a red intermediate in the adenine biosynthesis pathway. Using this technique, Kaeberlein et al. (98) confirmed that *SIR2* overexpression in the PSY316 strain leads to increased telomeric silencing, implying increased Sir2p activity, but this silencing was not seen after Resveratrol treatment. In the strain PSY316, increased Sir2p activity does not increase RLS, yet Resveratrol treatment does (92, 98); however, in W303R and BY472, the opposite appears to be the case (**Table 2**). This leads to the possibility that Resveratrol administration exerts its effect on life span through a Sir2p-independent mechanism. The in vitro data suggesting Resveratrol activates SIRT1 may depend on the specific substrate used for the assay; no SIRT1 activation by Resveratrol was seen using assays in which deacetylation substrates lacked the Fluor de Lys group (98, 99), although it has been argued that this is not the case if longer peptides are used as substrates (100).

In addition to the physiological effects of Resveratrol that depend on the sirtuins (92, 97), there remain alternate mechanisms via which the drug may increase life span other than as a sirtuin-activating compound, such as its ability to act as an antioxidant (100). Moreover, Resveratrol may also mimic the effects of DR through its effects on both insulin signaling and the AMP-activated protein kinase (AMPK). Resveratrol was shown to inhibit insulin signaling in rat hepatocytes in a SIRT1-independent manner (101), and mice fed high-calorie diets supplemented with Resveratrol showed increased levels of activated AMPK (96). AMPK is a key sensor of cellular energy levels and is an upstream regulator of both the target of rapamycin (TOR) and IIS pathways under low-nutrient conditions (102). AMPK may therefore play a

role in DR as a nutrient-sensing switch (102). The potential involvement of both TOR and AMPK in DR will be discussed below. The activation of AMPK by Resveratrol in cell culture and in mice was shown to be indirect and not the result of direct activation of AMPK kinase activity in vitro (96). Furthermore, a recent study reported that Resveratrol activation of AMPK in neurons requires the upstream kinase LKB1 but is completely independent of SIRT1 (103). It remains to be seen whether another sirtuin mediates Resveratrol activation of AMPK or whether Resveratrol activation of sirtuins and AMPK are parallel and fully independent. However, increased AMPK dosage extends the life span of *C. elegans* (104) and therefore potentially represents a sirtuin-independent mechanism through which Resveratrol may increase longevity.

## Metabolic Pathway

In 1956, Harman (105) proposed a mechanism of aging that suggested senescence was the result of the buildup of damage to DNA with age due to constant bombardment by free radicals. The free radical theory remains one of the most widely accepted proximate hypotheses as to why organisms age and one that links metabolism, and therefore potentially DR, to senescence. Much work has been done to test the free radical theory (also referred to as the oxidative stress theory of aging), yet more than 50 years since Harman's original hypothesis, results are still conflicting. One key prediction of the theory is that senescence cannot be retarded without corresponding reductions to oxidative damage/stress. In support of this, oxidative damage has been shown to increase with age in different tissues and in different species (106), and long-lived flies from artificial selection experiments have increased levels of superoxide dismutase (SOD) activity, a reactive oxygen species (ROS) scavenger (107).

Although initially it was thought that DR extended life span by reducing metabolism, it is now generally accepted that the metabolic rate of DR rodents per unit mass of metabolically active tissue is not decreased compared to controls (108) and that energy expenditure may even be increased by DR (109). Similarly in *Drosophila*, measurements of both oxygen consumption and heat production in DR and control flies reveal no significant effect of DR on mass-specific metabolic rate (110). It is therefore unlikely that a slowed rate of living can account for the increased life span associated with DR in this species.

Even though it does not decrease metabolic rate, DR could increase life span by reducing oxidative damage to macromolecules, either by decreasing the production of ROS or by increasing the repair of damage (106). In keeping with this idea, DR delays the accumulation of oxidative modification to various macromolecules, including protein carbonyls, peroxidized lipids and DNA (111). Possibly causally associated with the finding of reduced oxidative damage, production of ROS has been reported as lower in mitochondria isolated from several different tissues from DR rodents than from controls (112). Repair of ROS-damaged molecular species is also increased under DR; protein turnover is higher in DR rodents (113, 114) than controls, and this may increase life span (115, 116). Lens epithelial cells from DR individuals are more resistant to hydrogen peroxide in vitro than control cells, suggesting increased antioxidant defenses in DR animals (117). However, increased ROS defense systems in DR tissue was not observed in other studies (118–121), and measurements of ROS production, measured fluorometrically as hydrogen peroxide in mitochondria isolated from *Drosophila* of different ages, did not show any significant effect of DR (122).

Mitochondria produce the majority of ROS under normal cellular conditions because 1% to 2% of the oxygen molecules consumed are converted into superoxide anions (123). Therefore, the cellular components most likely to suffer the most oxidative

**Free radicals:** highly reactive molecular species containing unpaired electrons

**SOD:** superoxide dismutase

**ROS:** reactive oxygen species

damage will be the mitochondria themselves, and free radical damage to mitochondria may be one cause of senescence (124). The mitochondrial theory of aging states that deterioration in the fidelity of the mtDNA with age leads to cellular senescence through reduced ATP production, along with associated increases in damage to cellular macromolecules from increased ROS production and eventually programmed cell death from apoptosis (124). Accumulation of ROS damage to mitochondria is also thought to be responsible for many age-related pathologies, such as arteriosclerosis, cataracts, and neoplasia (124).

***Wolbachia*:** an intracellular microbe, found in up to 30% of laboratory *Drosophila* stocks, that can affect sex ratio, cost of reproduction, and survival

Genetic disruption of mitochondrial function has been suggested to increase longevity by reducing ROS production (125). Reduced function of electron transport chain (ETC) components in *C. elegans* results in dramatic increases in life span (126–128), and mutations in *clk-1*, which encodes an enzyme required for synthesis of ubiquinone, has been suggested to mimic DR because of the lack of additive life span extension in *eat-2 II; clk-1 III* double mutants compared to either single mutant (42). Similarly, heterozygous mutations in *Indy* (I'm not dead yet), which is 50% similar to mammalian renal sodium dicarboxylate cotransporters that act to take Krebs cycle intermediates into cells, were reported to double the median life span of *Drosophila* and induce a DR-like state (129). However, this link between disrupted ETC and DR in fruit flies is unconfirmed. The original finding of extreme longevity of *Drosophila Indy* mutants has recently been called into question by a report showing that when these flies are sufficiently outcrossed to remove background effects and infection by the cytoplasmic bacteria *Wolbachia*, the life span extension originally seen is completely diminished (130).

There are no conclusive data showing that the life span extension seen in worms with disruptions to the ETC is mechanistically similar to DR-induced longevity. In contrast, strong evidence suggests that they are separate pathways because the temporal requirements of the two interventions are strikingly different. RNAi of ETC components in the worm only extends life span if applied during development, with no effect seen when it is only applied in adulthood (126). Furthermore, by subjecting worms first to RNAi for components of the ETC from hatching and then to RNAi for *Dicer* (which is required for RNAi activity) when they reached adulthood, it is possible to limit the time period during which the ETC RNAi is effective to strictly during development and still generate the full longevity extension observed with continual inactivation during the animal's entire life cycle (126). These results are in stark contrast to DR, which still extends life span when applied late during adulthood in worms (9), flies (43), and mice (44).

Although it remains unproven if reduced ROS production and ROS damage is the causal mechanism behind the life span extension seen in DR animals, increases in ROS scavenger levels have been reported to increase the life span of worms (131), flies (132), and mice (133, 134). Furthermore, DR-induced changes to expression patterns of SODs in *C. elegans* are blocked in mutants that block longevity extension under food restriction (22). Although it would be compelling to inactivate the entire *sod* gene family to determine their role in DR, it is entirely possible that toxicity resulting from other forms of ROS (peroxidized lipids, for example) is at the heart of DR-induced longevity. Detailed analysis of the different types of ROS affected by DR in conjunction with gene family knockout studies in a readily tractable genetic model organism should prove informative.

## TOR/AMPK Pathway

Recently, focus has turned toward the amino acid-sensing TOR pathway as another candidate mechanism through which DR may act. TOR is a conserved serine/threonine protein kinase with at least one orthologue present in every eukaryote genome examined to date (135). TOR controls and regulates

protein synthesis and growth in response to nutrient intake. In mammalian cells, TOR is found within either a rapamycin-sensitive or -insensitive complex, both of which regulate distinct downstream targets and contain TOR bound to the coregulators LST8 and either Raptor or Rictor, respectively (135–137) (**Figure 2**). Reduction of amino acid intake is sufficient to extend life span in yeast (138), *Drosophila* (139), and rodents (140, 141), and similarly, genetically reduced TOR signaling has been shown to extend life span in yeast (138, 142), *C. elegans* (143, 144), and *Drosophila* (145). Furthermore, worms heterozygous for mutations in *daf-15*, the *C. elegans* orthologue of *Raptor*, are long-lived (146), and suppression of downstream targets of TOR that regulate translation also increases life span (80, 147). Reducing *dTor* expression specifically in the fat body in *Drosophila* acts non-cell autonomously to regulate growth in other tissues (148). Therefore, TOR signaling in specific tissues may represent a mechanism whereby altered nutritional intake could result in organism-wide changes in aging profiles.

Although TOR signaling likely plays a role in the life span extension seen under DR, this remains to be proven empirically. In an apparent confliction of results, reduced TOR signaling in *C. elegans* has been shown recently to extend (144) and to have no effect on the life span of *eat-2* mutants (80). However, the reduced food intake induced by *eat* mutation may not be the optimal level of DR that maximizes life span. Therefore, no conclusion can be made about whether the TOR pathway lies inside or outside of the DR pathway by further extension of *eat-2* mutant life span by TOR RNAi (**Figure 3**). Even if reduced TOR function does indeed genetically mimic DR, TOR RNAi could decrease, have no effect on or even increase the life span of an *eat-2* mutant depending on where those *eat-2* mutant worms are physiologically on the DR spectrum (**Figure 3**), and epistasis analysis with *eat-2* mutations alone is uninformative.

It is worth noting that, although mutants in the worm TOR orthologue *let-363* have been reported to extend life span (143), these worms are paralyzed and never reach adulthood, arresting at the larval stage three; therefore, the relevance of these data to the regulation of normal organismal aging requires further work. Also, *let-363* resides in an operon on chromosome 1 along with two other genes, one encoding a transcription initiation factor and the other a mitochondrial ribosomal subunit (149). RNA inhibition of genes within operons can (but does not always) result in downregulation of both the target sequence and the downstream genes in the operon (150). Disrupting mitochondrial function can extend life span in *C. elegans* (126), and indeed, RNAi of the mitochondrial ribosomal subunit encoding sequence (B0261.4) within the *let-363* operon has been reported to extend life span in a *daf-16*-independent manner (151). It is therefore difficult to determine if the life span extension seen using RNAi of *let-363* was caused by reduced TOR signaling, silencing of the other genes within that operon, or a combination of the two. Until this issue is resolved, any experiments involving *let-363* RNAi should be treated as preliminary.

A more conclusive experiment to test TOR's role in the DR pathway in *C. elegans* would be to examine the effect of reduced TOR flux across a range of bacterial food concentration levels (**Figure 1**). If long-lived TOR mutants are partially dietarily restricted by their genotype, the food concentration that maximizes their life span will be more concentrated than that of wild-type animals, yet the maximum life span achievable through reduced TOR flux and optimal DR in wild-type worms will be the same (**Figure 1*b***). However, if TOR signaling and DR function in separate pathways, TOR mutants will be longer lived at all food levels, similar to insulin-signaling mutants (8) (**Figure 1*d*** ). Indeed, this method is the most conclusive way to determine any intervention's interaction with the DR pathway (**Figure 1**). This technique was used for

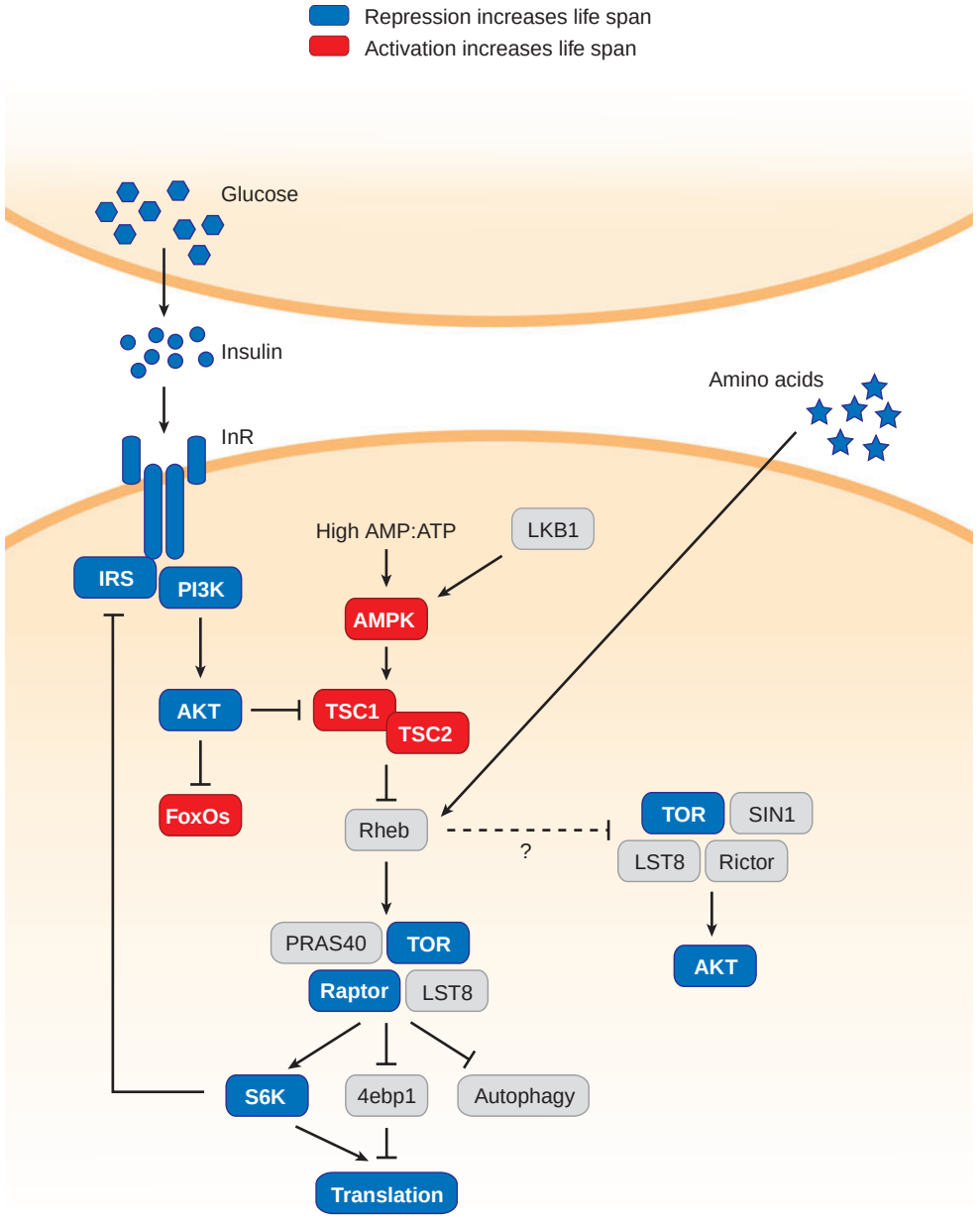


**Figure 2**

Life span extension by disruption of nutrient signaling components. This figure represents a schematic of the mammalian AMP-activated protein kinase (AMPK)/target of rapamycin (TOR) nutrient-sensing pathway. Blue coloration indicates components of the pathway whose reduction/downregulation of the orthologue in either *C. elegans* or *D. melanogaster* results in life span extension. Red coloration indicates components of the pathway whose overexpression/activation of the orthologue in either *C. elegans* or *D. melanogaster* results in life span extension. Gray coloration indicates either no change in life span or untested.

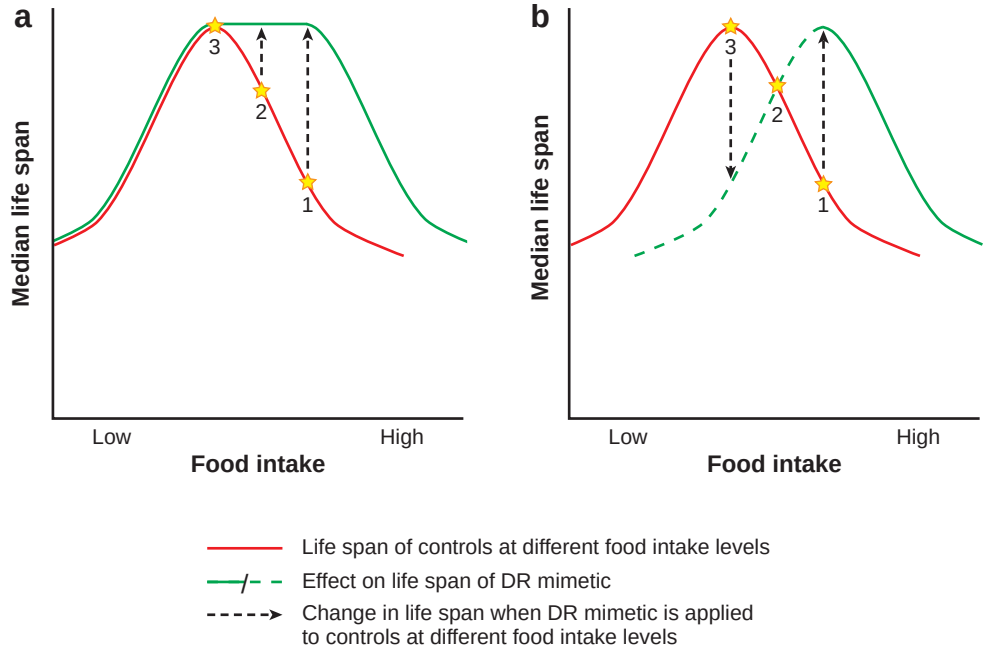


**Figure 3**

Potential pitfalls of epistasis analysis with nonoptimized dietary restriction (DR). Median life span (y-axis) increases as food intake (x-axis) is reduced from ad libitum levels (high food intake) until a nutrient regime that optimizes longevity is reached, past which further restriction results in reduced life span due to starvation (low food intake). Epistasis analysis of any intervention in combination with DR at one nutritional intake level is uninformative, as it can result in either life span extension, no change in life span or life span reduction depending on where on the x-axis that nutritional intake resides. Unless applied to animals on a dietary regime that already optimizes life span, an intervention functioning in the same pathway as DR can still result in life span extension. (*a*) If the intervention extends life span by upregulation of the same longevity assurance processes involved in the response to DR, epistasis analysis can result in life span extension if applied in conjunction with a food intake level that does not maximize life span (positions 1 and 2 on panel 3*a*), but no life span extension at the nutritional intake that maximizes life span of controls (position 3 on panel 3*a*). (*b*) If an intervention mimics DR by reducing nutrient uptake or cellular energy levels, it can increase life span in ad libitum-fed animals (position 1 on panel 3*b*), cause no additive effect at suboptimal DR (position 2 on panel 3*b*), and reduce life span by inducing starvation at the food level that optimizes life span of controls (position 3 on panel 3*a*).

studying the life span of a *Drosophila* strain with reduced TOR activity, and indeed, the maximum life span extension of the reduced TOR animals was greatest at the highest food levels, as would be predicted if this intervention mimics DR (145).

AMPK measures the energy status of cells and functions as a nutrient-sensing switch (102), and it has also been suggested as having a key role in DR. Under low-energy conditions (high AMP:ATP ratio), AMPK is activated and inhibits energy-consuming processes, such as protein synthesis, through the inhibition of TOR signaling (152, 153) (**Figure 2**). AMPK modulates a number of critical metabolic functions in mammals, acting in the hypothalamus to promote feeding rates (154) and regulating glucose homeostasis in the liver via deactivation of the cAMP-responsive element binding protein (CREB) regulated transcription coactivator 2 (CRTC2—also known as TORC2) (155, 156). AMPK phosphorylates CRTC2 and prevents it from shuttling to the nucleus

where it interacts with CREB to increase gluconeogensis (155). Similar to TOR, AMPK plays a role in aging, stress resistance, and tumorigenesis. Life span extension via reduced IIS is largely abolished in worms mutant for the AMPK α-subunit (*aak-2*), and overexpression of *aak-2* increases longevity in *C. elegans* (104).

Both decreased AMPK and increased TOR have been implicated in tumorigenesis. Tuberous sclerosis, a genetic disorder resulting from mutations in either of the TSC genes, results in hyperactivation of TOR (157), whereas another genetically inherited cancer predisposition syndrome, Peutz-Jeghers, is caused by inactivation of the tumor suppressor gene *LKB1*, an activator of AMPK (158). Adenoviruses and some tumor cell lines drive growth under nutrient-limited conditions by deregulation of nutrient-sensing pathways and, in particular, activation of TOR (158). One mechanism via which DR animals have reduced tumorigenesis may be reduced TOR activity. Indeed, rapamycin, which inhibits specific complexes of TOR, is in widespread clinical trials as an anticancer therapy, particularly in the treatment of epithelial carcinomas and hamartomas (159).

## Nutrient Sensing

If the longevity increase observed in animals subjected to DR is an active and adaptive response to altered food availability, the ability to rapidly sense changes in nutritional state is likely key to an organism's ability to respond to DR. Changes in nutrient status can be detected by changes to energy levels measured at the cellular level, for example by AMPK, which is activated when energy, determined by the AMP:ATP ratio, is low. Along with the downstream effects discussed in the previous section, constitutively active AMPK also results in behavioral changes such as increased feeding (154), and interestingly, mice under a DR regime show changes in feeding patterns and increased foraging behavior (2). However, chemosensory nutrient-sensing pathways act upstream of sensors of cellular energy levels in allowing an organism to detect and respond to changes in food availability. Worms in which either gustatory or olfactory neurons have been removed via laser ablation are long-lived (160, 161). However, the life span extension observed under these conditions is dependent upon *daf-16*, suggesting that nutrient sensing by these neurons is not required for the response to DR in *C. elegans*. However, flies exposed to the odor of nutrient-rich food but fed a restricted diet do not show the full life span extension of controls on DR, indicating that sensory perception may play as much of a role in DR-mediated longevity as changes to food ingestion in this species (162).

## Pha-4/Skn-1

Within the last 15 years, the field of aging research has witnessed an explosion of mechanistic-based experiments aimed at understanding the potential regulation of longevity and survival in whole animals. This increase is due in part to the identification of DAF-16 as a key transcription factor required for the extreme life span observed in worms with reduced insulin/IGF-1 signaling (31). The ability to increase life span by reducing IIS, and concomitantly suppress this extension by removing DAF-16 activity, has allowed researchers to accurately define several of the key networks required for IIS-mediated longevity extension. Life span extension by DR has been shown by multiple labs to not be dependent upon DAF-16 (8, 20, 22, 42, 46a), and studies of increased longevity via DR have been lacking such an internal control necessary and essential to deduce causal versus casual relationships between DR-induced phenotypes and longevity. Indeed, the idea that the longevity effects of DR would be exclusively dependent on any single protein seemed unlikely given the large number of nutrient signaling/sensing pathways impacted by wholesale changes to food availability. Remarkably, however, recent reports have identified two such transcription

factors, PHA-4 and SKN-1, found essential for and specific to DR-induced longevity extension in the worm (20, 22). These findings have provided the field with a new set of tools to decipher the causes of increased longevity via DR, described in the following paragraphs.

**Pha-4.** *pha-4* was originally identified because of its role in early pharyngeal development of the worm (163). Cloning of the gene by complementation revealed that *pha-4* was the single-worm orthologue of the mammalian Foxa family of forkhead transcription factors (Foxa 1, 2, and 3) (163). *pha-4* is essential for endodermal specification of pharynx and intestinal cells of the worm (163), and in mice, the Foxa family is required for endodermal specification of the foregut during development (164). Mice lacking Foxa family members die shortly after birth, are hypoglycemic, and have reduced glucagon expression from within pancreatic beta cells (164). The role of the Foxa proteins during adulthood has been characterized by utilization of loxP-flanked Foxa sequences in combination with tissue-specific Cre recombinase expression (164). This system facilitates tissue-specific knockout of the individual Foxa proteins via recombination at the loxP sites. Pancreatic-specific deletion of Foxa2 results in islets that do not secrete insulin in response to elevated glucose yet inappropriately secrete it in response to amino acids (164). Hepatic-specific deletion of Foxa2 affects glucose homeostasis by impaired activation of enzymes involved in gluconeogenesis, and both Foxa1 and Foxa2 are involved in regulating the expression of proglucagon (164). Therefore, the Foxa family of proteins is bifunctional, having an early developmental function but also a later role in maintaining metabolic homeostasis.

The role for PHA-4 in DR-mediated longevity in *C. elegans* was revealed by an RNAi-based screen testing all of the 15 forkhead transcription factors in the worm for their role in DR, motivated by the fact that deletion of just one forkhead factor encoding gene (*daf-16*) could entirely block the longevity effects of reduced IIS (31). Furthermore, the DAF-16 coregulator, SMK-1, was found essential for DR in a DAF-16-independent manner, suggesting that SMK-1 might interact with a different forkhead related transcription factor to mediate the response to DR (22).The forkhead DR screen revealed that RNAi toward a single forkhead, *pha-4*, can suppress the extended longevity of *eat-2* mutant animals (22). Additionally, loss of PHA-4 blocks the extended longevity of animals subjected to DR by direct reduction of food intake via bacterial dilution (22). Loss of PHA-4 does not affect the long life spans of either *daf-2* mutant animals or animals with reduced mitochondrial function, and therefore its role in life span extension is specific to the DR pathway (22). Because PHA-4 is essential for early pharynx development, it was imperative to determine whether the developmental function of PHA-4 was separable from its potential role during adulthood in modulating the response to DR. PHA-4 levels can be reduced specifically during adulthood either by using a temperature-sensitive mutant allele of *pha-4* or conditional RNAi. Crucially, such adult-specific reduction of *pha-4* expression, after development has completed, still blocks the response to DR (22). Therefore, like the Foxa family members, PHA-4 is also bifunctional, having an early role in development and an adult-specific role in life span extension by DR, which may be linked to the Foxa proteins' role in metabolic homeostasis in rodents.

PHA-4 shares overlapping DNA-binding sites with DAF-16 (165, 166) and may therefore regulate the same sets of longevity assurance genes. Analysis of one gene family involved in stress regulation and longevity, the *sod* gene family, suggests this is partially correct because *sod-1* and *sod-5* are both regulated by DAF-16 and PHA-4 in response to reduced IIS and DR, respectively (22). However, differential regulation by these two transcription factors also exists, as *sod-3* is specifically regulated by DAF-16 in response to reduced IIS, and both *sod-2* and *sod-4* are

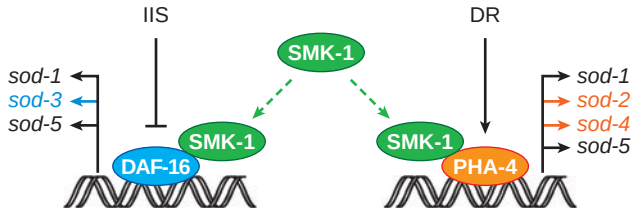


**Figure 4**

Forkhead box type O (FoxO) and Forkhead box type A (Foxa) are essential for insulin/IGF-1- and diet restriction-dependent longevity, respectively, in *C. elegans*. In response to reduced insulin/IGF-1 signaling (IIS), the transciption factor DAF-16/FoxO translocates to the nucleus to regulate expression of key stress response genes. DAF-16 regulates a subset of the *sod* gene family, with *sod-3* expressed specifically in response to reduced IIS. In response to diet restriction, a separate forkhead transcription factor, PHA-4/Foxa, regulates an overlapping, yet distinct, set of genes including members of the *sod* gene family. *sod-2* and *sod-4* are regulated specifically in response to dietary restriction (DR) by PHA-4. SMK-1, the worm orthalogue of the mammalian SMEK1 (suppressor of MEK null) is essential for increased longevity by both IIS and DR in the worm and may function as a transcriptional coregulator to mediate transcriptional activity of the forkhead proteins (22). Arrows indicate activation; solid bars represent repression. The signaling mechanism upstream of DAF-16 is the conserved insulin/IGF-1 pathway; the signaling mechanism upstream of PHA-4 is still unknown.

specifically regulated by PHA-4 in response to DR (**Figure 4**) (22). In the future, it will be imperative to assess the effect PHA-4 has on whole genomic transcriptional changes induced by DR and to determine the extent to which these changes overlap with those regulated by DAF-16.

It is not yet clear how PHA-4 activity is regulated in response to DR. PHA-4 is constitutively nuclear under all dietary conditions, however, ruling out nuclear/cytoplasmic trafficking (22). Interestingly, transcriptional levels of *pha-4* increase in response to DR, albeit only twofold, suggesting that tight regulation of *pha-4* expression may be essential for the DR response. This is also true for the regulation of the developmental role of *pha-4* since its expression increases twofold during pharyngeal development (166). It may be that protein turnover of PHA-4 also plays a role in its regulation during DR, although this has yet to be established. It is also not yet clear in which cells and tissues PHA-4 is required to regulate the response to DR. *pha-4* is predominantly expressed in the intestine but is also expressed in many cells of the head and tail (several of these are neuronal), and it may be that PHA-4 acts non-cell autonomously to regulate life span in response to changes in nutritional intake.

**SKN-1.** SKN-1 is the worm orthologue of NF-E2-related transcription factors, Nrf1 and Nrf2 (167). In mammals, the Nrf proteins induce expression of Phase II detoxification enzymes, such as *gcs* (glutamyl-cysteine synthetase) in the liver and digestive tract in response to oxidative stress and xenobiotics (168–170). Nrf proteins are also required early in development for mesendodermal differentiation. SKN-1, like the Nrf proteins, also appears bifunctional, having an early developmental role in endodermal and mesendodermal cell fate specification and a later role during adulthood involving resistance to oxidative damage (171, 172). *skn-1* is primarily expressed in the intestinal cells and two neurons (ASI) in the head of *C. elegans*. ASI neurons are essential for dauer entry and nutrient sensing (173). Interestingly, SKN-1 is constitutively nuclear in ASI neurons, but shuttles to the nucleus in intestinal cells under heat and oxidative stress conditions. The nuclear/cytoplasmic movement of SKN-1 in the intestine is dependent upon the p38 MAPK signaling pathway (171, 174).

The role of SKN-1 in oxidative stress management in animals, ranging from worms to mammals, suggested that it could be a key regulator of the response to DR, and indeed, strains harboring genetic knockouts of *skn-1* are unable to respond to DR but do have increased longevity if insulin/IGF-1 signaling is reduced (20). Interestingly, *skn-1* exists as several distinct isoforms (denoted *skn-1*a, b, and c) that are expressed in distinct cell and tissue types in worms. *skn-1b* is expressed in the ASI neurons, whereas *skn-1c* is expressed in the intestinal cells of *C. elegans* (20). In response to DR, SKN-1b levels increase in the ASI neurons, and transgenic expression of *skn-1b* (but not the other isoforms) is sufficent to

restore the ability of *skn-1* mutants to respond to DR. Furthermore, laser ablation of the ASI neurons also blocks the response to DR. It is not clear if *skn-1c* shuttles between the cytoplasm and nucleus of intestinal cells in response to DR or whether Phase II detoxification enzymes are induced in response to DR, but it is clear that restoration of *skn-1b* within the ASI neurons is essential for life span extension by DR (20). Interestingly, *skn-1* appears to establish a metabolic regulatory switch, as mitochondrial function is altered in response to DR, and this too is dependent upon *skn-1b*, specifically in the ASI neurons, but does not require *skn-1* function in the intestine (20).

Both PHA-4 and SKN-1 are needed during early development for pharyngeal and gut development, suggesting that their role in DR could be very indirect. However, loss of either *pha-4* or *skn-1* does not block the long life span of insulin/IGF-1 signaling mutants as would be expected if these mutants were under perpetual starvation due to defects in nutrient absorption through the gut. Furthermore, at least for *pha-4*, conditional gene inactivation during adulthood blocks the response to DR, and loss of *pha-4* does not block the long life span of animals with reduced mitochondrial ETC function (22). Therefore, it appears that SKN-1 and PHA-4 are indeed bifunctional, having evolved early developmental roles in gut development and later roles in sensing nutrient uptake. This is not too surprising given the early developmental role of DAF-16 and its later role during adulthood in IIS-mediated increases in longevity and stress resistance (45).

Although *pha-4* and *skn-1b* have established the ability to dissect the response to DR in the absence of IIS signaling, it is not clear how these factors are regulated in response to low nutrient uptake. It is intriguing to speculate that another key nutrient-sensing pathway, the amino acid and energy sensing AMPK/TOR signaling pathway, may be upstream of *pha-4/skn-1b*. In the future, it will be imperative to determine if life span extension via alterations to TOR/AMPK signaling is dependent upon either PHA-4/Foxa or SKN-1b/NF-E2.

## CONCLUSIONS

The original reports on life span extension via DR date back to the first half of the last century, and today, some 70 years later, we have yet to identify the downstream genetic pathways responsible for the effect. However, as discussed above, it seems no coincidence that lowering the flux through key nutrient signaling pathways extends longevity even in the presence of ample food, and much progress has been made in the past 15 years in investigating these effects. Direct reduction of food intake induces a host of physiological changes in an organism and is likely to impact on many nutrient signaling/sensing pathways, being neither independent of, nor as we have discussed above, exclusively dependent on any single one of them. The recent discovery of transcription factors whose activity is required for a DR response is of great interest and suggests that, under DR conditions, these upstream nutrient signaling pathways may converge on the same set of longevity assurance genes that are under tight transcriptional regulation. As yet, there is no evidence that these transcription factors play the same role in higher organisms that they do in *C. elegans*, but if this proves to be the case, the potential DR research has for the treatment of age-related pathology is manifold.

The advantage DR has over direct manipulations of genetic pathways, such as insulin signaling, is that it represents an environmental intervention to which organisms have been subjected and to which they have adapted a response to throughout evolution. Reducing insulin signaling in a nematode may increase longevity without any obvious detrimental effects in the laboratory, but in more natural conditions, these worms are less fit than wild types (175, 176) and would constitutively enter dauer arrest. Similar manipulations of insulin signaling in humans may

well result in severe detrimental side effects, such as diabetes, that would outweigh any potential benefits. If the response to DR is an adaptive strategy, the discovery of key downstream targets that regulate it may allow pharmacological interventions that improve human health by switching on mechanisms already within us, which originally evolved to increase longevity and disease resistance during periods of famine.

## SUMMARY POINTS

1. Dietary restriction (DR) extends the life span of a range of species, but the genetic pathways that regulate this response have yet to be identified and need not necessarily be conserved between them.
2. Elucidating the genetic pathways responsible for the DR effect in a range of species will allow us to determine the extent to which pharmacological DR mimetics will improve human health.
3. Unless the degree of DR that maximizes life span is first empirically determined, epistatic analysis of DR and any further intervention will become difficult to interpret.
4. In *C. elegans*, life span extension by reduced IIS is dependent upon *daf-16*, yet DR can extend the life span of *daf-16* null worms and is therefore not functioning exclusively through insulin signaling.
5. Overexpression of *SIR2* homologues extends life span in yeast, *C. elegans*, and *Drosophila*, but sir2p's role as a master regulator of DR in yeast is specific to particular strains and DR longevity assays.
6. Reduced nutrient intake reduces TOR signaling and increases AMPK activity, both of which increase the life span of model organisms, making them strong candidates for pathways involved in the DR response.
7. Although experimental alterations of many key nutrient signaling and energy sensing pathways/molecules have been shown to extend longevity in model organisms, DR has not yet been proven to function exclusively through any of them.
8. The recent discovery of transcription factors whose deletion blocks life span extension by DR in *C. elegans* may if conserved to other species provide powerful tools to further untangle the mechanisms by which DR improves health and increases longevity.

## FUTURE ISSUES

1. Further experimental analysis is needed of the requirement of sir2p homologues for life span extension by DR in *C. elegans*, *Drosophila*, and rodents using separate cohorts subjected to different degrees of DR.
2. The dependency of life span extension by reduced IIS or DR on members of the FoxO transcription family members in *Drosophila* and mammals should be investigated.
3. Evaluation of the conservation of the roles of *skn-1* and *pha-4* orthologues in life span extension by DR in other species is needed.

## DISCLOSURE STATEMENT

The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

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**Errata**

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