

Converging Pathways in Lifespan Regulation Review

Sri Devi Narasimhan^{1,3}, Kelvin Yen^{1,3},
and Heidi A. Tissenbaum^{1,2,*}

The processes that determine an organism's lifespan are complex and poorly understood. Yet single gene manipulations and environmental interventions can substantially delay age-related morbidity. In this review, we focus on the two most potent modulators of longevity: insulin/insulin-like growth factor 1 (IGF-1) signaling and dietary restriction. The remarkable molecular conservation of the components associated with insulin/IGF-1 signaling and dietary restriction allow us to understand longevity from a multi-species perspective. We summarize the most recent findings on insulin/IGF-1 signaling and examine the proteins and pathways that reveal a more genetic basis for dietary restriction. Although insulin/IGF-1 signaling and dietary restriction pathways are currently viewed as being independent, we suggest that these two pathways are more intricately connected than previously appreciated. We highlight that numerous interactions between these two pathways can occur at multiple levels. Ultimately, both the insulin/IGF-1 pathway and the pathway that mediates the effects of dietary restriction have evolved to respond to the nutritional status of an organism, which in turn affects its lifespan.

Introduction

Aging is a nearly universal process that involves the progressive deterioration of metabolic, muscular, reproductive, and cognitive functions, which ultimately affects lifespan [1]. In addition, in humans, the onset of several debilitating diseases that could potentially reduce overall lifespan — such as type II diabetes, cancer, and heart disease — is associated with the aging process. A complex array of genetic and environmental factors plays an important role in modulating lifespan. Studies in the past two decades have focused on elucidating the genetic determinants of longevity, using approaches such as genome-wide RNA interference (RNAi) and mutagenesis screens, microarrays, and protein arrays [2–5]. This work has led to the identification of several important genes across phylogeny that are involved in diverse cellular processes, including development, mitochondrial function, energy metabolism, protein translation, and the cell cycle.

The insulin/IGF-1 signaling pathway and modulation of food intake by dietary restriction have the most robust effects on lifespan across species. Insulin/IGF-1 signaling is a well-conserved and well-defined pathway that has been shown to regulate longevity in the nematode *Caenorhabditis elegans*, the fruitfly *Drosophila melanogaster*, and in several rodent models [6]. In contrast, dietary restriction was long thought of as an extrinsic intervention manifesting in

physiological changes that ultimately enhanced lifespan. Dietary restriction, where the actual number of calories ingested by the animal is unknown, differs from caloric restriction, where the exact number of calories consumed is known and the food is also supplemented with extra nutrients such as vitamins and minerals to prevent malnutrition. Recent studies have identified several genes that are necessary for lifespan extension mediated by dietary restriction, suggesting that, like insulin/IGF-1 signaling, the longevity-inducing effects of dietary restriction may be regulated by well-defined pathways [7–9].

In this review, we examine the insulin/IGF-1 signaling pathway and genetic components that are required for increased lifespan in response to dietary restriction. It is not entirely clear whether insulin/IGF-1 signaling and dietary restriction are independent, given that they are both major regulators of lifespan across phylogeny. We discuss the potential cross-talk between these pathways at multiple levels, and we suggest that the preferred pathway to ultimately control longevity may depend upon the nutritional status of the organism. The role of specific cell types and tissues as well as the spatio-temporal expression of genes involved in insulin/IGF-1 signaling and dietary restriction are undoubtedly important for understanding the regulation of lifespan. We refer the reader to some excellent reviews that discuss this aspect further [4,10].

Insulin/IGF-1 Signaling

Initial studies in *C. elegans* identified single gene mutations that resulted in a profound extension in lifespan [2,6]. Further genetic epistasis analysis showed that these genes, *age-1* and *daf-2*, were part of the same genetic pathway [2,6,11,12]. Subsequent cloning studies identified the *daf-2* gene as being equally homologous to both the mammalian insulin and the mammalian IGF-1 receptors and *age-1* as being homologous to the worm phosphoinositide (PI) 3-kinase catalytic subunit, thus revealing a *C. elegans* insulin/IGF-1 signaling pathway [2,6]. Since then the insulin/IGF-1 signaling pathway has emerged as the most well-characterized regulator of longevity across species. Indeed, no pathway has been identified with a more pronounced effect on longevity [2,6,13]. Furthermore, the components of insulin/IGF-1 signaling show remarkable molecular conservation from worms to humans (Table 1) and several studies have found a correlation between naturally occurring polymorphisms in this pathway and human longevity [6,14–16].

Insulin-Like Peptides and the Insulin/IGF-1 Signaling Pathway

At the cellular level, it is well-established that changes in blood glucose levels are the main trigger for insulin secretion in mammals. Similarly, in the context of the macroenvironment, organisms constantly sense their surroundings. In *C. elegans*, depending upon the availability of nutrients, several olfactory and chemosensory neurons are thought to regulate the secretion of insulin-like peptides through cyclic GMP and G-protein-coupled receptor signaling pathways [3,13]. Ablation of specific gustatory and olfactory neurons results in increased lifespan, consistent with a role for insulin/IGF-1 signaling in antagonizing longevity [4].

¹Program in Gene Function and Expression and ²Program in Molecular Medicine, University of Massachusetts Medical School, 364 Plantation Street, Worcester, MA 01605, USA.

*E-mail: heidi.tissenbaum@umassmed.edu

³These authors contributed equally.

Table 1. Modulators of insulin/IGF-1 signaling and dietary restriction.

Yeast	Worms	Flies	Mammals
-	INS-1-39 ¹ , DAF-28	DILP-1– DILP-7	Insulin and insulin-like growth factor (IGF-1)
-	DAF-2 ↑	dINR ↑	Insulin receptor (IR)/insulin-like growth factor-1 receptor (IGF-1R) ↑
-	IST-1	CHICO ↑	Insulin-receptor substrate (IRS) ↑
-	AGE-1 ↑	Dp110	Phosphoinositide 3-kinase catalytic subunit (p110)
-	AAP-1 ↑	p60	Phosphoinositide 3-kinase adaptor subunit (p55)
TEP1	DAF-18 ↓	dPTEN ↓	Phosphatase and tensin homolog (PTEN)
PKH1/PKH2	PDK-1 ↑	Pk61	Phosphoinositide-dependent kinase-1 (PDK-1)
SCH9 ↑	AKT-1/AKT-2 ↑	dAKT	Protein kinase B/AKT
YPK1/YKR2	SGK-1 ² ↑↓	-	Serum and glucocorticoid kinase (SGK)
RTS1	PPTR-1	Widerborst	Protein phosphatase 2A regulatory subunit B56β
FKH1/FKH2/FHL1/HCM1	DAF-16 ↓	dFOXO	Forkhead box O (FOXO) FOXO1, FOXO3a, FOXO4, FOXO6
TOR1 ↑	LET-363 ↑	TOR ↑	Target of rapamycin (TOR)
SCH9 ↑	RSKS-1 ↑	dS6K ↑	Ribosomal S6 kinase (S6K)
KOG1P	DAF-15 ↑	Raptor	RAPTOR (regulatory associated protein of TOR)
AVO3	RICT-1 ³ ↑	Rictor	RICTOR (rapamycin-insensitive component of TOR)
HSF	HSF-1	HSF1-4	Heat shock factor (HSF)
KEL1/KEL2/KEL3	HCF-1 ↑	dHCF	Host cell factor (HCF)
SNF1	AAK-2 ↓	SNF1A	AMP-dependent protein kinase (AMPK)
PSY2	SMK-1 ↓	Falafel	Suppressor of Mek-1 (SMEK-1)
SPS1/STE20	CST-1 ↓	Hippo	Mammalian Ste20-like kinase-1 (MST-1)
-	BAR-1 ↓	Armadillo	β-catenin
SIR2	SIR-2.1	SIR2	Sirtuin1–7 (SIRT)
HOG1	JNK-1 ↓	Basket	c-Jun N-terminal kinase (JNK)
-	SKN-1 ↓	Nrf-2	NF-E2-related factor (Nrf-2)
-	-	Keap-1 ↑	Kelch-like ECH-associated protein 1 (KEAP-1)
FKH1/FKH2/FHL1/HCM1	PHA-4 ↓	Fkh	Forkhead transcription factor box A (Foxa)
COQ7	CLK-1 ↑	dCLK-1	Clock-1 (mCLK-1) ↑

Arrows indicate the effect of mutations or RNAi on organismal lifespan in wild-type animals: upwards arrow, increased lifespan; downwards arrow, decreased lifespan. ¹Not all *ins* genes have been characterized: *ins-1* mutants are short-lived, *ins-7* and *daf-28* mutants show an extension in lifespan and *ins-18* RNAi/mutants show both phenotypes. ²*sgk-1* lifespan data are currently conflicting. ³*ric1-1* mutants show increased lifespan on HB101 bacteria but not OP50 bacteria.

Similarly, studies in flies show that ablation of neurosecretory cells expressing *Drosophila insulin-like peptide* (*dilp*) results in a 10–33% increase in median lifespan [10]. The *C. elegans* genome has an astounding 40 insulin-like (*ins*) genes [17,18] (<http://www.wormbase.org/WS198>), and *Drosophila* has 7 insulin-like peptides (*dilp*) [10]. Why lower organisms appear to have such an expanded family of insulins in comparison to mammals is still not entirely understood.

Although the precise ligand that binds to DAF-2 is still unknown, studies on insulins in *C. elegans* revealed that potential ligands may function either as antagonists (*ins-1*), agonists (*ins-7*, *daf-28*), or both (*ins-18*) [19]. While most studies on insulins in *C. elegans* have been genetic analyses, one study reported biochemical verification of INS-6 binding to the human insulin receptor [20]. The *in vivo* or *in vitro* binding of *C. elegans* insulins to DAF-2 therefore has yet to be validated. Studies in *Drosophila* have characterized *dilp2*, *dilp3* and *dilp5* as important regulators of growth and energy metabolism [10]. Many of these insulins in both worms and flies are expressed in distinct tissues, such as the intestine and muscle, and thus in simpler organisms these ligands may concertedly regulate a neuroendocrine signaling axis modulating development, metabolism, and longevity.

Insulin/IGF-1 Receptor

Although *C. elegans* and *Drosophila* contain a multitude of potential ligands, only a single receptor that bears homology to the insulin and the IGF-1 receptors has been identified in both organisms: DAF-2 in *C. elegans* and insulin/IGF-1 receptor (*dlnR*) in *Drosophila* [10,19]. Reduction-of-function mutations in *daf-2* result in lifespan extension ranging from

60–100%, indicating that under normal signaling conditions, insulin/IGF-1 signaling promotes growth and development while antagonizing longevity [21,22]. *dlnR* homozygous mutant flies are not viable; however, heteroallelic female flies live up to 85% longer than their wild-type counterparts [6,23]. Downstream of *dlnR*, mutations in the fly homolog of the insulin-receptor substrate (IRS) *chico* also extend lifespan up to 48% [10].

In mammals, although the insulin and IGF-1 receptors share high homology, they modulate distinct processes, such as metabolism and growth, respectively. Deregulation of insulin signaling in humans leads to the onset of age-associated debilitating diseases, for example type 2 diabetes and cancer. Insulin-receptor knockout mice have a drastically shortened lifespan due to ketoacidosis [24]. Studies using tissue-specific insulin-receptor knockout mice reveal a more complex picture, however; fat-specific insulin receptor knockout mice not only live almost 20% longer than control littermates, but also are leaner, have increased insulin sensitivity, and express normal IGF-1 levels [24]. Downstream of the receptor, there is conflicting evidence about whether mice heterozygous for brain-specific IRS2 have an increased lifespan (reviewed in [10]).

Several mammalian studies have shown that alterations to the IGF-1–growth hormone (GH) axis can increase longevity [6,24]. Mice bearing a mutation in the gene encoding GH and mice with mutation in either *Prop-1* or *Pit-1* (transcription factors involved in pituitary development) show enhanced longevity compared with wild-type littermates [6]. These long-lived mice lack several hormones, including prolactin and thyroid-stimulating hormone, and have diminished levels of GH and neuropeptide Y [24]. Furthermore, mice lacking GH receptor, GH-receptor-binding protein, or GH-releasing

Figure 1. Interactions between components of the insulin/IGF signaling and the TOR pathways.

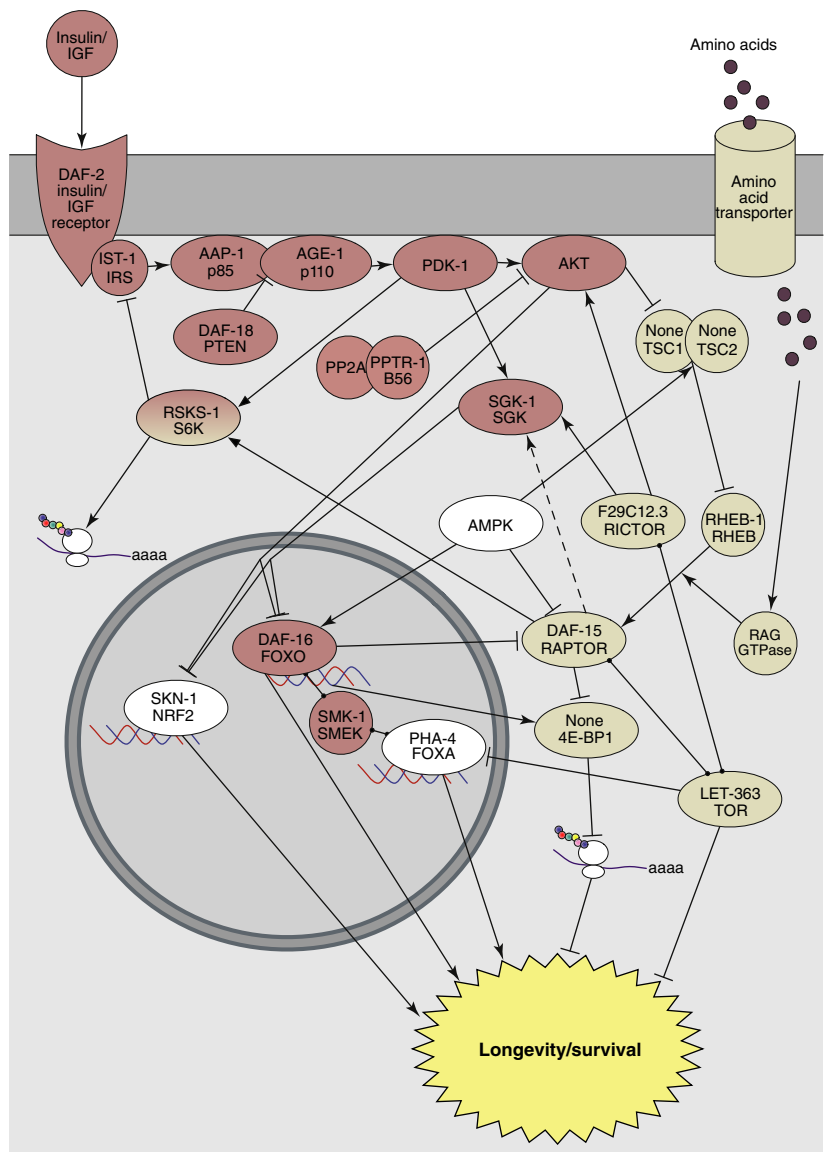
The insulin/IGF-1 signaling (red ovals) and TOR (beige ovals) pathways may interact at multiple levels to ultimately regulate organismal lifespan. Dual coloring indicates proteins implicated in both pathways. White ovals are proteins that are not typically viewed as being in one pathway or the other. The proteins are labeled with the worm name followed by the mammalian name.

hormone receptor show increased lifespan [6]. Interestingly, plasma IGF-1 levels are dramatically low in all of these long-lived mutant mice [25]. In dogs, a polymorphism in IGF-1 is a major determinant of size and a dog's size is inversely correlated with lifespan [26,27]. In humans, a specific polymorphism in the IGF-1 receptor has been associated with increased longevity [15]. Taken together, these data suggest that modifying either insulin receptor or IGF-1 receptor activity can result in changes in longevity across phylogeny.

Downstream Kinases

Similar to the signaling pathways in mammals, *C. elegans* has a well-conserved PI 3-kinase signaling pathway downstream of *daf-2* and *age-1*, the worm homolog of the PI 3-kinase catalytic subunit (Figure 1, Table 1) [2]. Downstream components, such as phosphoinositide-dependent protein kinase-1 (*pdk-1*), *akt-1*, *akt-2* and serum and glucocorticoid-inducible kinase (*sgk-1*), were identified by both forward and reverse genetic approaches, and mutations in these genes result in lifespan extension (Figure 1 and Table 1) [2,6]. In the budding yeast *Saccharomyces cerevisiae*, in which many aging studies have been performed, it is not clear whether a conserved insulin/IGF-1 pathway exists, although the conserved Akt homolog *SCH9* is important for regulating lifespan [28].

Remarkably, reduction-of-function mutations in the insulin/IGF-1 signaling pathway not only confer increased longevity but also enhance resistance to heat and/or oxidative stress. Studies in *C. elegans* revealed that these phenotypes ultimately depend upon the single forkhead box O (FOXO) transcription factor *daf-16* [3,4,13]. Loss-of-function mutations in *daf-16* result in a dramatic suppression of the lifespan extension and stress resistance phenotypes of *daf-2* mutants [3]. The insulin/IGF-1 signaling pathway activates AKT-1/2 and SGK-1 in a PI 3-kinase-dependent manner, and in turn, AKT-1/2 and SGK-1 negatively regulate DAF-16 by phosphorylation [2,4]. Under these conditions, DAF-16 is sequestered in the cytosol by its association with 14-3-3 proteins [8,13]. This regulation is conserved, because mammalian AKT and SGK also directly phosphorylate and negatively regulate FOXO proteins [29]. However, under low signaling conditions or in loss-of-function kinase mutants, such as *daf-2* and *age-1*, DAF-16 is able to



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translocate to the nucleus to transactivate or repress its target genes [2,4,29].

DAF-16/FOXO

What are the important target genes that DAF-16 regulates to confer significant increases in lifespan and stress resistance? A combination of approaches, including genome-wide screens, microarrays, and chromatin immunoprecipitation, has identified hundreds of genes that are under the control of DAF-16 [2–4,30], such as genes encoding molecular chaperones, superoxide dismutases, metabolic regulators, and regulators of the cell cycle. It is still unclear how DAF-16 regulates the activity of so many genes and whether all or a subset of these genes actively regulate the phenotypes mentioned above. As a transcription factor, DAF-16 may interact with other co-regulators, such as co-activators and repressors, to define particular biological processes. Indeed, the nuclear factor SMK-1, the *C. elegans* homolog of suppressor of MEK-1 (SMEK-1), associates with DAF-16 and is required for longevity, innate immunity and

resistance to oxidative stress, but not thermotolerance [2,19].

Several other additional transcription factors and co-activators (heat shock factor 1 (HSF-1), host cell factor 1 (HCF-1), the MST-1 homolog CST-1, and the β -catenin homolog BAR-1) have been shown or suggested to interact with DAF-16 in the insulin/IGF-1 signaling pathway (Table 1). The transcription factor heat-shock factor 1 (HSF-1) is an important regulator of thermal stress in eukaryotes. In response to heat stress, HSF-1 promotes the expression of heat-shock proteins in a DAF-16-dependent manner [4]. Recently, the *C. elegans* host-cell factor homolog HCF-1 was found to associate with DAF-16 in the nucleus and repress its transcriptional activity [31]. In addition, CST-1 and BAR-1 have been shown to regulate the transcription of genes involved in oxidative stress in a DAF-16-dependent manner [13]. Their respective mammalian homologs, MST-1 and β -catenin, also interact with mammalian FOXO, thereby showing the remarkable conservation from nematodes to higher mammals in adaptation to oxidative stress. Thus, depending upon the stressor, DAF-16/FOXO may not only associate with distinct transcriptional cofactors but also regulate the transcription of discrete sets of genes.

DAF-16 can be phosphorylated by multiple kinases at residues distinct from the AKT/SGK sites. The AMP-activated protein kinase (AMPK) positively regulates DAF-16/FOXO by phosphorylation [29,32]. Similarly, c-Jun terminal kinase (JNK) phosphorylates DAF-16 and promotes its nuclear localization, leading to increased lifespan and stress resistance [3,10].

Flies also have a single FOXO homolog (dFOXO) that is phosphorylated in response to signals transduced by dInsr. Overexpression of dFOXO in the adult fat body results in lifespan extension [33]. Similar to worms, a JNK-dependent increase in lifespan and stress resistance depends upon dFOXO, and JNK and dFOXO together can negatively regulate insulin/IGF-1 signaling by repressing the expression of *dilp2* [10].

Much work has focused on the role of DAF-16 and dFOXO in longevity. In mammals, there are four members of the FOXO family that show overlapping and distinct tissue expression patterns — Foxo1, Foxo3a, Foxo4, and Foxo6 — and their roles in longevity have not been studied [34]. Foxo1 plays an important role in angiogenesis and myoblast and adipocyte differentiation, and Foxo1-null mutant mice are embryonic lethal [34,35]. Foxo3a- and Foxo4-null mutant mice are viable and grossly similar to their littermate controls, but Foxo3a-null mutant female mice develop age-dependent infertility [34]. Foxo6 has been shown to be expressed in the developing brain in embryos as well as the adult brain, and knockout mice have not yet been examined. Although the correlation between FOXOs and longevity has yet to be clearly defined in mammals, these proteins have a well-established role as tumor suppressors [34]. In addition, they regulate the expression of stress-responsive genes, such as manganese superoxide dismutase and Gadd45, and may also regulate adult stem-cell proliferation [36].

To better understand the regulation of DAF-16/FOXO, it is equally important to elucidate the proteins that modulate insulin/IGF-1 signaling activity. While several kinases have been well studied in mammals and in simpler organisms, there are few known phosphatases that regulate insulin/IGF-1 signaling in the context of longevity. The best studied among these is *daf-18*, the *C. elegans* homolog of the

phosphoinositide 3-phosphatase PTEN. Mutations in *daf-18* can suppress the long lifespan of *daf-2* mutants and result in increased susceptibility to various stressors [2,11]. This shows that, similar to its mammalian homolog, DAF-18 acts to negatively regulate PI 3-kinase signaling [2]. More recently, the PP2A phosphatase regulatory subunit PPTR-1/B56 β was found to negatively regulate insulin/IGF-1 signaling by modulating AKT-1 phosphorylation, and thereby positively regulating DAF-16 [37]. In contrast, the calcineurin A serine/threonine phosphatase catalytic subunit TAX-6 and its regulatory subunit B CNB-1 were found to be negative regulators of insulin/IGF-1 signaling, as loss-of-function mutants in *tax-6* and *cnb-1* display enhanced longevity [5].

Given that a single transcription factor downstream of insulin/IGF-1 signaling, DAF-16, can be modulated by a multitude of kinases and adaptor proteins, it is evident that this signaling pathway is far more complex than previously appreciated. The identification of new regulators and interactors will undoubtedly shed more light on our understanding of how DAF-16/FOXO regulates longevity.

Dietary Restriction

Dietary restriction is a pan-species treatment that can extend an organism's lifespan and health [1]. While the majority of species on restricted diets show an increased lifespan, several species and even strains within a species appear to be insensitive to this treatment [38]. Caloric restriction, where the number of calories ingested is known, was originally shown to result in lifespan extension more than 60 years ago. Since then, the pathways and proteins involved in its beneficial effects have only recently been discovered.

Ins-ular View of Dietary Restriction

One attractive model is that the well-defined insulin/IGF-1 signaling pathway mediates the effects of dietary restriction. However, current data regarding this model are not definitive. In worms, at least six methods have been used to mimic dietary restriction and test its interaction with insulin/IGF-1 signaling. The effects of dietary restriction by semi-defined media, complete starvation, or dilution of *Escherichia coli* in liquid media all appear to be independent of insulin/IGF-1 signaling [7,39–41]. In contrast, dietary restriction using mutations that limit the amount of food consumed have both an additive and a non-additive effect with insulin/IGF-1 signaling, depending on the mutant allele [42,43]. The positive effects of dietary restriction by serial dilution of *E. coli* on solid media are dependent upon DAF-16 as well as AMPK [44]. In addition, the effects of an intermittent feeding paradigm are partially dependent on DAF-16 but fully dependent on Ras homolog enriched in brain (Rheb), a member of the small GTPase family of proteins [9]. Thus, data generated from studies in *C. elegans* generally show that the effects of dietary restriction are independent of insulin/IGF-1 signaling, although this depends on the method of dietary restriction.

Similar to *C. elegans*, the relationship between insulin/IGF-1 signaling and dietary restriction in *Drosophila* is unclear. In a study that exemplifies the importance of using a range of levels of dietary restriction, Clancy *et al.* [45] demonstrated that the lifespan of the long-lived *chico* mutant is not additive to an optimized dietary restriction regimen. These studies also showed that different strains of flies have distinct optimal nutrient intakes (i.e. the diet restriction level that maximally extends lifespan for one strain is not necessarily the same for all strains). A study of dFOXO

mutants found that these flies respond to dietary restriction, suggesting the opposite conclusion — that insulin/IGF-1 signaling and dietary restriction are independent pathways [46]. One possibility for the difference could be the methods used in these studies. Clancy *et al.* [45] used a diluted media-method for dietary restriction, while Min *et al.* [46] reduced the amount of yeast in the media. Therefore, as with *C. elegans*, studies in *Drosophila* do not conclusively show whether dietary restriction and insulin/IGF-1 signaling are entirely independent. These discrepancies may ultimately arise from the different methods used to administer dietary restriction.

Limited studies have examined the interplay between insulin/IGF-1 signaling and dietary restriction in mammals. In response to caloric restriction, the long-lived *Prop1* mutant mice show a further extension in lifespan [25]. In contrast, caloric restriction does not further increase the extended lifespan of GH receptor knockout mice [25]. Therefore, limited conclusions can be drawn from the data on the interplay of dietary restriction and insulin/IGF-1 signaling in mammals.

A TOR-Centric View

Of the different proteins implicated in dietary restriction, one of the most well-characterized is the target of rapamycin (TOR), a kinase that is part of a pathway highly conserved from yeast to humans that functions to integrate multiple nutrient signaling pathways [47]. As such, it is an appealing candidate as a central regulator of the effects of dietary restriction. TOR is active in two separate complexes containing both unique and common proteins. TOR complex 1 (TORC1) is composed of RAPTOR, PRAS40, LST8, and TOR. TOR complex 2 (TORC2) also contains TOR and LST8, with the additional components RICTOR, SIN1, PROTOR [48]. (Note that the TORC2 mentioned here should not be confused with the protein with an identical acronym ‘transducer of regulated CREB activity 2’.)

TOR — A Central Regulator of Dietary Restriction?

Evidence for a central role of the TOR pathway in dietary restriction comes from several species. In yeast, TOR can be required for the effects of dietary restriction [28]. In *C. elegans*, RNAi of TOR (*let-363*) produces a longevity phenotype that is independent of FOXO/DAF-16 [49]. On the other hand, a majority of studies show that reducing TOR signaling in dietary-restricted worms does not further increase lifespan [50–52]. Another link between dietary restriction and TOR is autophagy, a process by which the cell recycles its various components. Autophagy is required for the lifespan extension effects of dietary restriction, and TOR is a central regulator of this process [53–55].

As in worms [50], under normal laboratory conditions loss of TOR or TOR complex components extends lifespan in flies [56]. Mammalian studies linking TOR signaling and longevity have not been published but a connection between TOR signaling and nutrient sensing has been established. TOR regulates food intake, and the active, phosphorylated form of TOR is found in critical hypothalamic neurons in the arcuate nucleus [57]. Furthermore, TOR signaling is activated by leptin, a hormone released from adipose tissue in response to increasing fat levels, and inhibition of TOR activity reduces the anorectic effect of leptin administration [57].

Ribosomal S6 kinase (S6K), a downstream target of TOR, mediates hunger-driven behavior in flies when upregulated in specific neurons [58]. Further, S6K knockout mice are resistant to diet-induced obesity and age-related obesity

[59]. Therefore, under dietary restriction conditions, TOR signaling in mammals is probably reduced and may mediate the effects of dietary restriction, as in simpler model organisms. Taken together, these studies suggest that the TOR pathway may be the central regulator of the effects of dietary restriction ranging from yeast to mammals.

Cross-Talk between Insulin/IGF-1 Signaling and TOR

Genetic analysis in *C. elegans* indicates that the insulin/IGF-1 signaling pathway and the TOR pathway are independent of or converge downstream of DAF-16 because RNAi of TOR increases lifespan independent of FOXO/DAF-16, but reduced TOR signaling does not further increase the lifespan of insulin/IGF-1 signaling mutations [49,50]. Nevertheless, there is a substantial amount of cross-talk between the insulin/IGF-1 signaling pathway and the TOR pathway (Figure 1). Although both of these pathways monitor nutrients, the insulin/IGF-1 pathway is linked to an endocrine signal that monitors the nutrient status of the whole organism, whereas the TOR pathway monitors the intracellular nutrient status.

Insulin/IGF-1 signaling influences the TOR pathway by at least three separate mechanisms. In worms, decreased insulin/IGF-1 signaling inhibits the TOR pathway by reducing the transcription of *daf-15/RAPTOR* [60]. Additionally, in mammalian studies, insulin/IGF-1 signaling stimulates TOR activity by decreasing the ability of PRAS40 and tuberous sclerosis complex 2 (TSC2) to inhibit TORC1 (TSC2 and tuberous sclerosis complex 1 form a complex that inhibits Rheb-mediated activation of TORC1) [48]. Another connection between Rheb, TOR and insulin/IGF-1 signaling has been shown in worms, using an intermittent feeding paradigm [9].

The TOR pathway also influences insulin/IGF-1 signaling. In mice, TORC1 inhibits insulin/IGF-1 signaling via S6K-mediated phosphorylation of IRS-1, and this is likely to be conserved in humans [59,61]. TORC2, however, activates the insulin/IGF-1 signaling pathway by phosphorylating AKT at Ser473, which acts synergistically with phosphorylation by PDK-1 at Thr308 [62,63].

In addition, AMPK directly modulates both insulin/IGF-1 and TOR signaling. In worms AMPK phosphorylates DAF-16 to increase its activity, and this effect is conserved in mammals [44,64]. AMPK also phosphorylates RAPTOR and inhibits TORC1 [65]. Moreover, by phosphorylating TSC2, AMPK enhances the inhibitory effect of TSC2 on TOR activity [66]. These three functions of AMPK are likely to act in concert to strongly inhibit TOR signaling.

Insulin/IGF-1 and TOR also have several common downstream effectors. Both pathways regulate 4E-BP, an inhibitor of translation, and SGK-1 [67–70]. For insulin/IGF-1 signaling, SGK-1 negatively regulates DAF-16/FOXO under fed conditions [68]. TOR activates SGK-1 by direct phosphorylation, but it is unclear which TOR complex is responsible for the phosphorylation (shown in Figure 1) [69,70], although recent data from worms support the view that TORC2 is the complex that interacts with SGK [71,72].

Additional Players

Other proteins have been implicated in dietary restriction and longevity, but the pathways in which they are involved are not as well-defined as the insulin/IGF-1 or TOR pathways. This section summarizes the roles of several of these proteins: PHA-4/Foxa, SKN-1/Nrf2, and the sirtuins. In addition, the roles of odor and mitochondria in dietary-restricted longevity are also described.

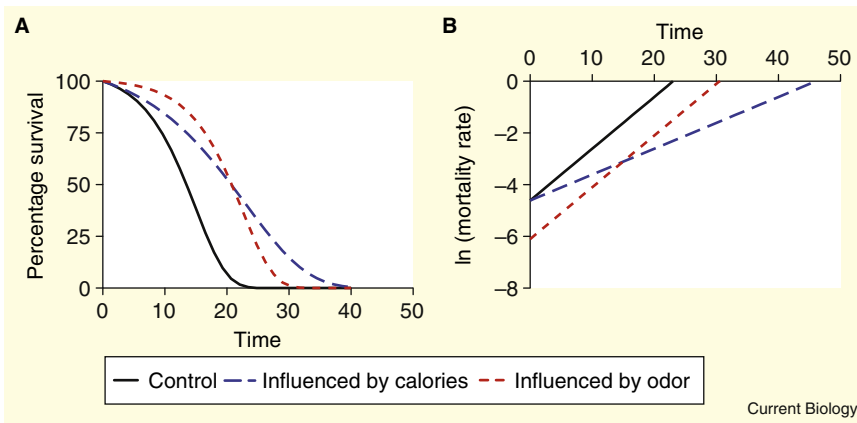


Figure 2. Mathematical differentiation of odor and caloric components of dietary restriction. (A) Idealized survival curves representing differential effects of odor and calories on lifespan. (B) Linearized mortality rate curves of the idealized survival curves. Lifespan changes due to odor and calories can be mathematically differentiated. Lifespan changes that are due to odor cause changes in the initial mortality rate, while lifespan changes due to caloric intake change the slope of the survival curve. These differences may be due to different mechanisms such as insulin/IGF-1 signaling or TOR signaling.

Additional Players: *SMK-1* and *PHA-4*

Panowski *et al.* [73] discovered that *SMK-1* was required to extend the lifespan of worms by dietary restriction. This was particularly interesting because *SMK-1* interacts with *DAF-16* and is required for extending the lifespan of insulin/IGF-1 signaling mutants [74]. Because *DAF-16* was not required in the models of dietary restriction investigated by Panowski *et al.* [73], they hypothesized that *SMK-1* was interacting with another transcription factor. RNAi studies carried out to reveal this transcription factor led to the identification of *PHA-4*, a Forkhead transcription factor box A (Foxa) protein, and found that it is required for lifespan extension by dietary restriction [73]. In worms, the TOR signaling pathway antagonizes *PHA-4*, and some of the effects of the TOR pathway on lifespan require *PHA-4* [75]. *DAF-16* and *PHA-4* have common consensus binding sites within promoters, and the regulation of at least two superoxide dismutases (*sod-1* and *sod-5*) depends upon both of these transcription factors [73]. Therefore, a complicated interaction between insulin/IGF-1 signaling, TOR signaling, and *PHA-4* begins to emerge, but because these interactions have only been shown in worms, confirmation in other organisms is still required.

Additional Players: *SKN-1/Nrf2*

SKN-1, the worm homologue of mammalian *Nrf2*, is required for lifespan extension by dietary restriction [7]. *Nrf2* is involved in the cellular response to toxins, and many studies in mice have examined its requirement in defending against oxidative, inflammatory, and electrophilic toxins [76]. *skn-1* has a critical role in insulin/IGF-1 signaling [77]. The insulin/IGF-1 signaling pathway directly inhibits *SKN-1*, suggesting that *SKN-1* may function as another point of intersection between the insulin/IGF-1 signaling pathway and dietary restriction [77]. It is possible that *SKN-1* is important for longevity mediated by either dietary restriction or insulin/IGF-1 signaling, depending upon the tissue in which it is expressed.

The role of *skn-1* homologs in lifespan extension so far appears to be conserved in simpler model organisms but not in mammalian models. A mutation in the *skn-1* repressor, *keap-1*, can extend the lifespan of male flies [78]. However, recent studies showed that *Nrf2* plays no role in dietary-restriction-induced longevity in mice, despite the mice having a higher susceptibility to tumors [79]. Given the role of *Nrf2* in detoxification, the observed effects on lifespan extension could also be explained if normal experimental conditions are slightly toxic to *Drosophila* or *C. elegans*. In fact, the *E. coli* that worms routinely feed on eventually infect

the worms as they age and also produce small quantities of cyanide [80,81].

Additional Players: Sirtuins

The sirtuins (*SIRT1–7*) are another family of proteins that have been implicated in dietary restriction and longevity. Sirtuins are a highly conserved group of NAD⁺-dependent de-acetylases and this dependence on NAD⁺ suggests that they may be possible mediators of dietary restriction. (NADH has been suggested to be more important to nutrient sensing than ATP, and moreover, AMPK activity does not significantly change in several tissues of dietary-restricted mice [82,83].) Interestingly, lifespan extension mediated by the *C. elegans* *SIRT1* ortholog *sir-2.1* depends upon *daf-16* [84], and *SIRT1* and *SIRT2* deacetylate *FOXO3a* in mammalian cell culture [84,85].

Thus far, the literature has been equivocal as to the requirement of sirtuins for dietary restriction. In yeast, sirtuins are required in some strains but unnecessary in others [8]. In worms, the requirement of *sir-2.1* for lifespan extension by dietary restriction depends on the method of dietary restriction [86]. The *Drosophila* ortholog of *SIRT1* (*dSIR2*) is required for the effects of dietary restriction [8]. Mammalian studies are underway, but initial papers show that sirtuins are required for some of the physiological changes associated with dietary restriction [87,88].

Two studies with *Sirt1* knockout mice indicate that *SIRT1* may be involved in dietary restriction-mediated lifespan extension. However, these results are complicated by the fact that *Sirt1* knockout mice are short-lived [88,89]. Other studies in mice have correlated the effects of dietary restriction with changes in sirtuin function. Caloric restriction induces *Sirt1* expression and *SIRT1* nuclear localization [90,91]. Therefore, *SIR-2* and its homologs may mediate cross-talk between dietary restriction and insulin/IGF-1 signaling.

Additional Players: Odor

In worms and flies, the smell of food alone can abolish the life-extending effect of dietary restriction [92,93]. Mutations in the odor receptors of worms result in lifespan extension that is mainly dependent on *DAF-16* [94]. These studies suggest that the majority of the effects of dietary restriction may be due to the lack of an odorant or sensation of odor.

An interesting possibility supported by mathematical analysis of dietary restriction in different species is that dietary restriction can be broken down into two mechanisms (Figure 2) [95]. The first is dominated by insulin/IGF-1 signaling

Figure 3. Mitochondrial signaling may mediate the cross-talk between the insulin/IGF-1 signaling and TOR pathways to regulate longevity.

Mitochondria are known to signal to the nucleus by at least two mechanisms: one is the retrograde signaling pathway (RTG) that involves LST8 and possibly TOR; the other uses reactive oxygen species and signals through the JNK-FOXO pathway. AMPK also receives input from mitochondria and is yet another protein involved in the mitochondrial control of lifespan.

and linked to odor and the anticipation of food by the whole organism. (In fact the mere anticipation of food can cause an insulin release in rats and humans [96,97].) The second is independent of insulin/IGF-1 signaling and is instead dependent on caloric intake and possibly regulated by the TOR pathway. These two mechanisms are not mutually exclusive and there are a number of different ways in which these mechanisms could interact.

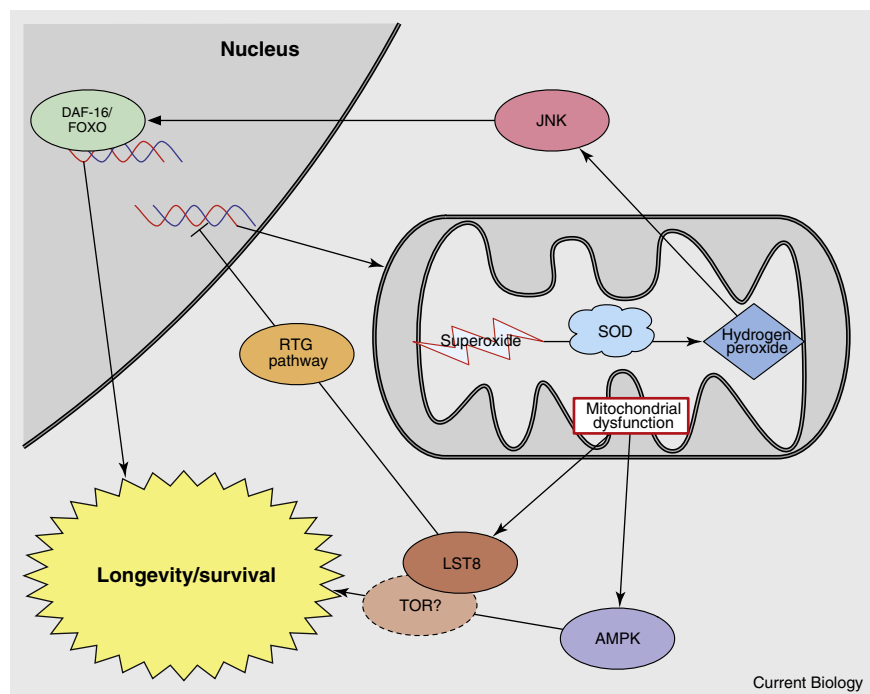
In worms, initial starvation causes DAF-16 to localize to the nucleus to induce a pro-survival pathway, but, as the starvation continues, DAF-16 is less nuclear and is observed in the cytoplasm [98]. Although DAF-16 is no longer found in the nucleus, the worms are nonetheless still resistant to free-radical damage. Furthermore, in the same study, it was observed that there is both DAF-16-dependent and DAF-16-independent resistance to free-radical damage ([98] and D. Weinkove, personal communication). Consistent with this view, complete removal of food can extend lifespan independent of DAF-16 [39,40]. It is plausible that the DAF-16-independent increase in lifespan is due to decreased TOR signaling or another yet unidentified pathway.

Additional Players: Mitochondria

Both caloric restriction and insulin affect mitochondrial metabolism [99,100]. On their own, specific mitochondrial mutations increase the lifespan of both worms and mice [42,101]. Although it may be tempting to think that the increased lifespan associated with these mitochondrial mutations is a result of decreased metabolism, metabolic rate does not determine lifespan [102].

It is currently unknown how mitochondrial mutations increase lifespan but mitochondria are known to signal to the nucleus through several possible pathways. One of the best established pathways is the mitochondrial retrograde signaling pathway [103,104]: this pathway has been primarily investigated in yeast and allows the mitochondria to signal to the nucleus [103]. LST8, a highly conserved member of both TOR complexes (TORC1 and TORC2), has been implicated in the regulation of this retrograde signaling pathway; furthermore, TOR signaling also regulates mitochondrial metabolism [103,105]. Although it seems likely that mitochondrial mutations signal through the retrograde signaling pathway, the effect of this pathway on lifespan has yet to be directly tested.

In *C. elegans*, the increased lifespan of two mitochondrial mutants can be partially reduced by a mutation in AMPK



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[106], but decreased ATP levels alone are not sufficient to increase lifespan [107]. In *Drosophila*, mitochondrial dysfunction signals to the nucleus via two mechanisms: one mediated by reactive oxygen species (ROS) and the other, similar to *C. elegans*, mediated by AMPK [108]. While the pathway mediated by ROS is controlled by FOXO and JNK, which implicates the insulin/IGF-1 signaling pathway, the AMPK-dependent pathway has implications for both insulin/IGF-1 and TOR signaling. Although this experiment did not test the effect of these pathways on lifespan, these data are in agreement with results from a number of longevity experiments in different species that show the role of mitochondrial components in modulating lifespan [109,110].

Studies in mice with a mitochondrial mutation in Mcl1, the mammalian homologue of the *C. elegans clk-1* gene (which encodes a protein involved in ubiquinone synthesis that reduces the flow of electrons in the electron transport chain [109]), have shown that this mutation increases the lifespan of heterozygous mice (homozygous mice are inviable) [110]. The heterozygous, long-lived mice have decreased ATP levels and decreased cytoplasmic ROS damage but increased signs of mitochondrial ROS damage [110].

Further supporting the importance of ROS signaling from the mitochondria, overexpression of the mitochondrial SOD, Mn-SOD, increases lifespan by approximately 20% in flies [111]. Although an increase in Mn-SOD could decrease mitochondrial free radicals, there were no detectable changes in markers of free-radical stress or the ability to resist free radical stress. A microarray analysis found a pattern suggestive of both hydrogen peroxide signaling from the mitochondria and decreased insulin signaling. In another study, glucose deprivation through the use of 2-deoxy-D-glucose (a non-metabolizable form of glucose) increased lifespan and stress resistance in worms [112]. The addition of antioxidants actually decreased the lifespan and suggested an important role of oxidative stress in the observed lifespan extension.

It is still unclear whether the lifespan extension seen in several mitochondrial mutants, such as *clk-1* and the iron

sulfur protein gene *isp-1*, requires the insulin/IGF-1 signaling pathway [113–115]. Together, the mitochondrial studies suggest the following possible scenario (Figure 3): mitochondrial dysfunction signals to the nucleus through a number of pathways; sub-lethal increases of ROS in the mitochondria signal through a JNK–FOXO pathway and extend lifespan; a second signal involves decreased AMP levels combined with another signal that may increase lifespan by a FOXO-independent pathway, possibly the retrograde signaling pathway, which may include TOR. Comprehensive experiments directly testing this hypothesis have not yet been conducted.

Conclusions

Both insulin/IGF-1 signaling and dietary restriction can increase lifespan in a number of different species. Additionally, these events are linked by their involvement in the nutritional status of the organism. Numerous studies have shown that these lifespan-extending methods may interact at several different levels. We suggest that the insulin/IGF-1 pathway and the pathway that mediates the effects of dietary restriction have evolved to respond to the nutritional status of an organism, which in turn impacts its lifespan. From an evolutionary point of view, these longevity pathways are likely to be used by an organism to survive unfavorable conditions in an effort to reproduce at a later time and should probably be more appropriately called survival pathways. Therefore, the lifespan extension is likely to be only a by-product of the primary function of these pathways. Several conditions, such as a decrease in nutrient availability or change in temperature, may cause a survival pathway to be activated. This requires that survival pathways monitor both external environment and internal homeostasis. Because the basic needs of most organisms are the same, survival pathways are largely conserved. Monitoring nutrient availability is therefore a central theme in most of these lifespan-regulating pathways. This is not surprising because the need to regulate nutritional status is surpassed only by the need for oxygen (in aerobic organisms) and the need for water. Studies using complex model organisms demonstrate the importance of endocrine signaling, but ultimately endocrine signaling is controlled at the cellular level. The feedback loops and intertwined nature of current studies of insulin/IGF-1 and TOR signaling are indicative of the need to regulate food and nutrition on multiple levels. The control of nutritional status on a number of different levels allows for many opportunities to manipulate this survival system, which may not only increase lifespan but may improve overall health.

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