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Review

The trifecta of aging in *Caenorhabditis elegans*

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Abstract

Insulin signaling, mitochondrial respiration, and dietary restriction share conserved roles not only in the regulation of lifespan, but also in the timing and control of diverse functions such as reproduction, stress resistance and metabolism. These autonomous pathways differ in their dependence on known transcription factors and in their temporal requirements, but converge to manipulate the core set of physiological systems necessary for extended lifespan in worms. Recent work suggests that components of these pleiotrophic pathways might be manipulated specifically for their effects on aging without affecting additional downstream functions. Examination of these findings will help us to understand how the molecular mechanisms of distinct pathways can unite in the regulation of longevity.

Keywords: Insulin/IGF-1 signaling; Aging; *Caenorhabditis elegans*; Dietary restriction; Mitochondria; Gluconeogenesis

1. Introduction

The discovery of genetic manipulations that regulate the aging process has revolutionized the field of molecular gerontology, moving it from a purely descriptive scientific endeavor to one of mechanistic, hypothesis-based experiments. This emergence resembles the early time period of developmental genetics when scientists first discovered that embryonic pattern formation was determined by the temporal ordering of early acting programs. In this regard, aging itself can be viewed as a developmental stage during which receptive cues initiate a progressive sequence of events that ultimately lead to the faithful execution of its processes. The identification of genetic pathways that regulate aging has suggested the realistic possibility of creating therapeutics that can target these pathways to circumvent the aging process. This in itself is a bold notion and has brought much excitement to the field. However, the focus of many aging researchers remains not only on the potential extension of human longevity, but also on the modulation of the aging process to retard or even prevent age onset diseases such as cancer, neurodegeneration and diabetes.

Since the discovery over 20 years ago that genetic or environmental manipulations cause worms to live over twice as long as wild type animals, (Johnson and Wood, 1982; Klass, 1977, 1983) many more genes have been identified and characterized as gerontogenes, or genes that play an active role in lifespan determination. Within a genetically tractable model organism with a short lifespan such as *Caenorhabditis elegans*, aging is often measured as the total lifespan of a population of age-synchronized animals. Despite the large number of mutations that influence aging, however, most gerontogenes can be grouped into one of three distinct pathways that regulate lifespan in worms. Reduced insulin signaling, dietary intake, or rates of mitochondrial respiration can increase lifespan via signaling mechanisms that are genetically regulated and autonomous of one another. All three pathways have been found to regulate longevity in *C. elegans* and in mice, suggesting an evolutionarily conserved mechanism for the determination of lifespan. Evidence for the independence of these pathways comes from three groups of observations regarding the temporal and epistatic requirements for their components with regards to longevity. First, simultaneous manipulation

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of more than one of these three pathways results in longer lifespan than is seen through the manipulation of a single pathway (Dillin et al., 2002b; Lakowski and Hekimi, 1998) (Fig. 1). Although this epistatic analysis lacks null alleles, it seems improbable that these effects are caused simply by a further downregulation of insulin signaling. While combinations of mutations between these parallel pathways results in a highly additive increase in lifespan, the combination of mutations in multiple genes downstream of IIS or of an IIS mutation in combination with *daf-2* RNAi yields results only in lifespan extension comparable to single mutations or to stronger *daf-2* alleles (Arantes-Oliveira et al., 2003; Dillin et al., 2002b; Dorman et al., 1995; Lakowski and Hekimi, 1998; Larsen et al., 1995). Secondly, the temporal requirements during the life cycle of the worm for each pathway are separable: loss of insulin signaling during adulthood regulates lifespan; mitochondrial respiration must be lost during early development in order to extend longevity; and dietary restriction appears capable of being imposed at any time point during the lifespan to increase longevity, at least in flies (Dillin et al., 2002a,b; Mair et al., 2003). Finally, transcription factors that are an absolute requirement for one pathway (such as *daf-16* for insulin signaling) are dispensable for the longevity of other pathways (Dillin et al., 2002b; Houthoofd et al., 2003; Houthoofd et al., 2005a; Lakowski and Hekimi, 1998). This collective evidence suggests at least three different mechanisms by which longevity is regulated in long-lived *C. elegans* mutants.

Although a clear separation of these signaling cascades in the regulation of longevity is documented, a central question remains as to whether a single, common downstream module is shared by all three pathways to regulate the aging process. Manipulation of the core components of these independent pathways results in overlapping effects that protect the organism from multiple toxic insults such as oxidative stress, thermal stress, and DNA damage (reviewed by Hekimi and Guarente, 2003). These pathways, along with the long-lived dauer pathways, also differentially regulate a wide range of metabolic functions, affecting the rates of glycolysis and gluconeogenesis, levels of glycogen and fat storage, ATP production, and the glyoxylate cycle (Hagopian et al., 2003; Holt and Riddle, 2003; Kimura et al., 1997; Lee et al., 2003a; Murphy et al., 2003). Additionally, inactivation of any one of the three pathways slows development and delays or protracts reproduction (Dillin et al., 2002b; Kenyon et al., 1993; Lakowski and Hekimi, 1998; Larsen, 1993; Lee et al., 2003a). While it remains unclear which of these core effects, if any, is an absolute requirement for extended lifespan, the number of overlapping physiological processes shared between the signaling networks indicates that the downstream mechanism for the regulation of aging might be shared. It makes intuitive sense, then, that beyond the level of autonomous transcriptional regulation lays a core and overlapping set of targets required to execute the genetic program of aging.

What, then, are the requirements for aging in worms? What are the most fundamental targets of these transcriptional pathways? Below we review the key components by which each of these pathways is regulated and discuss how each affects a set of basic physiological processes that can help determine the lifespan of the individual worm.

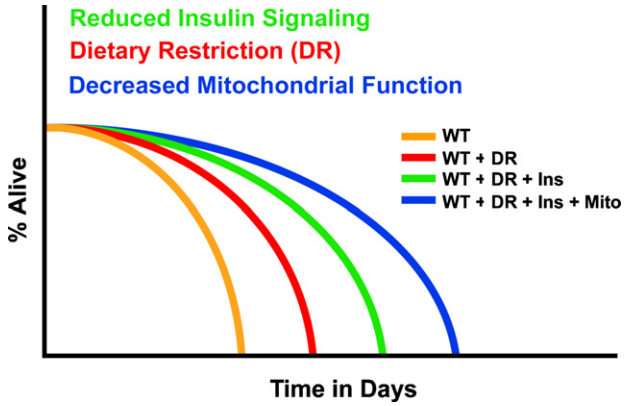

Fig. 1. Modulation of the three core pathways that affect aging act synergistically with each other. Dietary restriction (red line), reduced insulin signaling (green line), or impairment of the mitochondrial electron transport chain (blue line) can extend the lifespan of a wild type animal (orange line). Modulation of any two of these three pathways has an additive effect on lifespan, supporting arguments for their mechanistic autonomy. In theory, knockdown of all three pathways should create an animal that is far longer lived than an animal with reduced function in just two of the three pathways.

2. IIS dependent regulation of aging

The best-characterized mutations affecting the longevity of worms lie in the insulin/IGF-1 signaling (IIS) pathway, and many of the key components of this signaling network have been identified (Fig. 2). Lifespan extension in worms can be achieved by mutations in the sole insulin/IGF-1 receptor, DAF-2 (Kimura et al., 1997). Active DAF-2 initiates a subsequent downstream cascade that activates AGE-1, a phosphatidylinositol 3-kinase (PI(3)K) (Morris et al., 1996), produces PIP_3, and activates the AKT family kinases (Hertweck et al., 2004; Paradis and Ruvkun, 1998) in a PDK-1 kinase dependent manner (Paradis et al., 1999). These active AKT family kinases phosphorylate the forkhead transcription factor DAF-16 (Henderson and Johnson, 2001; Lee et al., 2001; Lin et al., 2001), preventing DAF-16 from entering the nucleus and rendering it incapable of promoting or repressing transcription of genes required for DAF-2 dependent functions (Lin et al., 1997; Ogg et al., 1997). Inactivation of this pathway occurs in part via the activity of the PIP_3 phosphatase, DAF-18 (Dorman et al., 1995; Ogg et al., 1997).

The DAF-2 pathway has pleiotrophic effects on the worm in addition to its effects on longevity, influencing divergent functions such as early developmental decisions, the timing and duration of reproduction, resistance to a variety of envi-

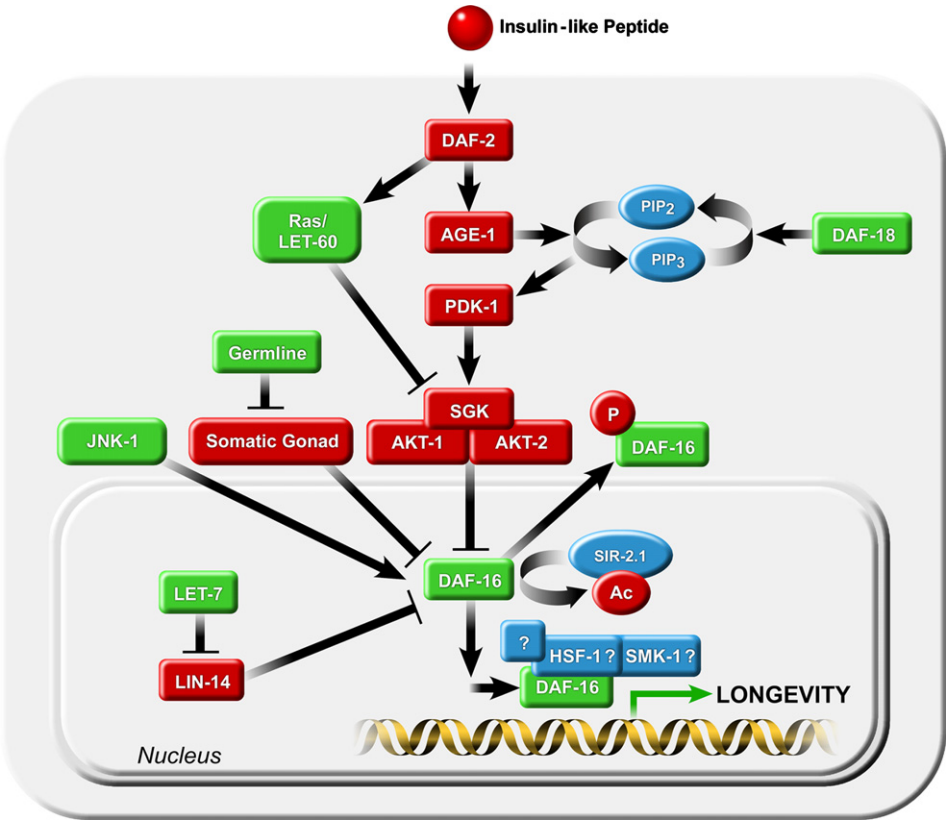

Fig. 2. DAF-16 regulates aging through IIS and IIS-independent modes of activation. The FOX3a transcription factor DAF-16 is primarily regulated through its state of phosphorylation. An unknown insulin-like peptide activates the DAF-2 IIS receptor and subsequent downstream kinases AGE-1, PDK-1, and AKT-1/AKT-2/SGK-1. Signaling bifurcation from IIS to the Ras/LET-60 MAPK pathway also helps to modulate DAF-16 activity. This signaling cascade results in the phosphorylation of DAF-16 and its subsequent export from the nucleus. DAF-16 can also be activated in an IIS-independent fashion via the histone deacetylase SIR-2.1, the LET-7/LIN-4 target LIN-14, germline ablation, or the Jun N-terminal kinase JNK-1. DAF-16 requires the activity co-regulators HSF-1 and SMK-1 for regulation of specific subsets of its target genes. Activators of DAF-16 are shown in green; repressors of DAF-16 are shown in red.

ronmental stressors, and fat metabolism. Attempts at targeted knockdowns of single genes downstream of *daf-16* have not resulted in the identification of a single gene capable of suppressing *daf-2* longevity (McElwee et al., 2003; Murphy et al., 2003). How does a seemingly simple signaling mechanism have control over such complex processes?

Although nucleo-cytoplasmic transport of DAF-16 plays a major role in its regulation, constitutive nuclear localization of DAF-16 does not increase lifespan, and additional regulators such SIR-2.1, HSF-1, LIN-14, and SMK-1 all play a role in modulating DAF-16's phenotypes once it is inside of the nucleus (Boehm and Slack, 2005; Hsu et al., 2003; Tissenbaum and Guarente, 2001; Wolff et al., 2006). Additionally, spatial and temporal activation of DAF-16 is necessary to specify its divergent functions (Dillin et al., 2002a,b; Libina et al., 2003; Wolkow et al., 2000). What is becoming a surprising suggestion, however, is that perhaps even the redistribution of DAF-16 into the nucleus is not a necessary prerequisite for all of its activity. For example, *age-1* mutant animals do not exhibit increased levels of nuclear DAF-16 localization but are still long lived (Henderson and Johnson, 2001). Examination of a well-characterized DAF-16 target, the *sod-3* promoter, using chromatin immunoprecipitation (ChIP) found equivalent amounts of DAF-16 bound in wild type and *daf-2* mutant animals, even though much higher levels of *sod-3* are produced in a *daf-2* mutant background (Oh et al., 2006). Worms that undergo starvation for extended periods of time retain *daf-16* dependent resistance to hydrogen peroxide even though DAF-16 eventually translocates back to the cytoplasm (Weinkove et al., 2006). Finally, DAF-16 binding sites in promoters do not correlate with increased probability that DAF-16 will actually be bound; further modulation is required for DAF-16 to select its specific targets *in vivo* (Oh et al., 2006). Interestingly, nuclear localization of DAF-16 is seen in response to certain stressors such as starvation (as opposed to dietary restriction) and heat shock, but some stresses such as UV irradiation or some forms of oxidative stress do not seem to cause nuclear localization of DAF-16 in wild type worms (Henderson and Johnson, 2001; Weinkove et al., 2006). This suggests that in some settings, the DAF-2 pathway is not the primary pathway invoked in order to provide a *daf-16* dependent response to environmental stressors.

One potential possibility is that a parallel pathway(s) regulates stress response in a DAF-16 dependent manner. Consistent with this hypothesis, overexpression of the his-

tone deacetylase SIR-2.1 extends lifespan in a *daf-16* dependent manner, and the long lifespan of *daf-2* mutants cannot be extended further by the overexpression *of sir-2.1* – but complete loss *of sir-2.1* does not affect the extended lifespan of *daf-2* mutant animals (Tissenbaum and Guarente, 2001; Wang and Tissenbaum, 2006). SIR-2.1 can also regulate the response to dietary restriction in a *daf-16* independent fashion; this pathway may be invoked to mediate both general stress response and the response to dietary restriction (Wang and Tissenbaum, 2006). Mammalian SIR2 can bind to and deacetylate FOXO transcription factors, suggesting a direct interaction between the two proteins (Brunet et al., 2004; Daitoku et al., 2004). However, in worms, DAF-16 is capable of increasing lifespan only in intestinal tissues, where SIR-2.1 is not present (Libina et al., 2003; Wang and Tissenbaum, 2006). In fact, SIR-2.1 and DAF-16 overlap in expression only in a small subset of neurons (Wang and Tissenbaum, 2006). This evidence may implicate SIR-2.1 in the upstream regulation of DAF-16 and the involvement of SIR-2.1 in regulation of a cell non-autonomous reaction to changing environmental conditions.

At least four other modulations also activate DAF-16 in an IIS-independent fashion. One, similar to overexpression of *sir-2.1*, worms with an ablated germline exhibit a *daf-16* dependent extension in lifespan, but the long lifespan caused by germline ablation functions in a synergistic manner with mutations in *daf-2* (Arantes-Oliveira et al., 2002; Hsin and Kenyon, 1999). In opposition to the lifespan extension caused by germline ablation alone, animals missing their entire gonad do not exhibit an extended lifespan except when combined with some classes of *daf-2* mutants, suggesting that this extension is again in part mediated by insulin signaling (Arantes-Oliveira et al., 2002). Both *daf-2* mutants and germline-ablated animals require DAF-16 nuclear localization in the intestine (Libina et al., 2003; Lin et al., 2001). In germline-ablated animals, however, the constitutive nuclear localization of DAF-16 is not sufficient to extend longevity (Lin et al., 2001); the activity of the nuclear hormone receptor *daf-12* is also required (Berman and Kenyon, 2006). Two, unlike germline ablation, overexpression of the JUN N-terminal kinase, *jnk-1*, increases lifespan in worms by increasing the nuclear localization of DAF-16 (Oh et al., 2005). This lifespan extension is additive with known mutations in IIS, but it is not known whether the propensity for nuclear localization of DAF-16 is synergistic with *DAF-2* mutations. Three, loss of the *lin-4* microRNA target, *lin-14*, causes a *daf-16* dependent increase in lifespan which further extends the long lifespan of *daf-2* mutant animals (Boehm and Slack, 2005). Finally, activation of the *let-60* Ras pathway may modulate the effect of IIS on longevity (Nanji et al., 2005). Gain-of-function *let-60* mutations extend the maximum lifespan of *daf-2* mutant animals, but it is not known whether this occurs in a *daf-16* dependent manner (Nanji et al., 2005). *let-7* is an upstream negative regulator of both *lin-14* and *let-60* (Boehm and Slack, 2005; Johnson et al., 2005). However, in one study, the *let-7* gain of function mutation extends *daf-2* lifespan by negatively regulating *lin-14* (Boehm and Slack, 2005), while the results of the second study suggest that the loss of *let-7* should theoretically extend *daf-2* lifespan by increasing expression of *let-60* (Nanji et al., 2005). Further analysis is needed before these interactions will be fully understood, but the involvement of tightly regulated, temporally-expressed microRNAs in the determination of longevity suggests an attractive new area for aging research and fits well with paradigms that define aging as a developmental program.

These collective findings strongly support the potential for the IIS-independent regulation of DAF-16 to delay aging. Although the upstream activation of DAF-16 occurs via multiple mechanisms, it does not appear that DAF-16's selection of its target genes differs in response to any of these pathways. With the possible exception of SIR-2.1 activation of DAF-16 via deacetylation, these pathways regulate DAF-16 primarily by changing its nuclear localization and thus cannot provide specificity for target activation. Additionally, most of these mutants seem to produce mild to moderate *daf-2*-like mutant phenotypes, and the extent to which they regulate downstream targets is minor when compared to *a daf-2* mutant. Furthermore, the evidence provided thus far does not clearly implicate these pathways in specifying the initiation of a particular DAF-16 program during its particular developmental stage. Perhaps, then, the activation of all of DAF-16's downstream core processes and stress-resistance pathways are required to regulate longevity. This would be an appealing and simple answer. However, the autonomy of many of these pathways, and their individual dispensability in specific long-lived mutants, suggests otherwise.

Multiple studies have helped to expose many of the physiological processes affected by reduced insulin signaling, but in turn, each of these processes also has proven dispensable for the lifespan of at least one specific long-lived mutant. It thus remains ambiguous as to which core modules are required for delaying the onset of the aging process, if any. For example, although IIS regulates the timing and the level of reproduction by the animal, the effects on fecundity appear to be dispensable for the long lifespan of *daf-2* mutants, as *daf-2* RNAi inactivation during adulthood only can extend longevity without affecting the reproductive schedule (Dillin et al., 2002a) and not all IIS mutants with extended lifespan exhibit protracted reproductive schedules (Johnson et al., 1993). The long lifespan of some *daf-2* mutants also can be suppressed with reduced expression of DAF-16 co-regulators without affecting the protracted reproductive schedule of *daf-2* mutants (Wolff et al., 2006). Propensity towards dauer formation likewise seems independent of the regulation of longevity; not all mutants that affect dauer formation increase adult lifespan, and the spatial and temporal requirements for DAF-16 activity to regulate this function are distinct from those in its regulation of longevity (Dillin et al., 2002a; Kenyon et al., 1993).

More than reproduction or activation of dauer programs, resistance to environmental stress may in part confer the longer lifespan of IIS mutants. *daf-2* mutants are resistant to multiple physiological challenges, such as immunity to infection, thermal stress, UV irradiation, DNA damage, and oxidative stress (Barsyte et al., 2001; Henderson and Johnson, 2001; Houthoofd et al., 2005b; Johnson et al., 2002; Larsen, 1993). However, resistance to all of these stressors is not required for the increased longevity in *daf-2* mutants. For example, mutations in the heat shock factor *hsf-1* render animals extremely susceptible to protein aggregation and thermal stress, but *daf-2* mutants with reduced *hsf-1* expression still exhibit elevated levels of some stress resistant genes, such as *sod-3* and *ctl-1* (Hsu et al., 2003). Thus, the upregulation of genes protecting worms from oxidative damage is not sufficient to confer longevity in *daf-2* mutants. Overexpression of *hsf-1* can also significantly increase lifespan in a *daf-16* dependent fashion. This would suggest that increased thermotolerance is sufficient to confer increased longevity. However, in an opposing fashion, reduced *smk-1* expression in *daf-2* mutants creates worms that are short lived but remain thermotolerant (Wolff et al., 2006). Thus, thermotolerance alone is also not sufficient for the increased longevity of *daf-2* mutants. As loss of *hsf-1* has been shown to cause a premature aging phenotype in wild type worms, and its loss can also decrease the lifespan of *eat-2* mutants (diet restricted animals, see below) by up to 50%, a phenotype that is non-specific to the IIS pathway (Garigan et al., 2002; Hsu et al., 2003). This could indicate that a loss of thermal stress resistance may generally mask increases in longevity seen as a result of other mechanisms (such as increased resistance to oxidative stress).

It seems, then, that DAF-16 dependent longevity initiates more than it truly needs to extend lifespan. It is not yet clear which of these specific functions is required, and which ones are sufficient. Perhaps by examining physiological processes regulated by DAF-16 independent of longevity we can determine whether any commonalties are shared between these autonomous mechanisms.

3. Dietary restriction dependent regulation of aging

The mechanism by which dietary restriction (DR) results in an extended lifespan is unknown but appears to be conserved across phyla. Studying DR in worms has been complicated by a failure to identify a single genetic component specific to dietary restriction; this remains true despite the relative ease for gene knockdown in this model organism. One obstacle in the characterization of the genetic requirements for DR lies in a lack of consensus as to what constitutes a valid method for DR in worms. Three main methods for implementing dietary restriction have been used, but differences in downstream physiological characteristics caused by these treatments have raised questions as to whether they allow for a true method of studying DR in worms. The most traditional model for DR comes from dilution of the bacteria fed to the worms (BDR). When bacteria are diluted, DR seems to increase lifespan maximally at an intermediate concentration in a parabolic curve (see Houthoofd et al., 2002b). Too little food results in starvation and sickness, while too much food results in organisms living a wild type lifespan or less. Like IIS mutants, worms fed diluted bacteria show increased expression levels of superoxide dismutase and catalase, but unlike IIS mutants, these worms do not exhibit resistance to paraquat or hydrogen peroxide treatment (Houthoofd et al., 2003). This could indicate that these worms are resistant to lower levels of oxidative stress not tested in these experiments. The effect of BDR appears to be independent of *daf-16*, as *daf-16* null mutant animals still exhibit a slight (although significantly diminished) parabolic curve in response to BDR and an optimal DR concentration at which the lifespan of *daf-16* mutants is increased (Houthoofd et al., 2003). However, as the optimal dilution of food concentration is neared, just a slight additional removal of food will cause DAF-16 to transport dramatically inside the nucleus, a point at which a starvation response may have been initiated (Houthoofd et al., 2003). Respiration of worms undergoing BDR remains constant, indicating that metabolism may not be a contributing factor in this lifespan extension as measured (Houthoofd et al., 2002b). Worms undergoing BDR display extended reproductive schedules and reduced brood sizes (Klass, 1977) consistent with DR in other organisms, but the change to fecundity does not correlate with increased lifespan. At high concentrations, brood size remains increased while lifespan is decreased below what is seen at optimal DR conditions (Klass, 1977).

Worms grown in the absence of bacteria, in liquid supplemented with nutrients known as axenic culture conditions, exhibit extended lifespan, and this lifespan extension may be caused by dietary restriction. Growth of worms on axenic culture enhances metabolism, levels of *sod*, and levels of catalase; additionally, these worms are resistant to thermal stress (Houthoofd et al., 2002a). The reduced fecundity and extended reproductive schedule of worms on axenic culture additionally suggests that this method is another surrogate for true dietary restriction (Houthoofd et al., 2002a). The physiological characteristics of these worms, including their longer lifespan, are independent of *daf-16*. As in BDR, these worms do not exhibit severely altered localization of DAF-16 (Houthoofd et al., 2003). The fact that worms grown in axenic culture are resistant to paraquat while worms grown on BDR are not resistant suggests that this may not be a true model for DR in worms. However, mice undergoing caloric restriction are more resistant to oxidative stress than mice fed *ad libitum*, a finding which argues that this increase in stress resistance may not be an artifact of the use of axenic culture (Bartke and Brown-Borg, 2004).

The genetic model most commonly used to mimic DR in worms involves a series of mutations that slow feeding rates – *eat* mutations. Of these, *eat-2* mutants exhibit the

most significant increases in lifespan, an effect thought to be a result of their decreased rates of feeding (Lakowski and Hekimi, 1998). Unlike other worms with chemosensory mutations, however, the effects of *eat-2* mutations on lifespan are independent of *daf-16* (Lakowski and Hekimi, 1998). *eat-2* worms, like BDR worms, do not exhibit increased metabolism but do exhibit increased sod and catalase activity (Houthoofd et al., 2002b). Unlike worms grown in axenic medium or BDR, however, *eat-2* mutant worms are not thermal stress resistant, a second example in which thermal stress can be uncoupled from longevity (Houthoofd et al., 2002b).

Each of these three methods offers unique benefits but comes with caveats beyond the potential differences in the downstream processes each one affects. Implementation of BDR in the measurement of lifespan is extremely time consuming and is not readily conducive to genome-wide screens. Additionally, creation of axenic media is expensive. Although use of the *eat-2* mutants offers a simple, convenient method for implementing DR and potentially screening for other genetic mediators of the extended lifespan, further research needs to help establish it as a true model for reduced dietary intake in worms. To date, however, much ambiguity remains as to which method should be used.

Can work in other model organisms shed light onto the genetic requirements of DR in worms? In the yeast *Saccharomyces cerevisiae* and the fruit fly *Drosophila melanogaster*, SIR2 has been implicated in the response to DR (Howitz et al., 2003; Wood et al., 2004). In worms, it remains ambiguous whether the SIR2 orthologue, *sir-2.1*, regulates DR-mediated longevity. Although overexpression of *sir-2.1* extends lifespan, this extension is dependent upon *daf-16*, a master regulator of insulin/IGF-1 signaling (Tissenbaum and Guarente, 2001). Since *daf-16* is not required for the long lifespan of *eat-2* mutant animals (Lakowski and Hekimi, 1998), a genetic surrogate for DR, it is possible that *sir-2.1* is part of the insulin signaling pathway instead of the DR pathway. However, deletion of *sir-2.1* suppresses the extended lifespan of *eat-2* mutants (and not *daf-2* mutants) (Wang and Tissenbaum, 2006). This evidence suggests that *sir-2.1* in worms may work through two parallel pathways to bridge the interaction between DR and IIS for the regulation of longevity.

Additional work on DR in yeast and flies suggests the possible role for TOR signaling in mediating the effects of DR on lifespan. *tor1* mutations in yeast increase both replicative and chronological lifespan but do not further increase the lifespan of yeast undergoing DR (Kaeberlein et al., 2005; Powers et al., 2006). In worms, however, like SIR-2.1, TOR (*let-363*) appears dependent on IIS for its activity. Loss of *let-363*, *tor*, results in increased lifespan, and research suggests that the TOR adaptor protein raptor (*daf-15*) is directly repressed by active DAF-16 (Jia et al., 2004; Vellai et al., 2003). Although the extended lifespan of *let-363* mutant animals cannot be suppressed by *daf-16* mutants, the loss of *let-363* in combination with *daf-2* mutation does not further extend lifespan (Vellai et al., 2003). This suggests that the downstream mechanisms and targets for TOR and *daf-2* extension of longevity are the same. While the potential remains that TOR and SIR-2.1 affect lifespan by mediating the response to DR, more definitive results are required before firmly placing either gene within the DR pathway in *C. elegans*.

A failure to identify genetic components regulating DR that work independently of IIS could suggest that IIS and DR in worms may not work autonomously of one another, but several pieces of evidence argue otherwise. For example, the extended lifespan of *daf-2* mutants can be greatly enhanced by *eat-2* mutants (Lakowski and Hekimi, 1998). The extended lifespan of *eat-2* mutants is *daf-16* independent, and extended lifespan seen in two other models for DR is independent of *daf-16* (Houthoofd et al., 2003; Lakowski and Hekimi, 1998). *daf-2* mutants undergoing DR exhibit a characteristic amplification in their mean survival when plotted against food concentration (Houthoofd et al., 2003). If DR worked via the same mechanisms as *daf-2*, one might expect a horizontal, rather than vertical, shift in this parabolic curve. Additionally, mutations in *eat-2* do not cause a nuclear localization of DAF-16 (Henderson and Johnson, 2001). In worms undergoing DR by axenic culture or by bacterial dilution, DAF-16 also remains largely cytoplasmic (Houthoofd et al., 2003). Finally, as mentioned earlier, *daf-16* mutations block the ability for chemosensory mutants to extend lifespan, but have no effect on the ability of *eat-2* mutants to extend lifespan (Apfeld and Kenyon, 1998; Lakowski and Hekimi, 1998). This suggests that unlike DR, IIS affects the worm's sense of the environment through neuronal sensing of nutrient availability.

We thus far have suggestions of a role for SIR2, TOR, and DAF-16 as potential regulators of DR in worms, but the role for any of these genes in the modulation of DR remains highly debatable. The differences in physiological outcomes of growth on BDR, axenic medium, and *eat-2* mutation suggest that care must be taken in interpreting downstream requirements for longevity. In each method tested, however, metabolic rates remain constant or increase when compared to wild type animals, while levels of ATP production are significantly diminished (Houthoofd et al., 2002a,b). In our examination of IIS mutants, we have seen that neither resistance to oxidative damage, nor thermal stress, seems an absolute requirement for longer life. Likewise, in our examination of methods for imposing DR, again, there is no clear stress-resistance pathway common to all forms of DR. Additionally, in opposition to the multiple studies examining downstream targets for IIS, no global screen for genes conditionally regulated by DR has been conducted in worms. The mechanism for lifespan extension in worms undergoing DR thus remains largely unknown.

4. Mitochondrial mediated regulation of aging

As the primary sites for oxygen consumption within the cell, mitochondria are also the primary sites for the production of reactive oxygen species (ROS). Proper control of ROS production, via modulation of mitochondrial activity, has been predicted to increase longevity. Consistent with this prediction, research in nematodes identified the mitochondrial electron transport and ATP synthase as regulators of the aging process (Dillin et al., 2002b; Feng et al., 2001; Lee et al., 2003b).

Three main studies demonstrate that the reduced function of several mitochondrial genes extends the lifespan of worms. Each of these three studies has identified mitochondrial perturbations that increase lifespan by decreasing respiration and electron transport chain activity. Mutations in the iron sulfur component of complex III, *isp-1*, increase longevity by decreasing oxygen consumption (Feng et al., 2001). Two additional independent RNAi-based screens indicate that reduced expression of components of the mitochondrial electron transport chain could increase longevity (Dillin et al., 2002b; Lee et al., 2003b). In one screen, RNAi inactivation of components of Complex I, III, IV and the ATP synthase increased longevity of wild type worms (Dillin et al., 2002b). In the second screen, a mutation in the mitochondrial leucyl-tRNA synthetase gene impaired mitochondrial function and was associated with longer lifespan (Lee et al., 2003b).

Mutations in another worm gene potentially affecting electron transport chain function, *clk-1*, lengthens lifespan (Wong et al., 1995). *clk-1* mutations prevent the synthesis of ubiquinone (Miyadera et al., 2001), which is required for respiratory-chain activity. *clk-1* mutants are viable because they acquire ubiquinone from the bacteria in their culture media (Jonassen et al., 2001). It does not appear that *clk-1* works to extend lifespan via the same mechanisms as other known ETC mutants. For instance, *clk-1* mutants do not reduce respiration (Braeckman et al., 1999; Miyadera et al., 2001). Unlike knockdown of other ETC components, *clk-1* mutant lifespan cannot further be extended by DR, and *clk-1* mutants grown on axenic culture require a supplement of exogenous CoQ, suggesting that the synthesis of ubiquinone is required to mediate the effects of DR on longevity and that a loss of ubiquinone synthesis models a form of DR in worms (Braeckman et al., 1999; Lakowski and Hekimi, 1998). Furthermore, the synergistic effect of a *clk-1* mutation on the *daf-2* mutant lifespan can be regulated during adulthood, suggesting a temporal requirement for *clk-1* that is disparate from the timing requirement of ETC knockdown in other known long-lived mitochondrial mutants (Burgess et al., 2003; Dillin et al., 2002b).

Again, it is possible that alteration of the mitochondrial electron transport chain regulates longevity through IIS pathway components. However, *isp-1* and *clk-1* mutations or knockdown of RNAi of respiratory-chain components can extend the lifespan of *daf-16* mutant animals (Dillin et al., 2002b; Feng et al., 2001; Lee et al., 2003b; Wong et al., 1995). Additionally, the already long lifespan of *daf-2(e1370)* mutants are further extended by *isp-1* (Feng et al., 2001), *clk-1* mutations or RNAi of respiratory-chain components (Dillin et al., 2002b; Feng et al., 2001; Lee et al., 2003b; Wong et al., 1995). Furthermore, unlike reduction of respiratory-chain activity, reduced IIS causes a significant increase in ATP levels (Braeckman et al., 1999; Dillin et al., 2002b). Finally, and most compelling, following the temporal requirements of the ETC by a conditional RNAi approach, inactivation of the ETC during larval development is sufficient to confer extended longevity, whereas inactivation during adulthood does not (Dillin et al., 2002b). Because *daf-2* and *daf-16* act exclusively in adults to regulate lifespan but the ETC is required during development, the temporal requirements of these pathways are separable (Dillin et al., 2002a). Together these findings indicate that respiratory-chain RNAi does not increase lifespan by inhibiting the DAF-2 pathway.

Mitochondria are key sites of metabolic output and ROS production, suggesting that core metabolic function/ROS production of the mitochondria might be responsible for lifespan regulation. If ROS production were a primary cause for premature aging in worms, decreased mitochondrial respiration during adulthood should extend longevity. However, although ETC activity can be reduced during this stage of the worm lifecycle, this decrease in activity cannot affect lifespan. In contrast, the early temporal requirements of the ETC suggest that a regulatory mechanism is established and maintained throughout the life of the animal. Additionally, equally long-lived ETC RNAi treated animals can respond differently to oxidative stress (Lee et al., 2003b). For example, RNAi towards Cytochrome C oxidase IV extends lifespan and increases resistance to hydrogen peroxide treatment, yet RNAi toward Cytochrome C heme lyase, which also extends longevity, does not increase resistance to oxidative stress (Lee et al., 2003b). Lastly, not all mitochondrial ETC lesions increase longevity. The *mev-1(kn1)* mutation is probably the best example of a mutation that decreases mitochondrial activity, but does not increase longevity. *mev-1* encodes the cytochrome b large subunit of Complex II (Ishii et al., 1998). *mev-1(kn1)* mutant animals are hypersensitive to paraquat, short lived and have reduced mitochondrial respiratory rates (Hosokawa et al., 1994; Ishii et al., 1998; Ishii et al., 1990). Additionally, *mev-1(kn1)* mutant animals have higher levels of oxygen free radicals compared to wild type animals (Senoo-Matsuda et al., 2001).

Reduced mitochondrial function, by either RNAi or mutation, can result in increased longevity. However, it is not clear that reduced mitochondrial metabolic function is responsible for the extended longevity in these experiments. Although many ETC perturbations do indeed result in increased resistance to external stresses, such as excess ROS, this can be uncoupled from the aging process since some perturbations that result in increased longevity are in fact hypersensitive to ROS. Therefore, it remains ambig-

uous as to whether which stresses, if any, unify this genetic regulatory switch that results in increased longevity.

5. Commonalities among the pathways?

Our examination of the three pathways towards longevity has failed to indicate a clear mechanism for the regulation of longevity. IIS mutants, worms undergoing DR, and worms undergoing decreased mitochondrial respiration all show divergent responses to the myriad of stressors tested, including oxidative stress, thermal stress, DNA damage, and innate immunity. While one long-lived mutant might be extremely resistant to paraquat, for example, another (such as worms treated with ETC RNAi) might be more sensitive.

If a unified stress response does not appear to unite these three pathways for the regulation of longevity, what then does? One suggestion is that each pathway, on its own and during its own temporal mode, functions to regulate a key metabolic alteration that drives the animal into a hyperprotective mode. One such alteration could include a general shift away from glycolytic pathways towards those involved with fat and glycogen storage. IIS mutants upregulate multiple classes of metabolic genes, suggesting that a metabolic shift may in part mediate its effects on longevity (Murphy et al., 2003; Oh et al., 2006; Vanfleteren and Braeckman, 1999). Multiple downstream targets of DAF-16 are required for the increased fat and glycogen storage of *daf-2* mutants, including well-placed genes in the metabolic pathways such as acetyl CoA synthetase (Kimura et al., 1997; Oh et al., 2006). However, increased fat storage in itself is not required for increased longevity, as TGF-β mutant animals accumulate fat, yet are not long lived (Kenyon et al., 1993). *daf-2* mutants downregulate the expression of the amino acid transporter *pep-2*, a gene which has been associated with TOR mediated increases in longevity, (Meissner et al., 2004; Murphy et al., 2003) and negatively regulate growth by downregulating the expression of rapTOR itself (Jia et al., 2004). It appears that active DAF-16 might also downregulate genes required for the metabolism of amino acids or to prepare for increased fatty acid oxidation, including aminopeptidases, carboxypeptidases, amino-oxidases, aminoacylases, and genes involved in the glyoxylate cycle (Murphy et al., 2003). Thus *daf-2* mutants may exhibit a general trend away from the usage of fat storage. IIS mutants also exhibit significant increases in ATP levels (Braeckman et al., 1999; Dillin et al., 2002b).

Less is known about the effects of DR on the metabolic pathways of worms. Mice undergoing DR, for example, exhibit the upregulation of multiple gluconeogeneic genes (Hagopian et al., 2003), and both worms and yeast exhibit increased storage of glycogen upon either IIS mutation or DR (Kimura et al., 1997; Powers et al., 2006). It is unknown whether long-lived worms with altered mitochondrial function require a metabolic shift towards storage in order to extend lifespan. Decreases in ATP levels would traditionally suggest an increase in activity of glycolytic regulators like phosphofructokinase in order to use up glucose and produce more energy. However, in situations in which feeding and glucose intake remain constant, the loss of ETC components would result in a move towards anaerobic respiration, away from oxidative respiration, and a subsequent lower return on glucose utilization. This shift could instigate an upregulation in gluconeogenesis in the efforts to keep glucose levels more constant in the organism. It would be interesting to determine whether this shift is required for ETC mutants to extend longevity. Alternatively, feeding worms a high caloric diet might suppress the extended longevity of these mutants. This may yield a parallel suppression among IIS mutants, ETC mutants, and worms undergoing DR, and would thus suggest the first commonality of a requirement for all known pathways affecting longevity.

It is evident that there is to date no clear mechanistic explanation for the increased longevity of IIS, DR, or ETC mutants. Unfortunately, changes to any of these three pathways result in pleiotrophic effects upon the organism. It then becomes imperative for aging researchers to focus their efforts on finding means to determine the specificity of these pathways, and, subsequently, the components that are absolutely required in order to increase health, stave off disease, and extend lifespan among these animals.

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