Dietary restriction involves NAD+-dependent mechanisms and a shift toward oxidative metabolism

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Dietary restriction involves NAD⁺-dependent mechanisms and a shift toward oxidative metabolism

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Introduction

The reduction of food consumption without malnutrition, termed dietary restriction (DR), is the most conserved intervention known to increase lifespan. DR promotes longevity in essentially all eukaryotes, including yeast, rotifers, spiders, Drosophila, C. elegans, many strains of mice, and possibly non-human primates (Fontana et al., 2010; Haigis & Sinclair, 2010; Guarante, 2013; Colman et al., 2014). In mammals, DR improves many physiological parameters that are associated with aging, and delays aging-associated disorders ranging from neurodegenerative disease to cancer. An understanding of how DR confers these benefits may allow development of interventions that mimic the effect of DR, but circumvent the difficulties of severely reducing nutrient intake.

The response to DR is thought to be an evolutionary adaptation to survive periods of low food availability, predicting that mechanisms through which DR extends lifespan are likely to be conserved. Indeed, genetic analyses of the model organisms S. cerevisiae, D. melanogaster, and C. elegans have implicated essential nutrient-sensing mechanisms in the benefits of DR. Considerable evidence indicates that DR acts on the nutrient-sensing mechanistic target of rapamycin complex 1 (mTORC1) kinase, which is activated by amino acid availability, oxygen, and growth signaling (Fontana et al., 2010; Johnson et al., 2013). Lower levels of mTORC1 activity, as would be expected to be encountered in DR, result in reduced protein and lipid synthesis, increased autophagy and stress-defense activity, enhanced regenerative capacity, and in longer life in various organisms.

Sirtuins, which modulate transcription and numerous metabolic processes, comprise another set of energy-sensing mechanisms involved in DR (Haigis & Sinclair, 2010; Guarante, 2013). Sirtuins are protein deacetylases or ADP-ribosyltransferases that convert nicotinamide adenine dinucleotide (NAD⁺) into nicotinamide (Nam), which in turn inhibits sirtuins. It has been proposed that DR promotes health and longevity by activating the nuclear sirtuin Sir2 (SIRT1 in mammals) (Haigis & Sinclair, 2010; Guarante, 2013). In mammals, SIRT1 and other sirtuins mediate DR effects on parameters such as mitochondrial biogenesis, metabolism, body weight, and glucose tolerance, and in mice overexpression of SIRT1 in the brain increases lifespan (Haigis & Sinclair, 2010; Guarante, 2013; Satoh et al., 2013). While these data suggest a major role for sirtuins in DR, the involvement of sirtuins in DR lifespan extension has remained a subject of debate. In lower organisms, the requirement for SIRT1 for DR longevity depends upon the experimental conditions, and in C. elegans, the SIRT1 ortholog sir-2.1 was required for lifespan extension by a weak mutation in eat-2, a genetic DR model, but not in other eat-2 experiments or DR methods that utilize bacterial food dilution (Mair et al., 2009; Kenyon, 2010; Burnett et al., 2011; Guarante, 2013).

From yeast to mammals, sirtuin activity is increased by conditions that increase NAD⁺ availability or reduce Nam levels (Haigis & Sinclair, 2010; Guarante, 2013). Interventions that boost NAD⁺ levels increase C. elegans lifespan and restore mitochondrial function in aging mice, in each case dependent upon sir-2.1/SIRT1 (Gomes et al., 2013; Mouchiroud et al., 2013b). C. elegans lifespan was also increased in a sir-2.1-dependent manner by reduced activity of PARPs (poly (ADP-ribose) polymerases, PARPs), which consume NAD⁺ (Mouchiroud et al., 2013a). Together, these findings support the idea that higher NAD⁺ levels
promote longevity by activating SIR-2.1/SIRT1. In yeast, DR longevity requires the nicotinamidase Pnc1, which acts in a salvage pathway that allows Nam generated by sirtuins and other NAD⁺ consumers to be recycled to NAD⁺ (Fig. S1, Supporting Information) (Anderson et al., 2003). The importance of NAD⁺ salvage in DR has not been assessed in a metazoan, but overexpression of the PNC-1 ortholog D-NAAM increases Drosophila lifespan in a Sir2-dependent manner (Balan et al., 2008). In yeast, DR shifts metabolism away from glycolysis and toward respiration, which generates NAD⁺ by consuming NADH (Guarente, 2013; Schleit et al., 2013). While DR reduces caloric availability, it has been proposed that DR paradoxically increases respiration by inducing this metabolic shift, leading to higher NAD⁺ levels that drive lifespan extension (Bishop & Guarente, 2007; Guarente, 2013).

Caenorhabditis elegans provides a powerful metazoan model for studying DR because it has a short lifespan and is amenable to genetic disruption of processes that might be essential in mammals. The genetic requirements for lifespan extension vary among C. elegans DR regimens (Greer & Brunet, 2009; Mair et al., 2009). This variation may arise because of differences in culture conditions, including differences between liquid and solid culture protocols with respect to oxygen exposure and the extent to which the animals move. This diversity has allowed identification of a wider range of DR-associated mechanisms than would be possible with one ‘standard’ protocol (Greer & Brunet, 2009, Mair et al., 2009). Here, we investigated requirements for stress defense and NAD⁺-associated mechanisms, using a new liquid feeding DR method thatRobustly extends lifespan and minimizes maintenance. DR lifespan extension depends upon stress-defense regulators that are regulated by mTOR and growth pathways, and also upon SIR-2.1. The NAD⁺ salvage pathway enzyme PNC-1 was required for DR lifespan extension but not some healthspan benefits, providing the first evidence in a metazoan that implicates NAD⁺ salvage in DR. Independently of pnc-1, DR reduced total oxygen consumption but increased the proportion of respiration devoted to ATP production. Apparently, DR drives the activity of key NAD⁺-associated mechanisms through a shift toward oxidative metabolism, but does not necessarily increase overall respiration rates.

Results

A simple liquid protocol for C. elegans dietary restriction

Among the approaches used to restrict C. elegans dietary intake, liquid DR protocols (Table 1, Table S1, Supporting Information) provide the advantage of allowing for: (i) variation of bacterial food availability, (ii) monitoring of food concentration over time, (iii) utilization of standard C. elegans strains, and (iv) scaling up for biochemical analyses. As C. elegans, liquid DR methods are generally labor intensive, we developed a protocol that requires relatively low maintenance (Fig. 1A). On day one of adulthood, worms are transferred to NGM plates seeded with bacteria that have been maintained in antibiotics for 1 week at 4 °C (treated OP50). On day three of adulthood, these worms are transferred to liquid cultures that contain treated OP50 at different concentrations (Fig. 1A). The treatment step prevents bacteria from re-entering growth phase (Fig. S2), eliminating the need for frequent food replacement.

To determine whether a gene is required for DR and does not simply alter the optimal response to DR, lifespan must be examined over a range of bacterial food concentrations (Mair et al., 2009). We designated 3 × 10⁵ C.F.U. ml⁻¹ (3.0 A₆₀₀) as ad libitum (AL) feeding, because at this concentration wild-type (WT) lifespan most closely resembled published observations on plates and in liquid (Fig. 1B,C, Table S3). A plot of mean lifespan vs. food would be parabolic if concentrations below that optimal for DR resulted in starvation-like effects (Mair et al., 2009). However, combined analyses of wild-type

Table 1 Comparison of C. elegans liquid DR methods

<table>
<thead>
<tr>
<th>Liquid DR method</th>
<th>Age at DR onset</th>
<th>Food source</th>
<th>Antibiotics present</th>
<th>Average lifespan of DR animals (days)</th>
<th>Average DR lifespan extension for WT at 20 °C (%)</th>
<th>Epistasis analysis</th>
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<tr>
<td>Klass*</td>
<td>48 hr. post-hatching</td>
<td>OP50</td>
<td>None</td>
<td>26</td>
<td>73</td>
<td>NA</td>
</tr>
<tr>
<td>Vanfleterenb</td>
<td>L4</td>
<td>E. coli 9001</td>
<td>None, 50uM FUdR</td>
<td>12*</td>
<td>140*</td>
<td>daf-2 independent, daf-16 partially required</td>
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<tr>
<td>Dillin et al.</td>
<td>Day-2 adult</td>
<td>OP50</td>
<td>50 µg mL⁻¹ Carb, 1 µg mL⁻¹ Tet, 10 µg mL⁻¹ Kan, 100 µg mL⁻¹ FUdR</td>
<td>42</td>
<td>60 (26 in smg⁻¹ ts)³</td>
<td>Requirement for aak-2 and daf-16 skn-1 required</td>
</tr>
<tr>
<td>Brunet*</td>
<td>Day-2 adult</td>
<td>OP50-1</td>
<td>50 µg mL⁻¹ Amp, 1 µg mL⁻¹ Tet, 10 µg mL⁻¹ Kan, 100 µg mL⁻¹ FUdR</td>
<td>42†</td>
<td>51†</td>
<td>skn-1 required</td>
</tr>
<tr>
<td>Guarenteb</td>
<td>L4/young adult</td>
<td>HT115</td>
<td>1 mg mL⁻¹ Erythro, 50 µg mL⁻¹ Amp, 12.5 µg mL⁻¹ FUdR, 1mM IPTG</td>
<td>33</td>
<td>22</td>
<td></td>
</tr>
<tr>
<td>Sinclair, Hart,</td>
<td>Day-3 adult</td>
<td>OP50</td>
<td>50 µg mL⁻¹ Amp, 10 µg mL⁻¹ Kan, 1 µg mL⁻¹ Tet, NYS, 100 µg mL⁻¹ FUdR</td>
<td>39 (42 DD in WT) (39 DD in smg⁻¹ ts)</td>
<td>60 (76 DD in WT) (45 DD in smg⁻¹ ts)</td>
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*Values approximated from graph.
†Average calculated from all presented data. ‡Animals were grown at 17 °C from hatching, at L4 were switched to 24 °C for remainder of lifespan. γ animals were grown at 25 °C from hatching, at first day of adulthood switched to 20 °C for 1 day, then 15 °C for remained of lifespan.

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DR involves NAD+ salvage and an oxidative shift, N. Moroz et al. 1077

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We compared our protocol to other methods by investigating whether lifespan extension required a set of regulators that were needed in some but not necessarily all C. elegans DR protocols in which they were examined. We first looked at the transcription factors DAF-16/FOXO, SKN-1/Nrf 1,2,3, and PHA-4/FOXA, each of which is regulated by mTORC1 signaling (Fig. S4) (Johnson et al., 2013). DAF-16/FOXO is required for longevity from reduced activity of insulin/GF-1 signaling (IIS) or mTORC1 (Fig. S4) (Kenyon, 2010; Robida-Stubbs et al., 2012). DAF-16 is completely or partially required for lifespan extension by some DR protocols (Greer et al., 2007; Greer & Brunet, 2009, Honjish et al., 2009), but appears to be dispensable in other DR regimens (summarized in Greer & Brunet, 2009). Our data are consistent with the former view: a lack of DAF-16 substantially reduced lifespan extension from either DR (from 58.2% to 26.3%) or DD (from 93.4% to 39%) (Fig. 2A,B, Table S4). SKN-1/Nrf has important functions in stress and starvation responses, proteostasis, and metabolism, and contributes to lifespan extension from downregulation of either IIS or mTORC1 (Fig. S4) (Kenyon, 2010; Robida-Stubbs et al., 2012). SKN-1/Nrf 1,2,3, and PHA-4/FOXA, each of which is regulated by mTORC1 signaling (Fig. S4) (Johnson et al., 2013). skn-1 was required for longevity in a liquid DR protocol that was less robust than this one, but not in a plate DR regimen (Table 1) (Bishop & Guarente, 2007; Greer & Brunet, 2009). Here, lack of skn-1 greatly impaired the lifespan increase from DR (from 48.3% to 20.9%) or DD (from 91% to 13.3%) (Fig. 2C,D, Table S5). PHA-4/FOXA is involved in autophagy and is required for lifespan extension from reduced TOR activity, the genetic DR model eat-2, and a liquid DR method (Panowski et al., 2007; Sheaffer et al., 2008), although it was not required in a DR protocol that involved solid media (Greer & Brunet, 2009). pha-4 was needed for DR lifespan extension in our protocol, although DR was less effective in the smg-1(ts) background in which the pha-4 mutation is maintained (Panowski et al., 2007; Sheaffer et al., 2008) (23.2% lifespan increase in smg-1 (ts) vs. 77.3% in WT from DR, and 44.6% vs. 77.3% from DD) (Fig. 2E, F, Table S6).

We also looked at the 5’ AMP-activated kinase (AMPK), which coordinates an adaptive response to low-energy availability that enhances oxidative metabolism, mitochondrial biogenesis, and autophagy, and is inhibited by mTORC1 in mammals (Mair et al., 2011). Increased activity of the AMPK catalytic subunit AAK-2 increases C. elegans lifespan (Apfeld et al., 2004; Mair et al., 2011) by inhibiting the transcriptional co-activator CRTIC (Mair et al., 2011) and activating DAF-16/FOXO (Greer et al., 2007; Greer & Brunet, 2009). AMPK was important for DR lifespan extension in Drosophila (Stenesen et al., 2013), and in C. elegans was required in some DR methods but not others (Greer et al., 2007; Greer & Brunet, 2009; Mair et al., 2009). In

Genetic analysis of DR-associated mechanisms

We compared our protocol to other methods by investigating whether lifespan extension required a set of regulators that were needed in some but not necessarily all C. elegans DR protocols in which they were examined. We first looked at the transcription factors DAF-16/FOXO, SKN-1/Nrf 1,2,3, and PHA-4/FOXA, each of which is regulated by mTORC1 signaling (Fig. S4) (Johnson et al., 2013). DAF-16/FOXO is required for longevity from reduced activity of insulin/GF-1 signaling (IIS) or mTORC1 (Fig. S4) (Kenyon, 2010; Robida-Stubbs et al., 2012). DAF-16 is completely or partially required for lifespan extension by some DR protocols (Greer et al., 2007; Greer & Brunet, 2009, Honjish et al., 2009), but appears to be dispensable in other DR regimens (summarized in Greer & Brunet, 2009). Our data are consistent with the former view: a lack of DAF-16 substantially reduced lifespan extension from either DR (from 58.2% to 26.3%) or DD (from 93.4% to 39%) (Fig. 2A,B, Table S4). SKN-1/Nrf has important functions in stress and starvation responses, proteostasis, and metabolism, and contributes to lifespan extension from downregulation of either IIS or mTORC1 (Fig. S4) (Kenyon, 2010; Robida-Stubbs et al., 2012). skn-1 was required for longevity in a liquid DR protocol that was less robust than this one, but not in a plate DR regimen (Table 1) (Bishop & Guarente, 2007; Greer & Brunet, 2009). Here, lack of skn-1 greatly impaired the lifespan increase from DR (from 48.3% to 20.9%) or DD (from 91% to 13.3%) (Fig. 2C,D, Table S5). PHA-4/FOXA is involved in autophagy and is required for lifespan extension from reduced TOR activity, the genetic DR model eat-2, and a liquid DR method (Panowski et al., 2007; Sheaffer et al., 2008), although it was not required in a DR protocol that involved solid media (Greer & Brunet, 2009). pha-4 was needed for DR lifespan extension in our protocol, although DR was less effective in the smg-1(ts) background in which the pha-4 mutation is maintained (Panowski et al., 2007; Sheaffer et al., 2008) (23.2% lifespan increase in smg-1 (ts) vs. 77.3% in WT from DR, and 44.6% vs. 77.3% from DD) (Fig. 2E, F, Table S6).

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our system, aak-2 loss reduced lifespan extension from DR (from 64.4% to 14.5%) or DD (from 66.4% to 30%) (Fig. 2G,H, Table S7).

Importance of SIR-2.1 and NAD+ salvage in DR

SIRT1 has been implicated in DR in various organisms, but was required for DR lifespan extension in only one of several previous C. elegans studies (Introduction; Table 1). SIRT1/SIR-2.1 functions both upstream and downstream of AMPK and activate DAF-16/FOXO (Canto et al., 2009; Rizki et al., 2011; Price et al., 2012; Guarente, 2013; Mouchiroud et al., 2013a), suggesting that it might be important in our DR regimen. Accordingly, a null sir-2.1 mutation significantly reduced lifespan extension from either DR (from 69.5% to 40.5%) or DD (from 89.4% to 47.4%) (Fig. 3A,B, Table S8). The importance of sir-2.1 for DR-associated longevity was particularly striking because the sir-2.1 mutant lived slightly longer than WT under AL conditions (Fig. 3B, Table S8).

We considered the possibility that the three additional C. elegans sirtuin genes might have DR-related functions that overlap with those of sir-2.1. sir-2.2 and sir-2.3 are nearly identical orthologs of the mitochondrial sirtuin SIRT4, and sir-2.4 is orthologous to the nuclear sirtuins SIRT6 and SIRT7. Each of these sirtuins modulates critical
metabolic processes (Haigis & Sinclair, 2010), and in C. elegans, SIR-2.4 acts non-catalytically to promote DAF-16 activity under stress conditions (Chiang et al., 2012). As sir-2.2 and sir-2.3 are located less than one kilobase apart (wormbase.org), making it difficult to disrupt them simultaneously, we analyzed the triple-null mutants sir-2.1; sir-2.2; sir-2.4 and sir-2.1; sir-2.3; sir-2.4. Each of these mutants responded to DR and DD comparably to sir-2.1 (Fig. 3C–F, Tables S9 and S10). The data do not reveal any additive requirement for these sirtuins and sir-2.1, but do not exclude the possibility that redundancy might mask a role for sir-2.2 and sir-2.3.

The importance of sir-2.1 for DR lifespan extension predicts that NAD+ availability would be a critical factor. Moreover, NAD+ has critical sirtuin-independent functions that could be important in DR (Pollak et al., 2007). NAD+ serves as a co-enzyme in numerous metabolic electron transfer reactions, and its reduced form NADH plays a central role in mitochondrial electron transport. NAD+ is also the precursor to NADP+ (NAD phosphate), the reduced form of which (NADPH) is essential for cellular oxidative defense and other reductive detoxification reactions (Pollak et al., 2007). We investigated the role of NAD+ in DR by examining requirements for PNC-1, the C. elegans ortholog of the NAD+ salvage nicotinamidase Pnc1 (Fig. S1) (Vrablik et al., 2009). A predicted null pnc-1 mutation dramatically reduced the lifespan increase associated with either DR (from 57.2% to 32.1% at OD 0.5 and 77.2 to 27 at OD 0.3) or DD (from 82% to 15%) (Fig. 3G,H, Table S11), suggesting that the NAD+ salvage pathway plays a major role in C. elegans DR.

**Fig. 3** The NAD+ salvage pathway and NAD+-dependent SIRT1/sir-2.1 regulate DR lifespan. (A, B) Reduced DR response in sir-2.1(ok434) mutants. Note that the sir-2.1 mutants live longer than WT worms under AL feeding. (C-F) Impairment of DR lifespan extension in sir-2.1;sir-2.2;sir-2.4 (C, D) and sir-2.1;sir-2.3;sir-2.4 (E, F) triple mutants. (G, H) pnc-1 is required for DR lifespan extension. Composites are shown, with data from individual experiments presented in Table S17 (Fig. 3A,B), Table S18 (Fig. 3C, D), Table S19 (Fig. 3E,F), and Table S20 (Fig. 3G,H). Two-way ANOVA analysis: **P = 0.0001.
In metazoa, DR not only extends lifespan but improves many parameters associated with health and resistance to chronic disease and slows their decline during aging (Fontana et al., 2010; Haigis & Sinclair, 2010; Guarente, 2013). This beneficial effect on ‘healthspan’ has been described not only in mammals but also in C. elegans, in which DR increases muscle activity (Greer et al., 2007). We investigated the importance of the NAD+ salvage pathway for muscle activity by comparing how DR affects movement in WT and pnc-1 animals. In pnc-1 mutants, body-wall muscle function is impaired (Vrablik et al., 2011), and under AL conditions their rate of spontaneous body bending was reduced compared to WT (Fig. 4A, Table S21). Surprisingly, however, DR comparably increased the frequency of bending in older WT and pnc-1 animals (Fig. 4A, B, Table S21). As a second healthspan measure, we examined thermotolerance, which is characteristically increased by DR in C. elegans (Kenyon, 2010). In WT animals, heat resistance declined with age, but this decline was reversed by day 15 under DR (Fig. 4C, D, Table S22). In pnc-1 mutants, DR increased thermotolerance similarly (Fig. 4C, D, Table S22). These improvements in healthspan parameters suggest that pnc-1 mutants are not refractory to DR lifespan extension because they are simply sick. Apparently, in C. elegans, the NAD+ salvage pathway is required for DR to increase lifespan, but not to confer these healthspan benefits.

DR reduces total oxygen consumption but proportionally increases productive respiration

Another source of NAD+ is conversion from NADH during mitochondrial respiration. Based upon data from yeast, it has been proposed that DR elevates NAD+ levels by increasing respiration (Guarente, 2013). However, two C. elegans studies reached opposite conclusions with respect to DR effects on respiration, and an analysis of Drosophila did not see an increase in respiration with DR (Hulbert et al., 2004; Bishop & Guarente, 2007; Houthoofd et al., 2007). Previous C. elegans studies of DR measured oxygen consumption in liquid culture using the Clark electrode, which analyzes large samples that are difficult to generate in an age-matched fashion. We circumvented this issue by measuring oxygen consumption rate (OCR) using the Seahorse XF24 Analyzer, which can precisely examine tens of animals per replicate.

We investigated how aging, DR, and the NAD+ salvage pathway influence respiration by examining WT and pnc-1 animals under AL and DR conditions at 9, 12, and 16 days after hatching (3, 6, and 9 days of DR). Under AL conditions, the OCR per worm decreased with age in WT animals (Fig. 5A, Tables S23 and S24), in close agreement with a recent study that used the XF24 (Mouchiroud et al., 2013b). C. elegans shrink in size post-reproductively, however, and with age exhibit profound sarcopenia in pharyngeal and body-wall muscles (Herndon et al., 2002). Respiration was therefore modestly elevated in older animals on a per-protein mass basis (Day 16; Fig. 5B, Tables S25 and S26). Surprisingly, DR markedly reduced the OCR per worm at each day examined on either a per-worm or per-mass basis (Fig. 5C, D, Tables S27 and S28). Similar trends were apparent in pnc-1 mutants. Under our conditions, therefore, DR lowered overall oxygen consumption, and the NAD+ salvage pathway was not required to maintain respiration rates during DR.

The evidence that DR promotes oxidative metabolism in yeast (Guarente, 2013; Schleit et al., 2013) predicts that this might also be true in C. elegans, even if DR reduces total oxygen consumption. This is an attractive model, because it seems logical that the efficiency of ATP production might need to be increased when food availability is reduced. To test this idea, we used the mitochondrial respiration uncoupler FCCP (Brand & Nicholls, 2011) to assess the extent to which oxygen consumption increases when it is uncoupled from ATP production. This increase reflects the proportion of respiratory capacity that was unused prior to uncoupling (Brand & Nicholls, 2011). The greater this increase, the more efficient the mitochondria were in generating ATP under basal conditions.

Fig. 4 DR increases movement and stress resistance independently of NAD+ salvage. (A,B) DR increased the rate of spontaneous movement comparably in aging WT and pnc-1(pk9605) animals. Body bends per minute were scored. Note that pnc-1 mutation did not affect the percentage increase associated with DR. (C,D) DR comparably increased thermotolerance (survival at 38 °C) in WT and pnc-1 animals. The time points indicated refer to days after hatching. Composites of all analyses are shown, with individual experimental data, mean, standard error, percent change, and statistical analysis presented in Table S21 (Fig. 4A, B), and Table S22 (Fig. 4C, D). *t-test vs. AL, P < 0.05; **t-test vs. AL, P < 0.001; $t-test vs. age 13, P < 0.01; @t-test vs. age 10, P = 0.065; #t-test vs. age 10, P < 0.025.

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conditions. At each day of life examined, the response to FCCP was dramatically higher in the DR group in either WT or \( pnc-1 \) animals (Fig. 5E,F, Tables S23 and S24). This suggests that under DR conditions, a greater proportion of oxygen consumption was devoted to ATP generation. Thus, although the overall respiration rate was not increased, DR induced a shift toward oxidative metabolism that might underlie the functions of NAD+-dependent mechanisms through an increase in respiration (Guarente, 2013; Johnson et al., 2013). Here, we used a new \( C. \) elegans liquid DR protocol to examine the importance of mechanisms that are associated with these two models. Our findings are consistent with some key predictions of each hypothesis, but suggest an important revision to the second model.

**Discussion**

It has been proposed that DR lifespan extension is mediated through reduction of mTORC1 signaling, and activation of sirtuins and other NAD+-dependent mechanisms through an increase in respiration (Guarente, 2013; Johnson et al., 2013). Here, we used a new \( C. \) elegans liquid DR protocol to examine the importance of mechanisms that are associated with these two models. Our findings are consistent with some key predictions of each hypothesis, but suggest an important revision to the second model.

**Importance of stress defense and nutrient-sensing pathways in DR**

We found that DR lifespan extension involved stress-defense regulators that are required in other longevity interventions (Kenyon, 2010), but...
were not necessarily essential in other *C. elegans* DR methods. One of these was DAF-16/FOXO (Figs 2A and 6), which some but not all other studies implicated in DR (Greer et al., 2007; Greer & Brunet, 2009; Honjo et al., 2009). Interestingly, the response to DR is improved by impairment of the insulin/IGF-1 receptor DAF-2, which inhibits DAF-16 (Bishop & Guarente, 2007). Together, these findings suggest that IIS, which senses nutrients, might be important in DR (Fig. 6). Our DR lifespan extension also depended upon SKN-1/Nrf (Figs 2C and 6), which is inhibited by IIS in parallel to DAF-16 (Tullet et al., 2008). Another study indicated that DR induces SKN-1 to increase respiration (Bishop & Guarente, 2007) but we observed that DR reduces oxygen consumption (Fig. 5C,D), suggesting that SKN-1 has additional functions in DR. PHA-4/FOXA was also required for DR longevity in our protocol, although the background *smg-1(tts)* mutation substantially impaired the response to DR (Fig. 2E). The SMG-1 kinase functions in nonsense-mediated decay, in which translationally stalled mRNAs are degraded (Sheaffer et al., 2008). Perhaps, clearing of stalled mRNAs by nonsense-mediated decay is important in DR.

Each of these transcription factors is functionally inhibited by mTORC1 signaling (Fig. 6) and required for longevity that results from inhibiting this pathway (Sheaffer et al., 2008; Robida-Stubbbs et al., 2012). The proposed importance of mTORC1 in DR (Johnson et al., 2013) also fits with the importance of the low-energy sensor AMPK (Greer et al., 2007; Greer & Brunet, 2009) (Figs 2G and 6), which inhibits mTORC1 in many species (Johnson et al., 2013). Our findings support the view that DR extends lifespan in part by strengthening stress defenses through a reduction in mTORC1 signaling, and possibly IIS (Fig. 6). The importance of *daf-16*, *skn-1*, and *aak-2* that we observed differed from results of some *C. elegans* studies that examined these genes individually (see above). Perhaps, these mechanisms may control overlapping and possibly compensatory processes. It is also possible that our DR method has more stringent requirements for lifespan extension than some other protocols because the animals spend more time on AL feeding prior to DR (Table 1).

**NAD*+-dependent mechanisms, respiration, and DR**

Our data also indicate the importance of NAD*-dependent mechanisms in DR and represent the first time that SIR-2.1/SIRT1 was required for DR lifespan extension in a *C. elegans* feeding protocol (Fig. 3A,B). DR increased lifespan in *sir-2.1* mutants, which were slightly long-lived, but this lifespan extension was consistently reduced compared to WT across four experiments that were performed in our three laboratories (Table S8). The importance of SIR-2.1 in our DR regimen is consistent with the evidence that SIRT1 mediates many metabolic effects of DR in mammals (Haigis & Sinclair, 2010; Guarente, 2013), and strongly supports the idea that SIR-2.1/SIRT1 plays a major role in DR.

DR lifespan extension appeared to be less effective in *pnc-1* than *sir-2.1* mutants, revealing for the first time in a metazoan that the NAD*+ salvage pathway is critical in DR, and suggesting that its importance might reflect the activity of NAD*+ consumers besides SIR-2.1/SIRT1 (Fig. 3A,G; Table 2). However, DR improved two healthspan parameters independently of *pnc-1* (Fig. 4; Tables S20 and S21), indicating that *pnc-1* mutants were not simply sick or completely refractory to DR. This result also indicates that some DR benefits may not require NAD*-mediated signals; therefore, that some effects of DR on healthspan can be uncoupled mechanistically from its longevity effects. Using *C. elegans* genetics, it may be possible to unravel how DR influences different parameters associated with health, and to elucidate how they contribute to long life.

It is an important question which NAD*+ consumers besides SIR-2.1/SIRT1 might contribute to DR lifespan extension. Poly-ADP ribose polymerase (PARP) proteins consume NAD*+ but are unlikely to play a positive role in DR because reducing their activity increases *C. elegans* lifespan (Mouchiroud et al., 2013a). However, we cannot exclude an important function for the predicted redundant sirtuins SIR-2.2 and SIR-2.3 (SIRT4; Fig. 3C,E), and in some tissues, NAD*+ generated by *pnc-1* might maintain proper levels of NADP (and NADPH) or activity of the many NAD*-dependent metabolic processes (Pollak et al., 2007). It might also be critical for *pnc-1* to metabolize NAM, excess levels of which recapitulate some developmental *pnc-1* phenotypes (Vrablik et al., 2009). Administration of exogenous NAM extends *C. elegans* lifespan by means of metabolites that increase ROS formation (Schmeisser et al., 2013), but a positive role for NAM in DR seems unlikely given that DR requires *pnc-1*, which metabolizes NAM.

Our surprising finding that DR sharply reduced the overall OCR (Fig. 5C,D; Tables S23–S28) would seem to be strong evidence against

**Table 2 Maximum affect of DR on lifespan across mutant strains**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Average max % increase lifespan vs. AL</th>
<th>SEM</th>
<th>Food conc. (g/AL)</th>
<th># Indep. expr.</th>
<th>F-value 2-way ANOVA vs. Ctrl.</th>
</tr>
</thead>
<tbody>
<tr>
<td>N2</td>
<td>85</td>
<td>0.01</td>
<td>0.01</td>
<td>21</td>
<td>NA</td>
</tr>
<tr>
<td>daf-16</td>
<td>39</td>
<td>0.2</td>
<td>0.00</td>
<td>3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>skn-1</td>
<td>21</td>
<td>1.3</td>
<td>0.50</td>
<td>6</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>aak-2</td>
<td>30</td>
<td>1.1</td>
<td>0.00</td>
<td>2</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>sir-2.1</td>
<td>47</td>
<td>1.3</td>
<td>0.00</td>
<td>4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>sir-2.1;2,2;2,4</td>
<td>36</td>
<td>2.7</td>
<td>0.00</td>
<td>2</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>sir-2.1;2,3;2,4</td>
<td>62</td>
<td>1.6</td>
<td>0.00</td>
<td>2</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>pnc-1</td>
<td>32</td>
<td>0.9</td>
<td>0.50</td>
<td>10</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>smg-1</td>
<td>45</td>
<td>2.5</td>
<td>0.00</td>
<td>4</td>
<td>NA</td>
</tr>
<tr>
<td>pha-4;smg-1</td>
<td>10</td>
<td>1.0</td>
<td>0.00</td>
<td>4</td>
<td>0.0004</td>
</tr>
</tbody>
</table>

This table presents the DR food concentration at which each strain experiences its maximal lifespan extension, in comparison to its lifespan on AL food, as well as what the percent increase in lifespan is. The wild-type N2 is the control for all strains except for *pha-4;smg-1*, whose genetic control is *smg-1*. The percent change experienced by the control strains is in bold.

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the view that DR exerts its beneficial effects by increasing respiration (Bishop & Guarente, 2007; Guarente, 2013). Importantly, however, DR increased the proportion of oxygen consumption that is devoted to ATP generation (Fig. 5E; Tables S23 and S24). This shift toward oxidative metabolism, which we documented for the first time in a metazoaon, seems to be a fitting response to lower food availability because respiration is more efficient than glycolysis. It will be of interest to determine whether this shift is also seen in other C. elegans DR protocols, including those involving solid culture media. Our data suggest a revised version of the respiration-based model for DR, whereby the driving force for activity of NAD+–dependent processes that delay aging is this respiratory shift (Fig. 6), not increased respiration per se. This respiratory shift might also influence the mitochondrial unfolded response and possibly other signals from mitochondria that could affect lifespan (Houtkooper et al., 2013; Mouchiroud et al., 2013b; Schleit et al., 2013; Schmeisser et al., 2013). The next challenge will be to explore these ideas by identifying mechanisms that are modulated by SIR-2.1 and other key NAD+–dependent processes in the setting of DR, and elucidating how they and other mitochondrial-associated processes are affected by the metabolic changes that are driven by DR.

**Experimental procedures**

Full methods and experimental procedures are available in Data S1 (Supporting Information).

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**Conflict of interest**

None declared.

**Author contributions**

N. M., J. J. C., and E. A. performed experiments, all authors designed and interpreted experiments, A. C. H., D. A. S. and T. K. B. directed the project, and N. M., A. C. H., D. A. S. and T. K. B. wrote the manuscript.

**References**


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DR involves NAD⁺ salvage and an oxidative shift, N. Moroz et al.


Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site.

Fig. S1 The NAD⁺ salvage pathway.

Fig. S2 Analysis of treated bacteria.

Fig. S3 Effects of the Mitochondrial Uncoupler FCCP on AL and DR animals.

Fig. S4 Regulation of longevity transcription factors by the IIS and TORC1 pathways.

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Table S28 Individual DR experiments performed on wild-type animals shown in Fig. 18A,B.

Table S29 Individual DR experiments performed on wild-type animals shown in Fig. 19A,B.

Table S30 Individual DR experiments performed on wild-type animals shown in Fig. 20A,B.

Table S31 Individual DR experiments performed on wild-type animals shown in Fig. 21A,B.

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Table S33 Individual DR experiments performed on wild-type animals shown in Fig. 23A,B.

Table S34 Individual DR experiments performed on wild-type animals shown in Fig. 24A,B.

Table S35 Individual DR experiments performed on wild-type animals shown in Fig. 25A,B.

Table S36 Individual DR experiments performed on wild-type animals shown in Fig. 26A,B.

Table S37 Individual DR experiments performed on wild-type animals shown in Fig. 27A,B.

Table S38 Individual DR experiments performed on wild-type animals shown in Fig. 28A,B.

Table S39 Individual DR experiments performed on wild-type animals shown in Fig. 29A,B.

Table S40 Individual DR experiments performed on wild-type animals shown in Fig. 30A,B.

Table S41 Individual DR experiments performed on wild-type animals shown in Fig. 31A,B.

Table S42 Individual DR experiments performed on wild-type animals shown in Fig. 32A,B.

Table S43 Individual DR experiments performed on wild-type animals shown in Fig. 33A,B.

Table S44 Individual DR experiments performed on wild-type animals shown in Fig. 34A,B.

Table S45 Individual DR experiments performed on wild-type animals shown in Fig. 35A,B.

Table S46 Individual DR experiments performed on wild-type animals shown in Fig. 36A,B.

Table S47 Individual DR experiments performed on wild-type animals shown in Fig. 37A,B.

Table S48 Individual DR experiments performed on wild-type animals shown in Fig. 38A,B.

Table S49 Individual DR experiments performed on wild-type animals shown in Fig. 39A,B.

Table S50 Individual DR experiments performed on wild-type animals shown in Fig. 40A,B.

Table S51 Individual DR experiments performed on wild-type animals shown in Fig. 41A,B.

Table S52 Individual DR experiments performed on wild-type animals shown in Fig. 42A,B.

Table S53 Individual DR experiments performed on wild-type animals shown in Fig. 43A,B.

Table S54 Individual DR experiments performed on wild-type animals shown in Fig. 44A,B.

Table S55 Individual DR experiments performed on wild-type animals shown in Fig. 45A,B.

Table S56 Individual DR experiments performed on wild-type animals shown in Fig. 46A,B.

Table S57 Individual DR experiments performed on wild-type animals shown in Fig. 47A,B.

Table S58 Individual DR experiments performed on wild-type animals shown in Fig. 48A,B.

Table S59 Individual DR experiments performed on wild-type animals shown in Fig. 49A,B.

Table S60 Individual DR experiments performed on wild-type animals shown in Fig. 50A,B.

Table S61 Individual DR experiments performed on wild-type animals shown in Fig. 51A,B.

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