ARTICLES

PHA-4/Foxa mediates diet-restriction-induced longevity of *C. elegans*

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Reduced food intake as a result of dietary restriction increases the lifespan of a wide variety of metazoans and delays the onset of multiple age-related pathologies. Dietary restriction elicits a genetically programmed response to nutrient availability that cannot be explained by a simple reduction in metabolism or slower growth of the organism. In the nematode worm *Caenorhabditis elegans,* the transcription factor PHA-4 has an essential role in the embryonic development of the foregut and is orthologous to genes encoding the mammalian family of Foxa transcription factors, *Foxa1*, *Foxa2* and *Foxa3*. Foxa family members have important roles during development, but also act later in life to regulate glucagon production and glucose homeostasis, particularly in response to fasting. Here we describe a newly discovered, adult-specific function for PHA-4 in the regulation of diet-restriction-mediated longevity in *C. elegans*. The role of PHA-4 in lifespan determination is specific for dietary restriction, because it is not required for the increased longevity caused by other genetic pathways that regulate ageing.

The insulin/IGF-1 signalling (IIS) pathway is a key regulator of the ageing process in worms, flies and mice, but its role in the regulation of diet-restriction-mediated longevity remains ambiguous[1–6]. Perfunctorily, it seems probable that the regulation of nutrient homeostasis and ageing by the IIS pathway might overlap with any regulatory networks affected by dietary restriction. However, prior research in worms suggests that diet-restriction-mediated increases in longevity can occur independently of the forkhead box O (FOXO) transcription factor DAF-16 (refs 7, 8), whereas the extended longevity of all known IIS mutants is completely dependent on DAF-16 (refs 4, 9–12); thus it seems unlikely that reduced food intake simply elicits an environment of reduced insulin signalling. From this hypothesis, we initially predicted that genetic components would not be shared across these two pathways.

Genetically, *smk-1* is an essential co-regulator of the longevity function of *daf-16*, and our previous data suggested a relationship in which these two genes cannot affect lifespan independently of each other[13]. *daf-16* is dispensable for the long lifespan of *eat-2(ad1116)* mutant animals (a genetic surrogate of dietary restriction exhibiting a reduced rate of pharyngeal pumping representative of eating)[8,14]. Thus, we were surprised to find that *smk-1* was required for the extended lifespan of *eat-2(ad1116)* mutant animals (see Methods, Fig. 1a and Supplementary Table 1). Consistent with previous results[7,8], we confirmed that *daf-16* is dispensable for diet-restriction-mediated longevity (Fig. 1b, Supplementary Fig. 1 and Supplementary Table 2). These data elicited the hypothesis that under conditions of low nutrient signalling, *smk-1* could interact genetically with a forkhead-like transcription factor other than *daf-16* to mediate the transcriptional response to dietary restriction. We systematically inactivated each of the fifteen forkhead-like genes found within the completed *C. elegans* genome[15] to examine their role in dietary restriction. RNA interference (RNAi) of only one, *pha-4*, completely suppressed the long lifespan of *eat-2(ad1116)* mutant animals (Fig. 1c and Supplementary Table 3). PHA-4 is orthologous to the human Foxa family of transcription factors (Supplementary Fig. 2)[16]. *Foxa1* homozygous mutant mice die shortly after birth, do not gain weight and are hypoglycaemic, suggesting an important role for *Foxa1* in pancreatic cell function and a central role in metabolic homeostasis[17,18]. *Foxa2* is also required for glucagon expression in the pancreas and induction of gluconeogenic genes during fasting in the liver[19]. *Foxa3* mutant mice become hypoglycaemic after a prolonged fasting[20,21]. The bifunctional role for Foxa family members in development and metabolic homeostasis of mammals prompted us to investigate further a potential role for *pha-4* in the regulation of metabolism and diet-restriction-mediated longevity of the adult worms in addition to its known role in development of the worm[22,23].

pha-4 is required for multiple forms of dietary restriction

Because *pha-4* is required for development of the worm pharynx, and *eat-2* mutations affect pharyngeal pumping rates, we tested whether a loss of *pha-4* suppressed dietary restriction in a non-genetic model. In the worm, dietary restriction can also be achieved by limiting the concentration of bacteria fed to worms in culture by bacterial dietary restriction (BDR)[24]. At high and extremely low food concentrations, wild-type animals are short lived, whereas conditions of optimal food intake result in increased longevity (see Methods, Fig. 1b and Supplementary Table 2). Much like wild-type animals, and in agreement with previous results[7], *daf-16(mu86)*-null mutant animals were longer lived at the optimal concentration and shorter lived at lower and higher concentrations, exhibiting a parabolic curve (Fig. 1b and Supplementary Fig. 1). In contrast, *pha-4(zu225);smg-1(cc546ts)* mutant worms, but not *smg-1(cc546)* control mutant worms (Supplementary Fig. 3), were short lived at all concentrations and did not exhibit a parabolic curve in response to varying food concentrations. A loss of *pha-4* fully blocked the entire response of lifespan to dietary restriction, as would be expected of a gene essential for diet-restriction-mediated longevity (Fig. 1b). In all experiments, transfer to restrictive temperatures to inactivate *pha-4* (ref. 22) as well as dietary restriction treatment itself was delayed until the first day of adulthood to avoid possible developmental abnormalities[22] (see

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Methods). We concurrently confirmed that a loss of *smk-1* using this method suppressed the extended lifespan under conditions of optimal dietary restriction (data not shown).

pha-4 is specific to diet-restriction-induced longevity

A loss of *pha-4* suppressed any potential lifespan extension across a spectrum of bacterial concentrations, suggesting that it was not causing a general sickness in the animal. However, to determine more conclusively whether *pha-4* was acting specifically to affect the dietary restriction pathway, we examined its effect on other pathways that influence longevity. Reduced IIS, by mutation of the insulin/IGF-1 receptor *daf-2* increases longevity. We found that *pha-4* was not required for the long lifespan of *daf-2* mutant animals. RNAi knockdown of *daf-16*, but not *pha-4*, completely suppressed the long lifespan of *daf-2(e1368)* (Fig. 1d), *daf-2(mu150)*, and *daf-2(e1370)* mutant animals (Supplementary Fig. 4a, b, respectively). Additionally, we tested whether *pha-4* was required for the long lifespan of animals with reduced mitochondrial electron transport chain activity[25,26]. Neither RNAi of *pha-4* nor the *pha-4(zu225);smg-1(cc546ts)* allele shortened the long lifespan of *cyc-1*-RNAi-treated animals (Fig. 1e and Supplementary Fig. 5a, respectively) or *isp-1(qm130)* mutant animals (Supplementary Fig. 5b) any more than reduction of *pha-4* in a wild-type background (Fig. 1f and Supplementary Table 1) and to a lesser extent than the loss of *daf-16* in *cyc-1*-RNAi-treated animals (Supplementary Fig. 5c). We thus conclude that *pha-4* is a specific requirement in the regulation of longevity in worms undergoing dietary restriction and that its loss does not simply cause a general sickness.

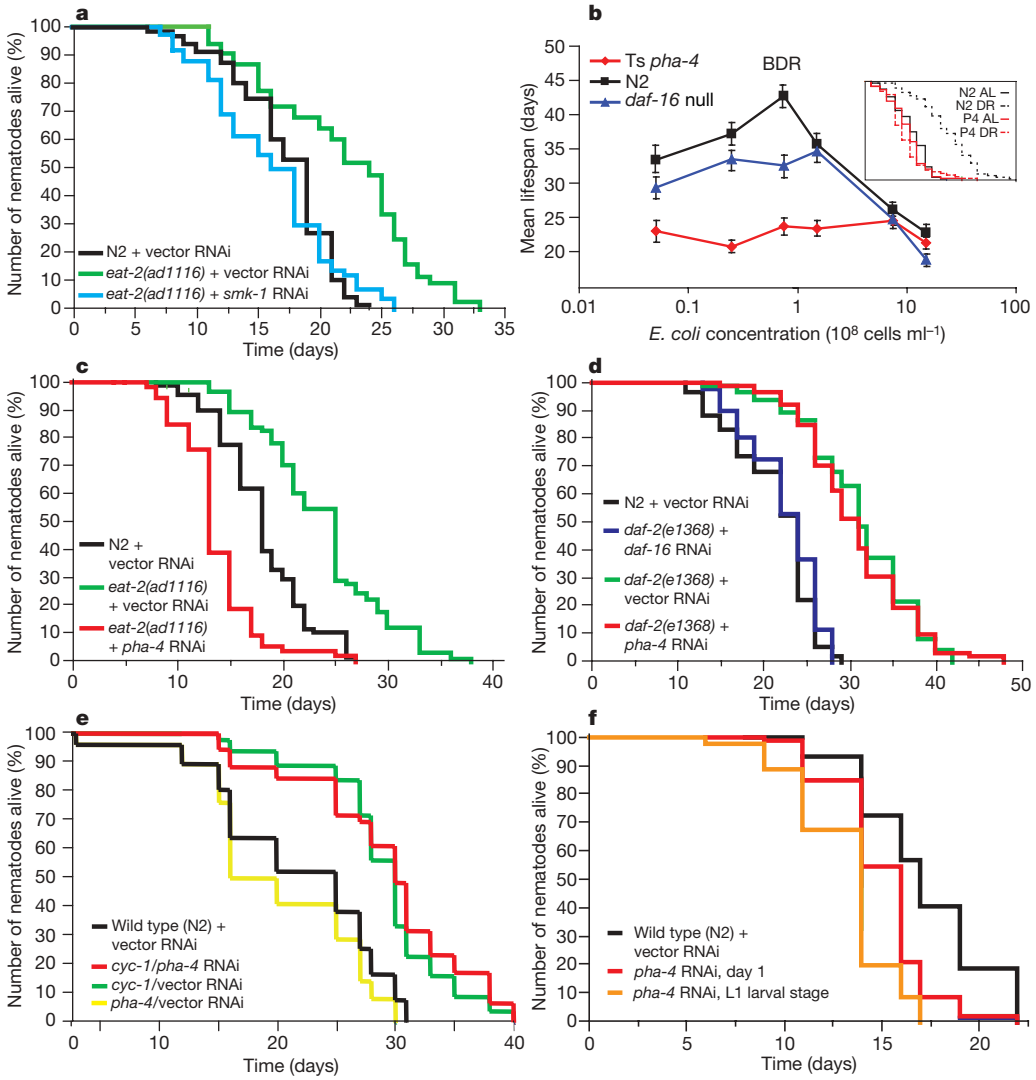

Figure 1 | *smk-1* and *pha-4* are required for diet-restriction-mediated longevity. All statistical data can be found in Supplementary Tables 1 and 2. Black lines indicate N2 worms grown on empty vector RNAi bacteria unless noted. **a**, *eat-2(ad1116)* worms fed empty vector RNAi bacteria (green line) lived significantly longer than *eat-2(ad1116)* worms fed *smk-1* RNAi bacteria (light blue line). **b**, Dietary restriction using non-RNAi bacterial dilution (BDR) results in a parabolic curve for wild-type worms (black line) and *daf-16(mu86)*-null mutant animals (blue line), but not *pha-4(zu225);smg-1(cc546ts)* temperature sensitive (ts) mutant animals (red line). Inset, lifespan plot of wild-type (N2; black line) and *pha-4(zu225);smg-1(cc546ts)* (P4; red line) worms with 7.5×10^8 cells ml^{-1} (*ad libitum*; AL) or 7.5×10^7 cells ml^{-1} (dietary restriction; DR). Error bars, s.e.m. **c**, *eat-2(ad1116)* mutant animals fed *pha-4* RNAi bacteria from the L1 larval stage (red line) were shorter lived than animals fed vector RNAi bacteria (green line). **d**, *daf-2(e1368)* mutant animals fed either vector RNAi bacteria (green line) or *pha-4* RNAi bacteria (red line) lived significantly longer than when fed *daf-16* RNAi bacteria (blue line). **e**, Wild-type (N2) animals fed 50% *cyc-1* and 50% vector RNAi bacteria (green line) or 50% *cyc-1* and 50% *pha-4* RNAi bacteria (red line) showed a similar lifespan extension compared to N2 animals fed vector RNAi bacteria alone (black line). N2 animals fed 50% *pha-4* and 50% vector RNAi bacteria (yellow line) had a slightly shorter lifespan. Lifespan analyses of animals with reduced mitochondrial electron transport chain were performed at 15 °C. **f**, Wild-type worms fed *pha-4* RNAi bacteria starting from either day 1 of adulthood (red line) or the L1 larval stage (orange line) lived significantly shorter than worms fed vector RNAi bacteria (black line).

The role of *pha-4* in development and longevity is separable

pha-4 has an essential early role during embryo development in the morphogenesis of the pharynx, and inactivation of *pha-4* up to the first larval stage, L1, can result in lethality[23]. We tested whether the early developmental function of *pha-4* can be temporally separated from its role in dietary restriction during adulthood. We allowed *eat-2(ad1116)* mutant animals to develop through the larval stages (L1–L4) and grow on normal bacteria and then shifted the animals on the first day of adulthood to bacteria expressing *pha-4* double-stranded-RNA, thereby only inactivating *pha-4* during adulthood—long after pharyngeal development had completed[23]. RNAi of *pha-4* during only adulthood suppressed the long lifespan of *eat-2(ad1116)* mutant animals to wild-type levels (Fig. 2). In support of these data, *pha-4(zu225);smg-1(cc546ts)* mutant worms in our BDR experiments were not shifted to the restrictive temperature to inactivate *pha-4* (ref. 22) until adulthood.

We additionally considered whether reduction of *pha-4* could suppress diet-restriction-mediated longevity by altering pharyngeal function during adulthood by indirectly affecting the feeding rates of animals and pushing diet-restriction animals towards starvation. We found this hypothesis inconsistent with multiple observations. First, the pumping (feeding) rate of wild-type animals grown on control bacteria was very similar to the pumping rate of wild-type animals treated with *pha-4* RNAi (wild type treated with vector RNAi, 242 ± 10 pumps per min (± s.d.); wild type treated with *pha-4* RNAi, 238.2 ± 11.5 pumps per min (± s.d.)). Additionally, *eat-2(ad1116)* mutant animals treated with *pha-4* RNAi did not exhibit altered feeding rates (*eat-2(ad1116)* treated with vector RNAi, 50.1 ± 7.1 pumps per min; *eat-2(ad1116)* treated with *pha-4* RNAi, 48.4 ± 5.5 pumps per min). We confirmed that RNAi of *pha-4* was activated by the time pumping rates were monitored by following the green fluorescent protein (GFP) signal of *pha-4–gfp* transgenic animals treated with *pha-4* RNAi (see Methods and Supplementary Fig. 6). In agreement with this observation, *pha-4* RNAi did not increase longevity of wild-type animals, as would be expected if feeding rates were reduced[8]. In fact, RNAi of *pha-4* slightly shortened wild-type longevity, even when applied specifically to adult animals (Fig. 1f). As noted previously, the *pha-4(zu225)* mutation did not change the parabolic relationship observed between BDR and longevity: it blocked the entire response (Fig. 1b). Finally, *pha-4* RNAi did not further increase the long lifespan of *daf-2* mutant animals (Fig. 1d and Supplementary Fig. 4a, b), as is observed with *eat-2;daf-2* mutant animals that live longer than either single mutation[8], and did not enhance the long lifespan of animals treated with RNAi or mutation resulting in reduced electron transport chains (Fig. 1e and Supplementary Fig. 5a, b).

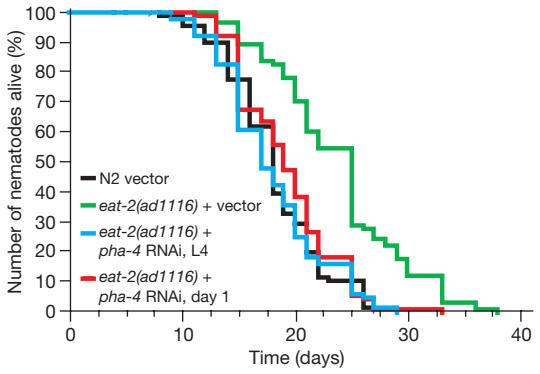

Figure 2 | *pha-4* is required during adulthood to regulate longevity in response to dietary restriction. *eat-2(ad1116)* mutants were transferred to *pha-4* RNAi bacteria at the L4 larval stage (blue line; mean lifespan 18 ± 0.5 days; mean ± s.e.m.) or day 1 of adulthood (red line; mean lifespan 19.2 ± 0.4 days) and in both cases showed a decreased lifespan compared with *eat-2(ad1116)* animals fed vector RNAi bacteria (green line; mean lifespan 23.8 ± 0.6 days). Mean lifespan of N2 worms fed vector RNAi bacteria (black line) was 18.1 ± 0.4 days.

pha-4 expression is increased in response to dietary restriction

pha-4 is expressed in the developing pharynx and intestine during embryogenesis and larval stages[23,27]. We asked whether the expression pattern of *pha-4* during adulthood was different from its developmental expression pattern. Using a red fluorescent protein (RFP) transcriptional fusion to the *pha-4* promoter, we observed strong expression in the developing pharynx and in the intestine, as noted previously[23,27]. In the adult animal, expression was lacking in the pharynx, but still present in the intestine (Fig. 3a). Using a full-length *pha-4* complementary DNA translation fusion to GFP under the *pha-4* promoter, we observed nuclear localization of PHA-4 during development and adulthood within the same cells (Fig. 3b and see Methods) and also found *pha-4* expression in the adult worm expanded to a few neuronal cells in the head and tail, which were not found in the developing animal (Fig. 3b). This expression pattern did not change in response to dietary restriction (data not shown), and PHA-4 seemed constitutively nuclear under all conditions tested (Fig. 3c).

During embryogenesis, levels of PHA-4 expression determine its binding specificity: low levels of PHA-4 bind high-affinity sites in promoters during early embryogenesis; PHA-4 does not bind to low-affinity sites until late in embryogenesis when *pha-4* expression levels increase[22]. Following this paradigm, we reasoned that expression of *pha-4* might increase during dietary restriction to facilitate its binding to diet-restriction-specific genes. Using both semi-quantitative PCR with reverse transcription (RT–PCR) and quantitative real-time RT–PCR (Q-PCR), expression of *pha-4* increased by more than 80% in response to dietary restriction (Fig. 3d).

Overexpression of *pha-4* extends longevity in the absence of *daf-16*

Because expression levels of *pha-4* were increased in response to dietary restriction, we tested whether overexpression of *pha-4* was sufficient to extend longevity under normal feeding conditions. Eleven independent lines overexpressing *pha-4* were established (see Methods). In nine lines, *pha-4* overexpression increased longevity of wild-type animals, but only slightly (Supplementary Table 4). However, when the same *pha-4* expression construct was used to overexpress *pha-4* in a *daf-16(mu86)*-null mutant strain, we observed a statistically significant increase in lifespan (Fig. 4 and Supplementary Table 4). There seem to be at least two explanations for this result. One, an inherent competition between *daf-16* and *pha-4* in wild-type animals may exist; or two, the role of *daf-16* and *pha-4* may be partially redundant in determination of longevity of wild-type animals. In any event, the relative increase in lifespan by *pha-4* overexpression was greatest in the complete absence of *daf-16*.

The *sod* gene family is differentially regulated by DAF-16 and PHA-4

In analysing the potential competition among *daf-16* and *pha-4*, we noticed that the consensus DNA binding sites for DAF-16 and PHA-4 overlap: PHA-4, T(A/G)TT(T/G)(A/G)(T/C) (ref. 22) versus DAF-16, T(A/G)TTTAC (ref. 28). This observation raised the hypothesis that DAF-16 and PHA-4 regulate expression of the same genes either directly or indirectly. *sod-3*, a mitochondrial Fe/Mn superoxide dismutase[29–31], is the best characterized DAF-16 target gene and contains three DAF-16 DNA binding sites within the promoter region[32]. All three DAF-16 sites overlap with the consensus PHA-4 DNA binding site. Therefore, we used Q-PCR analysis to examine *sod-3* expression in diet-restricted, *eat-2(ad1116)* mutant animals. Surprisingly, we found no increase in expression levels of *sod-3* in response to dietary restriction (Fig. 5a, see Methods). Furthermore, the basal level of *sod-3* expression was not altered in diet-restricted animals lacking *pha-4* (Fig. 5b).

The *sod-1* promoter contains four consensus PHA-4 binding sites. Furthermore, the mouse *sod-1* orthologue, *sod-1*, has been shown to be a transcriptional target of *Foxa1* (ref. 33). *sod-1* is a cytoplasmic Cu/Zn superoxide dismutase[34]. We tested whether the *C. elegans*

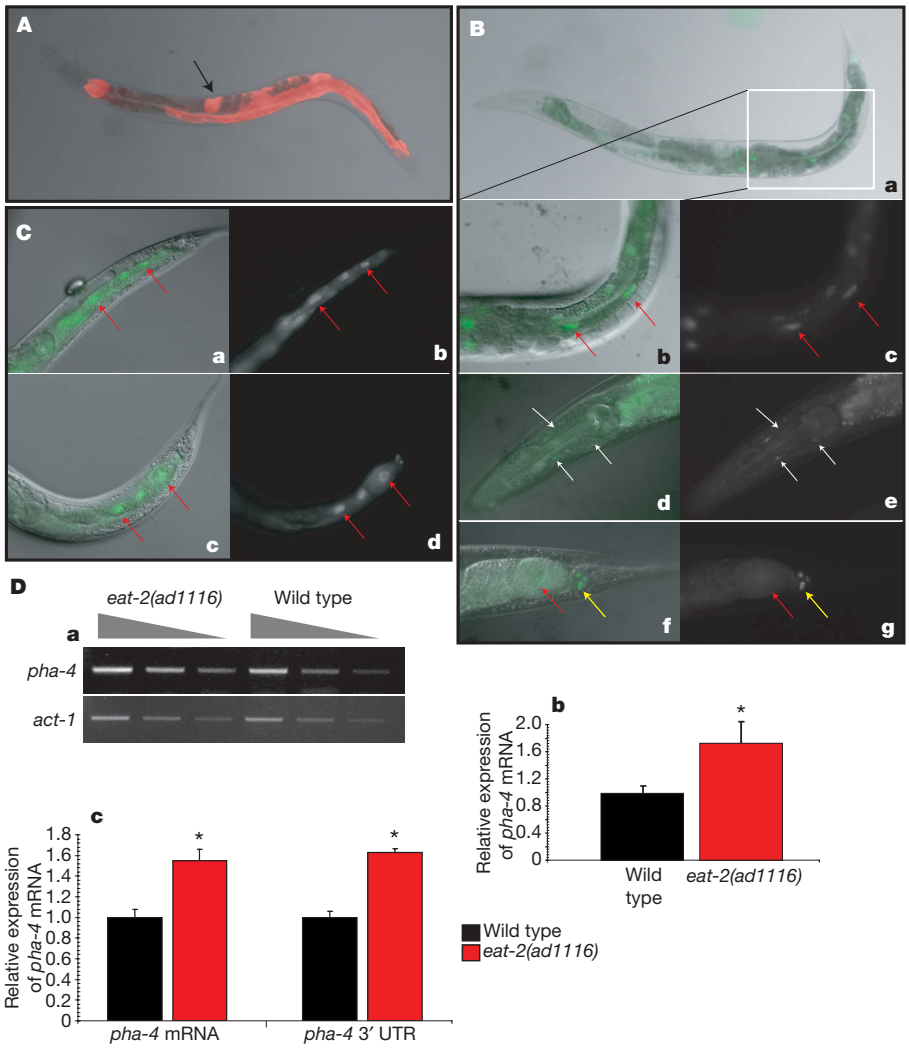

Figure 3 | Regulation and localization of *pha-4* in response to dietary restriction. **A**, A *pha-4–rfp* transcriptional fusion construct (see Methods) reveals *pha-4* expression in the intestine and somatic gonad (spermatheca, black arrow) of the adult worm. **B**, **a**, AD84 worms reveal PHA-4–GFP nuclear localization in intestinal cells (**b** and **c**, red arrows), head neurons (**d** and **e**, white arrows) and tail neurons (**f** and **g**, yellow arrows). **C**, AD84 worms carrying the *pha-4–gfp* transgene were placed in high-food cultures (7.5×10^8 cells ml^{-1}; **a**, **b**) or low-food cultures (7.5×10^7 cells ml^{-1}; **c**, **d**). Nuclear localization of PHA-4–GFP in intestinal nuclei (red arrows) remained constant under both conditions (see Methods). Images were taken on day four of adulthood. **D**, Semi-quantitative RT–PCR (**a**) reveals an increase in *pha-4* messenger RNA levels in *eat-2(ad1116)* animals compared with wild-type animals of ~80% (unpaired two-tailed *t*-test *P*-value < 0.001; error bars, s.d.; **b**). Q-PCR analysis of the *pha-4* gene and the *pha-4* 3′ UTR confirms that *pha-4* mRNA levels were increased in *eat-2(ad1116)* worms (red bars) compared with wild-type worms (black bars) (unpaired two-tailed *t*-test *P*-value < 0.0001; errors bars, s.d.; **c**). $n = 3$ for both semi-quantitative RT–PCR and Q-PCR experiments.

sod-1 was transcriptionally regulated in *eat-2(ad1116)* mutant animals. By Q-PCR analysis, *sod-1* expression was greatly upregulated in response to dietary restriction (Fig. 5c). In diet-restricted animals, *sod-1* expression was decreased in the absence of *pha-4*, but was slightly increased in the absence of *daf-16* (Fig. 5d). Therefore, expression of *sod-1* requires PHA-4, but not DAF-16, in *eat-2(ad1116)* mutant animals.

The *C. elegans* genome contains five *sod* genes including *sod-3* and *sod-1* (ref. 15). We further investigated the expression patterns of each of the *sod* gene family members under conditions of dietary restriction or reduced IIS signalling. Interestingly, we found that the expression level of every *sod* gene except for *sod-3* was increased under dietary restriction (Fig. 5e). The increases were *pha-4*-dependent (Fig. 5f). In response to reduced IIS, *sod-1*, *sod-3* and *sod-5* expression levels were increased (Fig. 5g); this increase was *daf-16*-dependent (Fig. 5h). Taken together, we thus find that *sod-2* and sod-*4* expression is specific to dietary restriction and dependent on *pha-4*, whereas *sod-3* expression is specific to reduced IIS and dependent on *daf-16*. Common to both dietary restriction and reduced IIS, expression of *sod-1* and *sod-5* are increased by PHA-4 and DAF-16, respectively (Fig. 5i). Although each *sod* gene contains respective predicted DAF-16 and PHA-4 binding sites within their promoters, regulation by additional factors cannot be ruled out at this time.

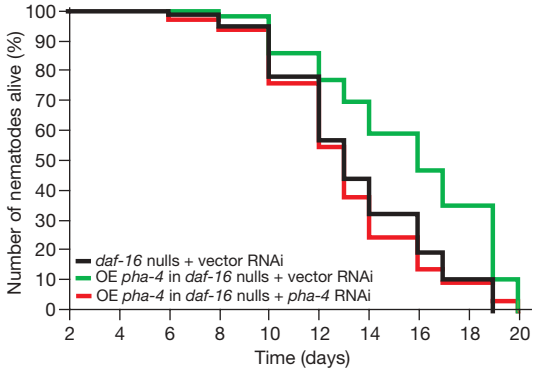

Figure 4 | Increased dosage of *pha-4* extends lifespan. Transgenic *daf-16(mu86)*-null mutant worms carrying an overexpressor (OE) *pha-4* transgene (AD115, green line) were long-lived compared with *daf-16(mu86)* worms (AD105, black line). This lifespan extension was fully suppressed by *pha-4* RNAi (red line). Statistical data can be found in Supplementary Table 4.

Discussion

In worms, PHA-4 is bifunctional, having an early developmental function in pharyngeal determination during embryogenesis and the L1 larval stage[22,23], and a later function during adulthood in regulating the response to dietary restriction. This dual mode of action of PHA-4 is similar to that of DAF-16, which is required during early larval stages to regulate the dauer developmental decision and reproductive status of the animal, and later during adulthood to regulate the response of ageing to IIS[35]. In mammals, a parallel regulation of insulin levels by FOXO proteins[36], and glucagon levels by *Foxa1* and *Foxa2* (refs 18, 19), supports a model in which, under continually low nutrient signalling, PHA-4/Foxa may mediate levels of glucagon or other changes in hormones ultimately capable of regulating the ageing process. In contrast,

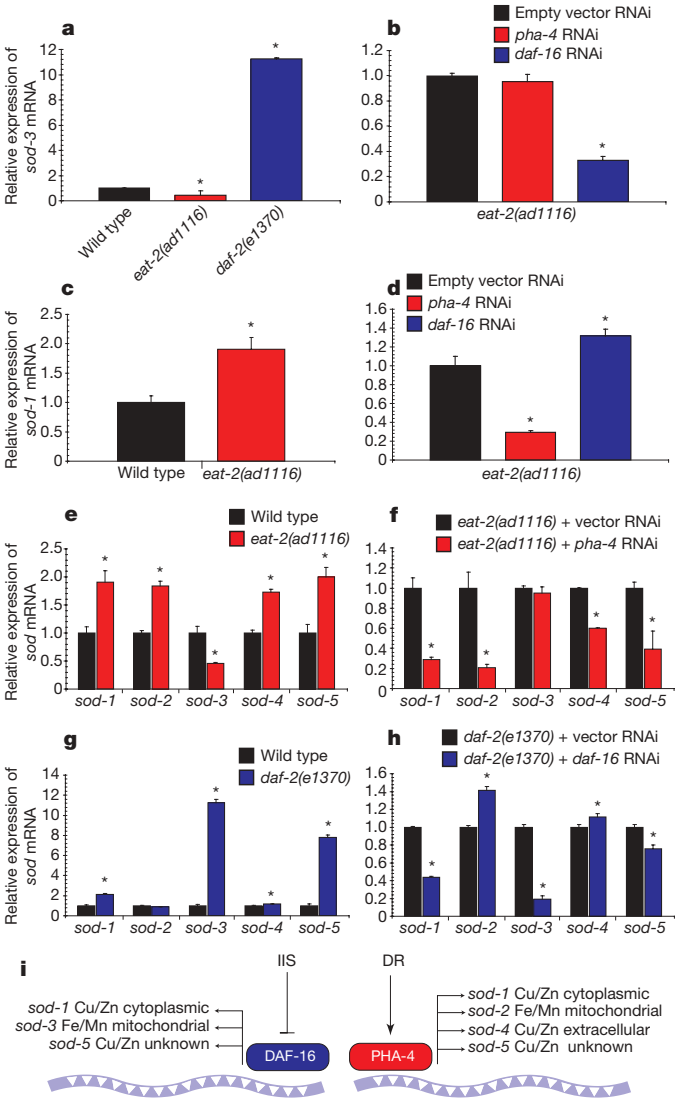

Figure 5 | Differential transcriptional regulation of *sod*s by *pha-4* and *daf-16* in response to dietary restriction and IIS. All mRNA expression levels were determined using Q-PCR analysis and performed in parallel (see Methods). Q-PCR reactions were run in quadruplicate and averages from one representative set of reactions are depicted in graphs. Error bars represent s.d. for the reaction depicted and asterisks indicate a change in expression with an unpaired two-tailed *t*-test *P*-value <0.005 as compared with black bars of the same graph and gene. **a**, **c**, **e**, **g**, mRNA levels of worms fed vector RNAi bacteria. **a**, *sod-3* expression levels were increased in *daf-2(e1370)* animals (blue bar) compared with wild-type N2 (black bar) and *eat-2(ad1116)* animals (red bar). **b**, *sod-3* mRNA expression levels in *eat-2(ad1116)* mutant worms were unaffected by *pha-4* RNAi (red bar), but were decreased in response to *daf-16* RNAi (blue bar). **c**, *sod-1* mRNA levels were increased in *eat-2(ad1116)* worms (red bar) compared with wild-type worms (black bar). **d**, *sod-1* mRNA expression levels were decreased in *eat-2(ad1116)* worms fed *pha-4* RNAi (red bar). **e**, All *sod*s, except *sod-3*, were upregulated in *eat-2(ad1116)* mutant animals (red bars) compared with wild-type animals (black bars). **f**, *sod-1*, *sod-2*, *sod-4* and *sod-5* mRNA expression in *eat-2(ad1116)* worms was greatly decreased when worms were fed *pha-4* RNAi bacteria (red bars) compared with *eat-2(ad1116)* worms fed vector RNAi bacteria (black bars). **g**, *daf-2(e1370)* mutant worms (blue bars) had elevated levels of *sod-1*, *sod-3* and *sod-5* mRNA when compared with wild-type worms (black bars). **h**, *daf-2(e1370)* mutant animals fed *daf-16* RNAi bacteria (blue bars) had reduced levels of *sod-1*, *sod-3* and *sod-5* compared with *daf-2(e1370)* mutant animals fed vector RNAi (black bars). **i**, Model depicting differential regulation of *sod*s in response to IIS and dietary restriction (DR) mediated by DAF-16 and PHA-4, respectively. Types (Fe/Mn, Cu/Zn) and location, if known, of superoxide dismutases are listed next to the genes[30,31,45,46].

in times of severe stress or starvation, DAF-16/FOXO will mediate the response to decreased insulin signalling. Although *C. elegans* does not contain an obvious glucagon orthologue, it does contain a full complement of insulin-like peptides[37], suggesting that a conserved functional regulation of glucose homeostasis may be present. The finding that some insulin-like peptides work as agonists[38], whereas others are antagonists[39], to insulin signalling in worms indicates that glucose homeostasis could be more directly regulated by expression of insulin-like peptides in response to dietary restriction. In the future, it will be imperative to understand whether the Foxa family is required for diet-restriction-mediated longevity in mammals and what part glucagon production plays in this process.

The response to IIS involves the DAF-16-dependent regulation of *sod-1, sod-3* and *sod-5*, whereas dietary restriction involves the PHA-4-dependent expression of *sod-1, sod-2, sod-4* and *sod-5*. The disparate transcriptional outcomes of these treatments on oxygen radical scavenging genes could suggest that a different form of reactive oxygen species production may be induced under conditions of reduced IIS than is induced under conditions of dietary restriction. This may indicate divergent underlying metabolic consequences stemming from the manipulation of these independent pathways. Alternatively, as the expression patterns for most of these *sod*s remain unknown, the differential transcriptional regulation of *sod*s under IIS and dietary restriction could indicate distinct tissue-specific requirements for IIS and diet-restriction-mediated longevity. In *C. elegans*, IIS is required in the neurons and intestinal cells to regulate lifespan[40–42]. Although expression patterns of *pha-4* overlap with those of *daf-16* in the intestine and some neuronal cells, it is not known which tissues integrate and respond to reduced dietary intake. It is possible that the same tissues that exhibit increased levels of oxidative-stress response genes also will require DAF-16 or PHA-4 to affect longevity. It is likely that *sod* gene regulation is not the sole target of DAF-16 and PHA-4 for longevity assurance, but rather these transcription factors orchestrate a larger regulatory network that has been previously proposed[38,43].

Many of the physiological outcomes of animals with reduced IIS compared with animals undergoing dietary restriction are similar, including reduced body size, lower plasma IGF-1 and insulin levels, and increased insulin sensitivity. Furthermore, transcriptional profiling of long-lived dwarf mice, having reduced IGF-1 signalling, in combination with dietary restriction, additively increased expression of multiple liver-specific genes[44]. However, compelling genetic analysis indicates that many key differences among IIS- and diet-restricted mice exist as well. For example, long-lived growth-hormone-deficient mice can still respond to dietary restriction, and IGF-1R long-lived heterozygous mice do not show protracted or reduced reproduction[3].

Therefore, given the discrepancy between the mode of action elicited by reduced IIS and dietary restriction that results in increased longevity of an organism, it is important to note that *pha-4* is exceptionally specific for the longevity induced by dietary restriction. Reduction of *pha-4* does not suppress the long lifespan of *daf-2* mutant animals or animals with defective electron transport chains. We conclude, in agreement with previous reports[7,8], that there exists an independent pathway for the regulation of dietary restriction in worms. Consistent with this observation, worms undergoing dietary restriction do not require *daf-16*. Our results instead suggest that dietary restriction impinges on an independent mechanism that ultimately increases the activity of PHA-4. Overexpression of *pha-4* extends longevity in the absence of *daf-16*, and *pha-4* expression is increased under conditions of dietary restriction. We thus report the first findings of a forkhead transcription factor that acts independent of and with a parallel mechanism to *daf-16* and IIS to regulate the ageing process in diet-restricted worms.

METHODS SUMMARY

A detailed description of all experimental methods is provided in the Methods section.

C. elegans strains, growth, imaging, lifespan analysis, Q-PCR and RNAi application were performed as previously described[13]. For bacterial restriction

studies, each lifespan consisted of 4 wells, with 1 ml of culture and 15 worms per well ($n = 60$). Lifespans were scored and worms transferred to new cultures every 3–4 days. Liquid cultures were prepared using an overnight culture of OP50 *Escherichia coli* grown at 37 °C. Bacteria were washed three times in S-Basal medium. The bacterial concentration was adjusted to 1.5×10^{-9} cells ml^{-1} in S-Basal medium containing cholesterol, carbenicillin, tetracycline and kanamycin (for concentrations see Methods). Serial dilutions were performed to achieve bacterial concentrations of 7.5×10^{8}, 1.5×10^{8}, 7.5×10^{7}, 2.5×10^{7} and 5×10^{6} cells ml^{-1}. Cultures contained Fluorodeoxyuridine (FUDR) at 100 μg ml^{-1} for the first twelve days of lifespan analysis to block worm reproduction. For analysis of the temperature-sensitive *pha-4(zu225)* mutant allele, *pha-4(zu225);smg-1(cc546ts)*[22] double mutant worms were grown at 25 °C to inactivate *smg-1* and allow production of functional *pha-4*. *pha-4* was inactivated by shifting double mutants to 15 °C, restoring *smg-1* activity, which results in degradation of the *pha-4(zu225)* allele after the first day of adulthood, thus avoiding any developmental defects owing to loss of *pha-4* during larval stages. All control worms were treated identically.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions A.D., S.W., H.A. and S.P. conceived the framework of the manuscript. A.D., S.W. and S.P. wrote the paper. A.D. oversaw the entire project. S.W. and H.A. screened all forkhead-related genes for their role in dietary restriction. J.D. performed the *isp-1(qm150)* experiments. S.P. created all transgenic lines, performed overexpression, localization, BDR and Q-PCR experiments.

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METHODS

***C. elegans* methods and generation of transgenic lines.** CF1037 (*daf-16(mu86)I*), CF1041 (*daf-2(e1370)III*), DR1572 (*daf-2(e1368)III*), CF512 (*fer-15(b26)II;fem-1(hc17)IV*), CF1379 (*daf-2(mu150)*), PD8120 (*smg-1(cc546ts)I*) and wild-type *C. elegans* (N2) strains were obtained from the *Caenorhabditis* Genetic Center. SM190 (*pha-4(zu225);smg-1(cc546ts)*)[22] was provided by S. Mango. Nematodes were maintained and handled using standard methods[47]. For generation of transgenic animals, plasmid DNA containing the construct of interest and pRF4(*rol-6*)[48] was microinjected into the gonads of adult hermaphrodite animals, using standard methods[48]. F_1 progeny were selected on the basis of the roller phenotype. Individual F_2 worms were isolated to establish independent lines.

For generation of worm strains AD137–AD141 and AD143–AD148 (N2, *pha-4*; N2 worms overexpressing a *pha-4* transgene), the plasmid DNA mix consisted of 50 ng μl^{-1} pSP15(*pha-4*) and 50 ng μl^{-1} pRF4(*rol-6*). Wild-type (N2) worms were injected. Worms used as controls in lifespan experiments for AD137–AD141 and AD143–AD148 were generated by microinjecting wild-type (N2) worms with 50 ng μl^{-1} of pRF4(*rol-6*) alone.

For generation of AD115 (*daf-16(mu86);pha-4*), the plasmid mix consisted of 50 ng μl^{-1} pSP15*(pha-4)* and 50 ng μl^{-1} pRF4(*rol-6*). *daf-16(mu86)* worms were injected. *daf-16(mu86)* animals injected with 50 ng μl^{-1} of pRF4(*rol-6*) alone were used as a control in lifespan experiments and termed AD105.

For AD150 (N2, *pha-4-rfp*), wild-type (N2) worms were microinjected with plasmid DNA containing 75 ng μl^{-1} pSP23(*rfp*) and 75 ng μl^{-1} pRF4(*rol-6*).

For AD84 (N2, *pha-4–gfp*), wild-type(N2) animals were microinjected with 75 ng μl^{-1} pSP1(*pha-4–gfp*) and 75 ng μl^{-1} pRF4(*rol-6*). An extrachromosomal array was integrated as described[49] and outcrossed seven times.

Creation of *pha-4* constructs. All constructs were sequence verified.

pSP23: to construct the plasmid expressing tdTOMATO(RFP) driven by the *pha-4* endogenous promoter, the *gfp* transcript in the worm expression vector pPD95.77 was replaced with *tdTomato*[50]. The 2.7 kilobase sequence upstream of the *pha-4* coding region was amplified from genomic DNA by PCR and inserted upstream of *tdTOMATO* in pPD95.77.

pSP1: to construct the plasmid expressing PHA-4–GFP driven by the *pha-4* endogenous promoter, full-length *pha-4* cDNA (1,521 base pairs) was cloned from first-strand worm cDNA by PCR amplification and inserted in frame and upstream of the *gfp* sequence in the worm expression vector pPD95.77. The 2.7 kb sequence upstream of the *pha-4* coding region was amplified from genomic DNA by PCR and inserted upstream of the *pha-4* cDNA.

pSP15: the plasmid containing untagged PHA-4 driven by the endogenous *pha-4* promoter was constructed by amplifying the *pha-4* promoter and cDNA sequence from pSP1 by PCR amplification and inserting it in place of the *gfp* sequence in pPD95.77. A stop codon was added to the 3′ end of the *pha-4* cDNA sequence by PCR.

Lifespan analyses. Lifespan analyses were performed as described previously[35]. All lifespan analyses were conducted at 20 °C unless otherwise stated. JMP IN 5.1 software was used for statistical analysis to determine means and percentiles. In all cases, *P*-values were calculated using the log-rank (Mantel–Cox) method. Lifespan per cent decreases were determined by dividing the shorter lifespan by the longer lifespan, subtracting 1, and multiplying by 100. Mean lifespan data were used for per cent decreases.

RNA isolation, semi-quantitative RT–PCR and quantitative RT–PCR. Total RNA was isolated from synchronized populations of approximately 10,000 day-1 reproductive adults. Total RNA was extracted using TRIzol reagent (GIBCO). cDNA was created using the Quantitec Reverse Transcriptase kit (Qiagen). For semi-quantitative RT–PCR, serial dilutions of 5×, 10× and 20× were used for PCR reactions. For each primer pair, cycle times and primer concentrations were optimized to ensure linear amplification. Quantification was performed on 10× dilution reactions using Gel-Doc software and levels were normalized to *act-1* cDNA. SybrGreen real-time Q-PCR experiments were performed as described in the manual using ABI Prism79000HT (Applied Biosystems) and cDNA at a 1:20 dilution. All Q-PCR experiments were normalized to *act-1* mRNA levels.

GFP localization. GFP localization analysis was performed as described previously[13]. Worms were grown on OP50 *Escherichia coli* and images were taken on day 1 of adulthood unless otherwise noted. GFP is shown in green and is merged with differential interference contrast images. Black and white images represent only the GFP channel with fluorescence shown in white.

Pumping rate assays. Pumping rates of wild-type (N2) worms and *eat-2 (ad1116)* mutant worms on various RNAi bacteria were determined by counting pumps of the terminal pharyngeal bulb for one-minute intervals to determine pumps per min. The pumping rates of ten worms per condition were determined and averaged to determine the rates represented in the text. Worms were synchronized by transferring and growing eggs on empty vector RNAi until day 1 of adulthood. On day 1 of adulthood, worms were transferred to RNAi treatments and pumping rates were determined after forty-eight hours of RNAi treatment. Functionality of RNAi was determined by examining AD84 (*pha-4–gfp*) animals using fluorescence microscopy (Supplementary Fig. 5).

Bacterial dietary restriction (BDR) lifespan analysis. Synchronized populations of eggs were hatched and grown at 25 °C on NG agar plates containing OP50 *E. coli* until day 1 of adulthood. At day 1 of adulthood, animals were transferred to new plates of OP50 *E. coli* containing FUDR at 100 μg ml^{-1} and shifted to 20 °C. At day 2 of adulthood, worms were transferred into liquid culture (discussed below) and placed on a gentle rocker at 15 °C for the remainder of the lifespans. Liquid cultures were done in 12-well cell culture plates containing 1 ml of culture per well. Each lifespan consisted of 4 wells with 15 worms per well ($n = 60$). Lifespans were scored and worms transferred to new cultures every 3–4 days.

Liquid cultures were prepared using an overnight culture of OP50 *E. coli* grown at 37 °C. Bacteria were washed three times in S-Basal medium containing 5 μg ml^{-1} cholesterol, carbenicilln (50 μg ml^{-1}), tetracycline (1 μg ml^{-1}) and kanamycin (10 μg ml^{-1}). The bacterial concentration was adjusted to 1.5×10^9 cells ml^{-1} in S-Basal medium containing cholesterol, carbenicillin, tetracycline and kanamycin at the concentrations above. Serial dilutions were performed to achieve bacterial concentrations of 7.5×10^8, 1.5×10^8, 7.5×10^7, 2.5×10^7 and 5×10^6 cells ml^{-1}. Cultures contained FUDR at 100 μg ml^{-1} for the first twelve days of lifespan analysis to block worm reproduction.

Temperature-sensitive inactivation of *pha-4(zu225);smg-1(cc546ts)*. *pha-4(zu225);smg-1(cc546ts)*[22] double mutant worms are grown at 25 °C to inactivate *smg-1* and allow functional *pha-4* to be made. *pha-4* was inactivated by shifting double mutants to 15 °C, restoring *smg-1* activity, which results in degradation of the *pha-4(zu225)* allele, after the first day of adulthood, thus avoiding any developmental defects owing to loss of *pha-4* during larval stages. All control worms were treated identically.

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