HIF-1 modulates longevity and healthspan in a temperature-dependent manner

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Summary

The hypoxia-inducible factor HIF-1 has recently been identified as an important modifier of longevity in the roundworm *Caenorhabditis elegans*. Studies have reported that HIF-1 can function as both a positive and negative regulator of life span, and several disparate models have been proposed for the role of HIF in aging. Here, we resolve many of the apparent discrepancies between these studies. We find that stabilization of HIF-1 increases life span robustly under all conditions tested; however, deletion of *hif-1* increases life span in a temperature-dependent manner. Animals lacking HIF-1 are long lived at 25°C but not at 15°C. We further report that deletion or RNAi knockdown of *hif-1* impairs healthspan at lower temperatures because of an age-dependent loss of vulval integrity. Deletion of *hif-1* extends life span modestly at 20°C when animals displaying the vulval integrity defect are censored from the experimental data, but fails to extend life span if these animals are included. Knockdown of *hif-1* results in nuclear relocalization of the FOXO transcription factor DAF-16, and DAF-16 is required for life span extension from deletion of *hif-1* at all temperatures regardless of censoring.

Key words: *Caenorhabditis elegans*; DAF-16; healthspan; HIF-1α; hypoxic response; longevity.

Introduction

Appropriately altering cellular physiology and gene expression in response to changing oxygen availability is essential for survival. The hypoxia-inducible factor (HIF) is a highly conserved transcriptional regulator of genes involved in this process (Maxwell *et al.*, 2001; Webb *et al.*, 2009). HIF exists as a heterodimer of the regulated HIF-α subunit and the constitutively expressed HIF-β subunit (Wang *et al.*, 1995). Under normoxic conditions, HIF-α is ubiquitinated by a cullin E3 ligase complex containing the von Hippel Lindau protein (VHL) substrate recognition subunit and targeted for proteasomal degradation (Maxwell *et al.*,

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1999). This ubiquitination reaction is inhibited by hypoxia, leading to stabilization of HIF-α and induction of the hypoxic response (Kim & Kaelin, 2003). The mechanism of ubiquitin-mediated regulation of HIF-α stability is conserved from nematodes to humans.

The *Caenorhabditis elegans* hypoxic response has been characterized through a series of genetic, biochemical, and microarray studies (Shen & Powell-Coffman, 2003; Shen *et al.*, 2005). Loss of HIF-α (*hif-1*) causes sensitivity to hypoxia, and many mRNAs have been shown to be induced by hypoxia in a HIF-1-dependent manner (Jiang *et al.*, 2001). Deletion of the gene coding for the von Hippel Lindau homolog, VHL-1, results in stabilization of HIF-1 because of its failure to be ubiquitinated and proteasomally degraded (Epstein *et al.*, 2001). Similar to wild-type animals subjected to hypoxia, *vhl-1(ok161)* animals show elevated levels of HIF-1 and activation of HIF-1 target genes (Bishop *et al.*, 2004; Shen *et al.*, 2005; Mabon *et al.*, 2008). Interestingly, a relationship between longevity and resistance to hypoxic stress has been noted: several mutant alleles of *daf-2* confer resistance to hypoxia (Scott *et al.*, 2002; Mabon *et al.*, 2009), and a genome-wide RNAi screen identified 11 previously known longevity genes as regulators of hypoxia resistance (Mabon *et al.*, 2008).

We have recently reported a direct connection between HIF-1 and aging, with the finding that stabilization of HIF-1 by RNAi knockdown of *vhl-1*, or by deletion of *vhl-1*, leads to a significant increase in life span (Mehta *et al.*, 2009). Life span extension from deletion of *vhl-1* is suppressed by deletion of *hif-1* and is genetically distinct from the insulin-like signaling pathway and from dietary restriction (DR). Stabilization of HIF-1 also enhances healthspan, as measured by resistance to polyglutamine toxicity, resistance to amyloid beta toxicity, and accumulation of autofluorescent age pigment (Mehta *et al.*, 2009).

Since our report, four additional studies from different groups have implicated the hypoxic response in nematode aging. Chen *et al.* (2009) first described an opposite role for HIF-1 by showing that deletion of *hif-1* can also increase life span, a finding which has been independently confirmed by two additional studies (Bellier *et al.*, 2009; Zhang *et al.*, 2009). Chen *et al.* (2009) reported that life span extension from deletion of *hif-1* occurs independently of DAF-16 and proposed that HIF-1 acts as a negative regulator of life span downstream of DR by modulating the response to ER stress. This model differs from that of Zhang *et al.*, (2009) who report that life span extension from deletion of HIF-1 requires DAF-16.

In addition to showing that loss of HIF-1 can increase life span, Zhang *et al.*, (2009) also confirmed that stabilization of HIF-1 increases life span. They reported that transgenic overexpression of either wild-type HIF-1 or a nondegradable allele of HIF-1 is

sufficient to enhance longevity by a DAF-16-independent mechanism. The pro-longevity role of HIF-1 was then further validated by Muller *et al.*, (2009) who verified that deletion of *vhl-1* increases life span in a DAF-16-independent manner. Under their conditions, however, the life span extension from *vhl-1(RNAi)* appeared to be partially HIF-1 independent, leading them to propose that *vhl-1* modulates longevity by both HIF-1-dependent and HIF-1-independent mechanisms.

The data described in these five studies define a key role for HIF-1 and the hypoxic response in aging, but they also raise an important question: How can HIF-1 play both a pro- and anti-longevity role at the same time? Here, we address this question by showing that life span extension from stabilization of HIF-1 is independent of temperature between 15 and 25°C, but life span extension from loss of HIF-1 is temperature dependent, occurring only at higher temperatures. We further report that knockdown of HIF-1 results in nuclear localization of the FOXO transcription factor DAF-16 and that life span extension resulting from deletion of *hif-1* is suppressed by mutation of *daf-16*. The failure of *hif-1* deletion to increase life span at lower temperatures arises, at least in part, from a variable-penetrance, age-associated vulval integrity defect (Vid), resulting in diminished healthspan and longevity.

Results

Life span extension from hif-1 deletion, but not HIF-1 stabilization, is temperature dependent

To clarify the apparent contradiction between our observation that deletion of *hif-1* has no effect on life span (Mehta *et al.*, 2009) and the report by Chen *et al.* (2009) that deletion of *hif-1*

increases life span, we explored potential explanations for this difference. One difference that we identified between the two studies was the temperature at which life span was examined; we used 20°C, while the other study used 25°C (Kaeberlein & Kapahi, 2009). To test the possibility that experimental temperature might influence the effect of *hif-1* deletion on life span, we measured survival of N2 wild-type and *hif-1(ia4)* animals maintained at either 25, 20, or 15°C. Consistent with prior data (Chen *et al.*, 2009), we observed that *hif-1(ia4)* animals lived significantly longer than N2 animals at 25°C (Fig. 1; Table S1, Supporting Information). At 20 or 15°C, the life span of *hif-1(ia4)* animals was not significantly different from N2 animals.

Because deletion of *hif-1* modulated longevity in a temperature-dependent manner, we next asked whether life span extension from stabilization of HIF-1 was also temperature dependent. In contrast to *hif-1(ia4)* animals, *vhl-1(ok161)* animals were long lived at 15, 20, and 25°C, relative to N2 (Fig. 1; Table S1). At all three temperatures, *vhl-1(ok161)* animals were also significantly longer lived than *hif-1(ia4)* animals. Deletion of *hif-1* largely suppressed life span extension from deletion of *vhl-1*; however, *hif-1(ia4); vhl-1(ok161)* animals were slightly longer-lived than *hif-1(ia4)* animals at 25 and 20°C (Fig. 1; Table S1). Our prior study (Mehta *et al.*, 2009) failed to detect a statistically significant difference in life span between *hif-1(ia4)* and *hif-1(ia4); vhl-1(ok161)* animals, perhaps because of a slight variation in experimental temperature between these two studies.

Loss of HIF-1 causes a temperature-dependent increase in vulval integrity defects

While performing the life span experiments on *hif-1(ia4)* animals, we noted elevated frequencies of vulval protrusion (Pvl,
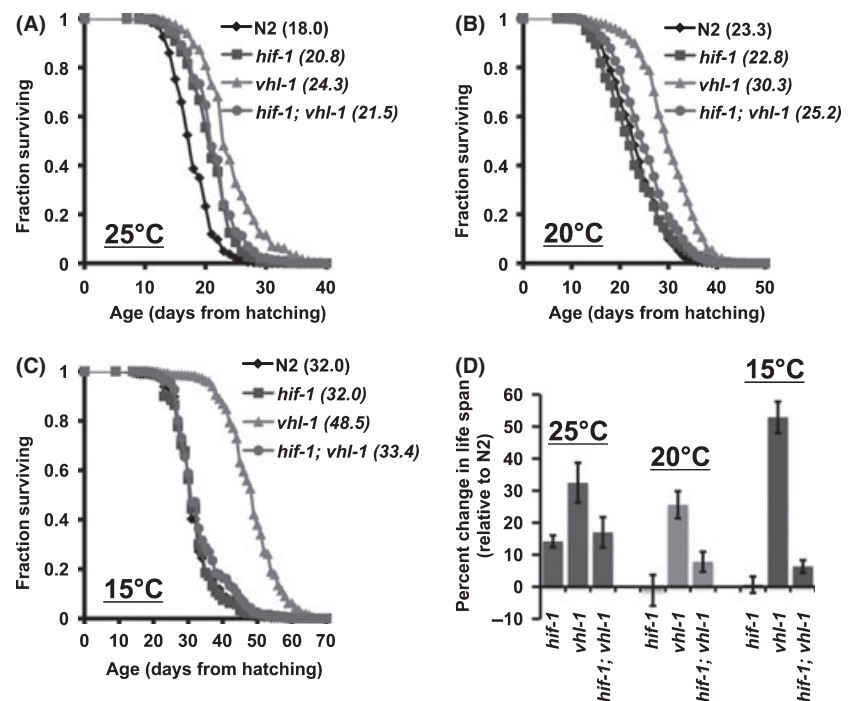

Fig. 1 Temperature influences the effect of HIF-1 deletion but not HIF-1 stabilization. Life span from hatching was determined for N2, *vhl-1(ok161)*, *hif-1(ia4)*, and *hif-1(ia4); vhl-1(ok161)* animals at (A) 15°C, (B) 20°C, and (C) 25°C. Mean life span is shown in parentheses and is summarized in (D). Deletion of *vhl-1* significantly increased median life span at all three temperatures, while deletion of *hif-1* only significantly increased life span at 25°C. A minimum of 350 animals and three replicate experiments were examined for each genotype at each temperature. Pooled data are shown. Summary data and statistics for individual and pooled experimental data are provided in Table S1.
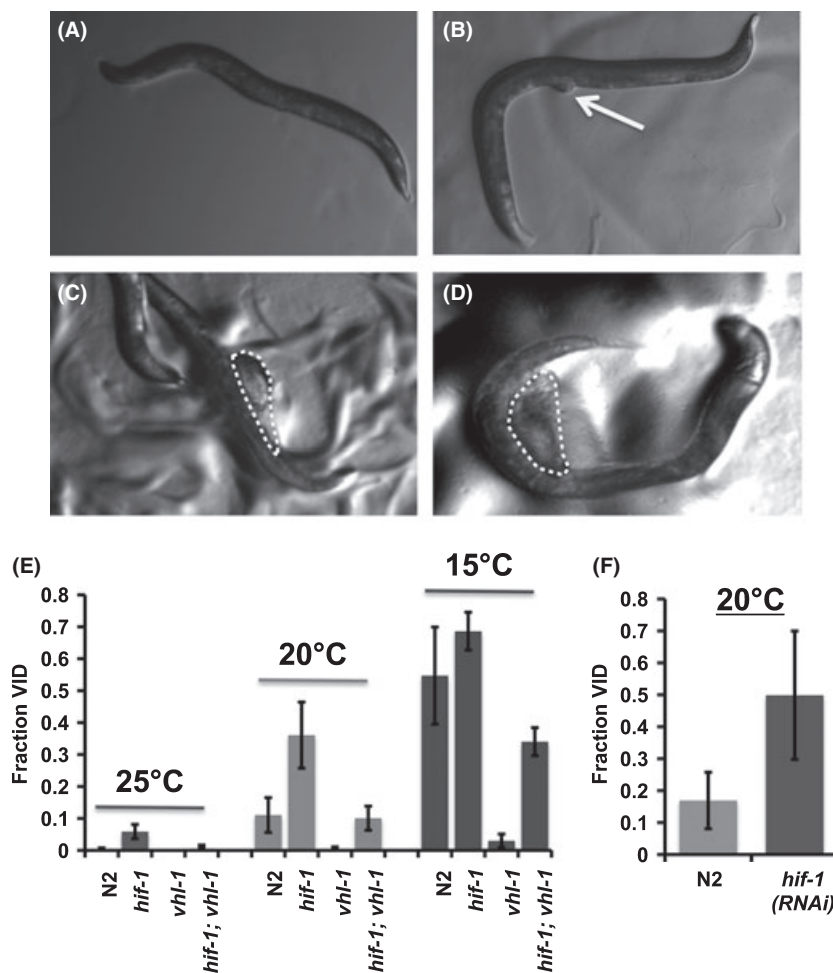
Fig. 2 Loss of HIF-1 impairs healthspan because of a defect in vulval integrity. Panels A–D show a representative example of the vulval integrity defect (Vid) observed in *hif-1(ia4)* animals across their entire life time. The Vid is typically first detected morphologically when (A) healthy worms begin to show a (B) protruding vulva. This usually progresses through stages of (C) early rupture and (D) late rupture prior to death. Panel E shows the total fraction of N2, *hif-1(ia4)*, *vhl-1(ok161)*, and *hif-1(ia4); vhl-1(ok161)* animals displaying a Vid prior to death at 25, 20, and 15°C, respectively. Panel F shows the increase in Vid of *hif-1(RNAi)* treatment from egg at 20°C. A, B, and C are viewed at 70× magnification, and D is viewed at 75× magnification. Error bars represent SEM. Summary data and statistics for individual and pooled experimental data are provided in Table S2.

Wormbase phenotype WBPhenotype:0000697) which progressed in a majority of animals soon thereafter to vulval rupture (Rup, Wormbase phenotype WBPhenotype:0000038). Vulval rupture (also referred to as 'exploded through vulva') can be observed when the animal displays an extrusion of the internal organs through the vulva (Fig. 2a–d). For simplicity, we will refer to both of these phenotypes as a Vid hereafter. The molecular basis of Vid is not well understood; however, Pvl and Rup are relatively common phenotypes, having been annotated on Wormbase for 1403 and 449 RNAi experiments, respectively (Harris *et al.*, 2010). To the best of our knowledge, deletion or RNAi knockdown of *hif-1* has not been previously described to cause Vid nor has this phenotype been previously associated with aging.
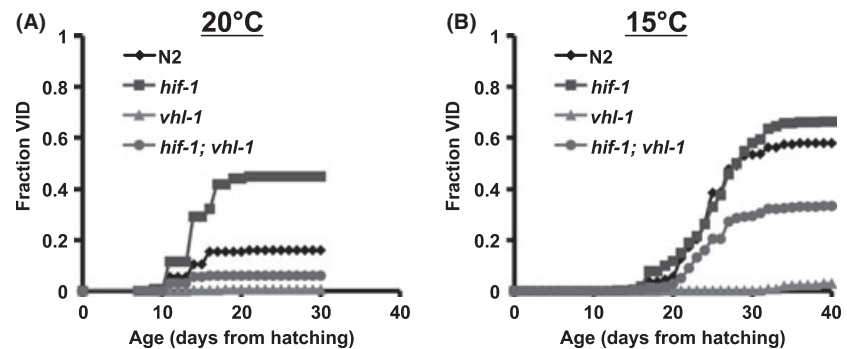
The penetrance of Vid was clearly temperature dependent, with higher frequencies of Vid occurring at 15°C, compared to 20°C (Fig. 2e; Table S2). Vid occurred rarely at 25°C. Although the frequency of Vid varied from experiment to experiment (Table S2), it was always the case that *hif-1(ia4)* animals showed a significantly greater frequency than N2 animals, while *vhl-1(ok161)* animals had a reduced level of Vid (Fig. 2e). Animals with both *vhl-1* and *hif-1* deleted had an intermediate level of Vid, indicating that *hif-1* is only partially epistatic to *vhl-1* for this

phenotype. RNAi knockdown of *hif-1* resulted in a similarly elevated level of Vid (Fig. 2f), demonstrating that this phenotype results from reduced HIF-1 abundance.

The appearance of Vid showed a striking age dependence. At 20°C, Vid was rarely observed during the first week of life, when animals are developing and reproductively active (Fig. 3). During the second week of life, the percentage of animals showing Vid increased and then leveled off. Vid was never observed after the 20th day of life at this temperature, even though a majority of animals were still alive and lived for several additional days. The same trend was observed at 15°C, but occurred about 1 week later, likely due to the slower rate of development and aging at lower temperatures. Based on these observations, we propose that Vid represents an age-dependent loss of healthspan that is modulated by both temperature and HIF-1.

Many laboratories censor 'ruptured' or 'exploded' animals from life span experiments because this phenotype is not considered to be a part of the normal aging process; however, it has been our policy not to exclude animals from our longevity experiments whenever possible. Interestingly, when Vid animals are censored from our data set, deletion of *hif-1* results in a significant, albeit modest, increase in life span at 20°C (Fig. 4; Table S3). Censoring of Vid animals failed to yield a significant

Fig. 3 Loss of vulval integrity is an age-dependent healthspan phenotype. The fraction of animals with observable vulval integrity defects are shown as a function of age from hatching for N2, *hif-1(ia4)*, *vhl-1(ok161)*, and *hif-1(ia4); vhl-1(ok161)* at (A) 20°C and (B) 15°C.

life span extension in *hif-1(ia4)* animals at 15°C (Fig. 4; Table S3).

DAF-16∕FOXO relocalizes to the nucleus in response to loss of HIF-1 and is required for life span extension

We next explored the nature of the temperature-dependent life span extension from deletion of *hif-1*. As life span extension
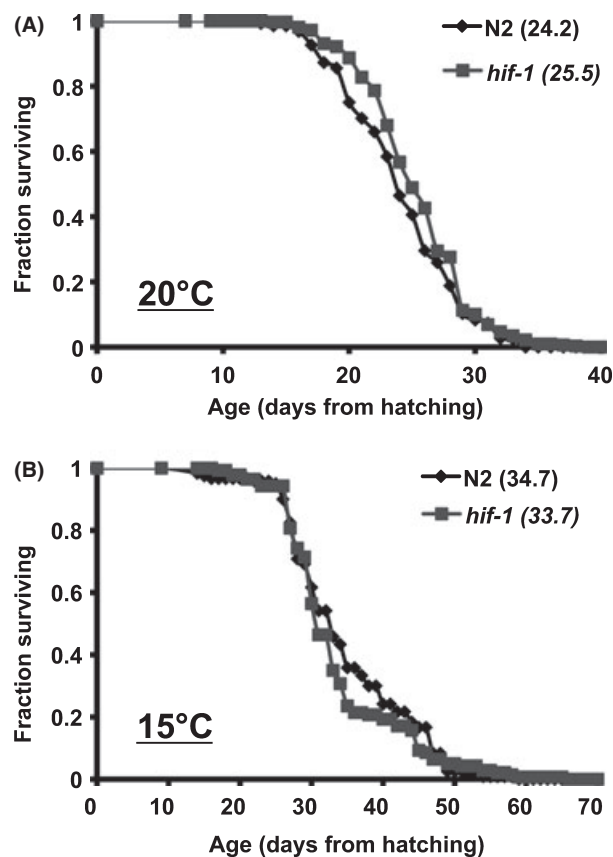

Fig. 4 Deletion of *hif-1* increases life span when animals exhibiting a vulval integrity defect are censored. Reanalysis of our longevity data removing animals displaying loss of vulval integrity. Censored life span from hatching is shown for N2 and *hif-1(ia4)* animals at (A) 20°C and (B) 15°C. Censoring results in a significant increase in life span of *hif-1(ia4)* animals at 20°C but not at 15°C. Summary data and statistics for individual and pooled experimental data are provided in Table S3.

from stabilization of HIF-1 is independent of DAF-16 (Mehta *et al.*, 2009), we wondered whether deletion of *hif-1* would also extend life span in a DAF-16-independent manner. Relative to *daf-16(mu86)* single mutants, *daf-16(mu86); hif-1(ia4)* double mutant animals were not long lived at any of the three temperatures tested (Fig. 5; Table S4). Life span extension from *hif-1* deletion at 20°C when Vid animals were censored was similarly DAF-16-dependent (Fig. 6; Table S5). The elevated frequency of Vid caused by mutation of *hif-1* was only partially suppressed by mutation of *daf-16* (Table S2).

Although suppression of life span extension by mutation of *daf-16* is consistent with the idea that DAF-16 acts downstream of HIF-1, it may also be the case that loss of DAF-16 prevents life span extension by indirect mechanisms. To determine whether DAF-16 function is altered in animals lacking HIF-1, we used a previously described DAF-16::GFP reporter strain to monitor localization of DAF-16 (Henderson & Johnson, 2001). Similar to mammalian FOXO transcription factors, translocation of DAF-16 from the cytoplasm to the nucleus is required for activation, and has been observed in animals with reduced insulin-like signaling and in response to stresses such as heat shock or starvation (Calnan & Brunet, 2008). Interestingly, RNAi knock-down of *hif-1* resulted in translocation of DAF-16 to the nucleus to an extent comparable to knock-down of the insulin-like receptor *daf-2* (Figs 7 and S1, Supporting Information). These experiments were performed at atmospheric oxygen levels, indicating that basal HIF-1 activity promotes cytoplasmic localization of DAF-16 under normoxic conditions and supporting the idea that temperature-dependent life span extension from loss of *hif-1* results from activation of DAF-16. In contrast to loss of *hif-1*, stabilization of *hif-1* by deletion of *vhl-1* had no effect on nuclear localization of DAF-16::GFP. This is consistent with prior reports that life span extension from deletion of *vhl-1* does not require DAF-16 (Mehta *et al.*, 2009; Muller *et al.*, 2009).

Discussion

HIF-1 has recently been reported to both promote and repress longevity in *C. elegans*, leading to confusion regarding the role of the hypoxic response in aging (Leiser & Kaeberlein, 2010). These apparent contradictions can be partially explained by the observation that experimental temperature and censoring of Vid animals both influence the effect of *hif-1* deletion on life span.
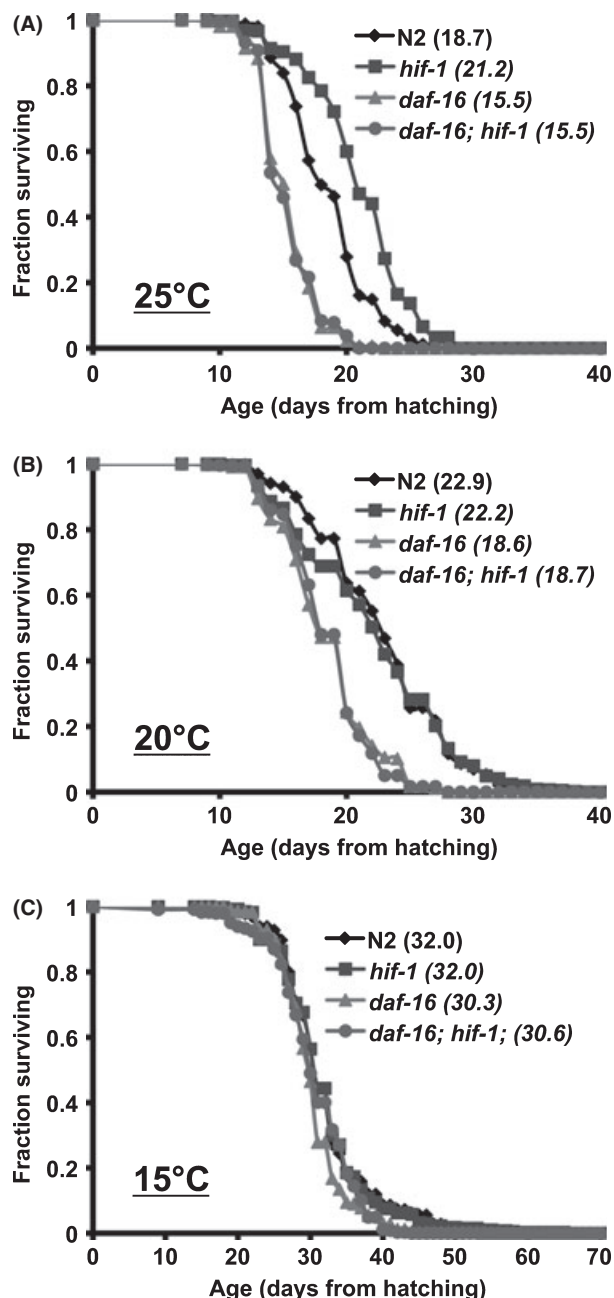
Fig. 5 DAF-16 is required for life span extension from deletion of *hif-1*. Life span from hatching was determined for N2, *daf-16*(*mu86*), and *daf-16*(*mu86*); *hif-1*(*ia4*) animals at (A) 25°C, (B) 20°C, and (C) 15°C. Mean life span is shown in parentheses. Deletion of *hif-1* failed to significantly increase life span of *daf-16*(*mu86*) animals at any temperature. Summary data and statistics for individual and pooled experimental data are provided in Table S4.

Fig. 6 DAF-16 is still required for life span extension from deletion of *hif-1* when animals with vulval integrity defects are censored. Reanalysis of the longevity data from Fig. 5 removing animals displaying loss of vulval integrity at (A) 20°C and (B) 15°C. Mean life span is shown in parentheses. Summary data and statistics for individual and pooled experimental data are provided in Table S5.

Fig. 7 *hif-1* RNAi causes nuclear relocalization of DAF-16::GFP. (A) Fraction of animals showing distinct DAF-16::GFP puncta following 2 -h treatments of either empty vector RNAi (EV), 37° heat shock (heat), *daf-2*(*RNAi*), *hif-1*(*RNAi*), or *vhl-1*(*RNAi*) at 15°, 20°, and 25°. *P*-value of < 0.05 relative to EV by one-way ANOVA. Representative images of animals are provided in Fig. S1.

Our observation that RNAi knockdown of *hif-1* causes relocalization of DAF-16 to the nucleus suggests that HIF-1 acts as a repressor of DAF-16 under normoxic conditions and is consistent with suppression of life span extension in *hif-1*(*ia4*) animals by a null allele of *daf-16*. In contrast to deletion of *hif-1*, stabiliza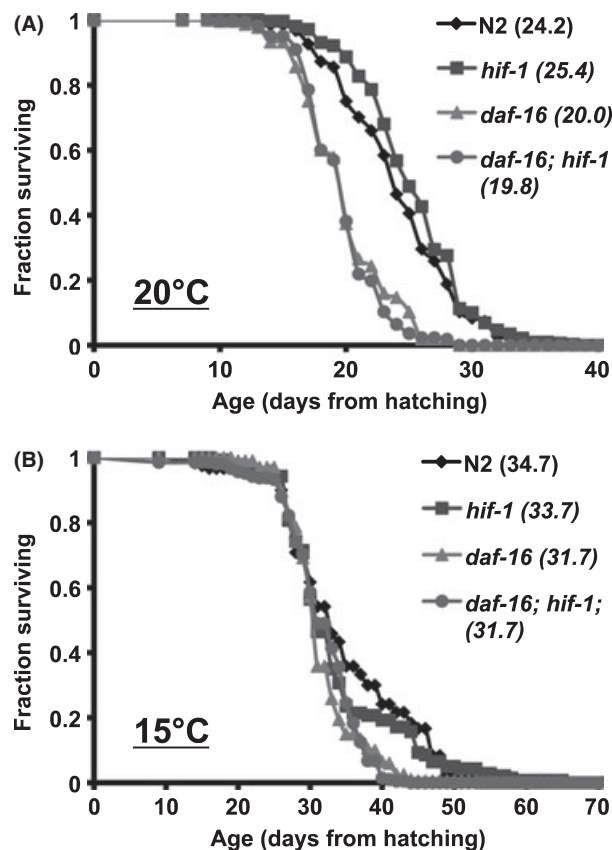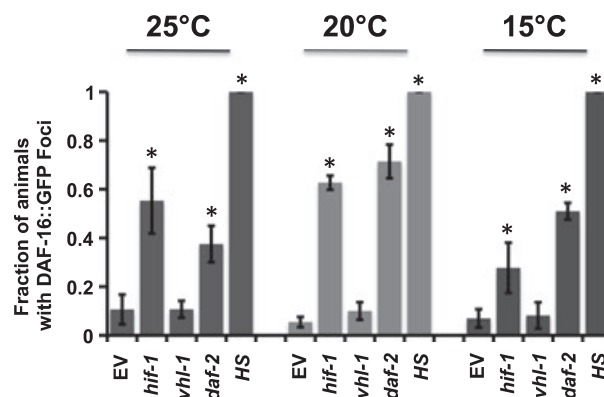tion of HIF-1 by deletion of *vhl-1* robustly increases life span at all three temperatures tested, and prior studies have indicated that this life span extension is genetically distinct from insulin-like signaling and DR (Mehta *et al.*, 2009; Muller *et al.*,
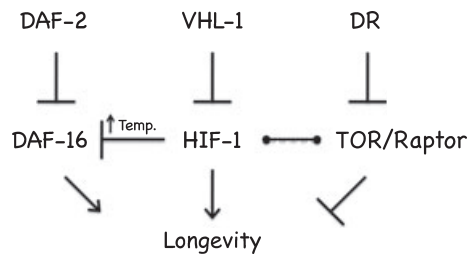
Fig. 8 Model for HIF-1 modulation of longevity in *C. elegans*. Stabilization of HIF-1 promotes longevity by a mechanism distinct from both insulin-like signaling and dietary restriction (DR). HIF-1 also negatively impacts longevity by repressing nuclear localization and activation of DAF-16, in a temperature-dependent manner. HIF-1 also interacts with TOR∕Raptor and the DR pathway, but in a complex manner dependent on additional experimental variables that are currently unknown (represented by dashed lines).

2009; Zhang *et al.*, 2009). Taken together, these data suggest a model in which stabilization of HIF-1 can promote longevity and healthspan regardless of temperature by inducing hypoxic response target genes under normoxic conditions, while deletion of *hif-1* can promote longevity by activation of DAF-16 at high temperature (Fig. 8).

Although we have reconciled the contradictory data regarding life span extension from deletion of *hif-1*, our model still differs from that of Chen *et al.* (2009), who proposed that deletion of *hif-1* increases life span by a mechanism related to DR and involving the ER stress response factor IRE-1. This model was based, in part, upon their observation that *hif-1(RNAi)* increased the life span of *daf-16(mgDF47)* animals (Chen *et al.*, 2009). In contrast to this, we found that the *hif-1(ia4)* mutation did not increase the life span of *daf-16(mu86)* animals, consistent with the data of Zhang *et al.*, (2009). Although both *daf-16* alleles are thought to be null alleles, it is possible that different *daf-16* alleles interact differently with loss of HIF-1. It is also possible that *hif-1(RNAi)* and *hif-1(ia4)* reduce HIF-1 activity to a different extent or in a different manner. For example, it may be the case that RNAi knockdown reduces HIF-1 activity to a variable extent in different cell types, while deletion of *hif-1* results in loss of HIF-1 activity in all cells. It will be of interest to determine which cell types are most important for life span extension from loss of HIF-1 as well as stabilization of HIF-1.

DAF-16 and HIF-1 interact in a complex manner

DAF-16 and HIF-1 are both stress-responsive transcription factors that promote longevity. Our data suggest that HIF-1 impairs nuclear localization of DAF-16::GFP under normoxic conditions, providing a previously unexpected connection between these two proteins. An important question is whether this effect of HIF-1 on DAF-16 is direct or indirect. We cannot currently differentiate between these two possibilities, but we speculate that loss of basal HIF-1 activity is perceived as a stress signal even under normoxic conditions, and that this signal is sufficient to cause DAF-16 to become activated.

Similar to mammalian FOXO transcription factors, DAF-16 localization is regulated by at least three post-translational modifications: phosphorylation, acetylation, and ubiquitination (Calnan & Brunet, 2008). Several aging-related factors are known to influence DAF-16 localization and activity. For example, the *C. elegans* AMP kinase catalytic subunit AAK-2 is able to phosphorylate at least 6 residues on DAF-16 (Greer *et al.*, 2007). Likewise, Cst1, Akt, and Jnk are all known to phosphorylate DAF-16 to modulate its localization and activity (Lehtinen *et al.*, 2006; Mukhopadhyay *et al.*, 2006). Polyubiquitination of DAF-16 is modulated by the RLE1 ubiquitin ligase (Li *et al.*, 2007), and acetylation of DAF-16 is influenced by the SIR-2.1 protein deacetylase (Berdichevsky *et al.*, 2006; Wang *et al.*, 2006). This latter interaction may be particularly relevant here, as the mammalian SIR-2.1 ortholog SIRT1 has recently been reported to deacetylate both HIF-1α and HIF-2α (Dioum *et al.*, 2009; Leiser & Kaeberlein, 2010; Lim *et al.*, 2010). It will be of interest to determine how post-translational modification of DAF-16 is altered by reduced expression of *hif-1* and the extent to which this regulation is conserved in other species.

It is also noteworthy that despite the fact that *hif-1* RNAi induces DAF-16::GFP nuclear localization to an extent similar to *daf-2* RNAi at 20 and 15°C, deletion of *hif-1* does not extend life span at these temperatures. One possible explanation is that DAF-16 target genes important for longevity are not activated by loss of *hif-1* at 15°C, despite nuclear localization of DAF-16. It is also possible that DAF-16 activity is regulated differently in *hif-1(ia4)* animals that completely lack HIF-1 activity from hatching, when compared to animals subjected to RNAi knockdown of *hif-1*. We note that there are additional examples of interventions that induce DAF-16::GFP nuclear localization without extending life span, demonstrating that nuclear localization of DAF-16 is not always sufficient to promote longevity in *C. elegans* (Yamawaki *et al.*, 2008).

Vulval integrity and healthspan in *Caenorhabditis elegans*

Healthspan can be defined as the period of life spent in relatively good health, and maintenance of healthspan is recognized as an important component of aging (Tatar, 2009). Measures of healthspan have been proposed in *C. elegans*, including retention of motility during aging, accumulation of autofluorescent age pigment, and resistance to proteotoxic stress (Huang *et al.*, 2004; Gerstbrein *et al.*, 2005; Steinkraus *et al.*, 2008). We have previously reported improved healthspan in *vhl-1(ok161)* animals by two of these measures, reduced accumulation of age pigment and enhanced resistance to proteotoxic stress (Mehta *et al.*, 2009).

We propose that maintenance of vulval integrity is another potentially important measure of nematode healthspan that should be considered during longevity studies. In wild-type animals, loss of healthspan because of Vid occurs postreproductively in a relatively small percentage of the population. The frequency of Vid is temperature dependent, occurring more

often at lower temperatures, at least within the range of 15–25°C. Loss of healthspan because of Vid is significantly greater in *hif-1(ia4)* animals than N2 animals and may explain some of the differences observed in prior studies with respect to effect of *hif-1* deletion on life span. Consistent with this possibility, we note that Zhang *et al.* (2009) excluded animals that 'burst at the vulva' and observed life span extension at 20°C. The frequency of Vid is significantly reduced in *vhl-1(ok161)* animals, relative to N2, at both 20 and 15°C, providing further evidence that loss of *vhl-1* enhances both life span and healthspan in *C. elegans*.

Reduced healthspan because of Vid has been previously associated with RNAi knockdown of mRNA translation factors, although this relationship has not been carefully examined. Specifically, Hansen *et al.*, (2007) reported that 'translation-associated RNAi clones, in particular TOR RNAi, often induced a slightly higher level of rupturing'. Similar to Zhang *et al.*, (2009), ruptured animals were censored from the life span analysis in these experiments (Hansen *et al.*, 2007). It is also unknown whether the enhanced frequency of Vid associated with translation-related RNAi clones is temperature dependent as in the case of *hif-1(ia4)*, because Hansen *et al.*, (2007) performed all of their life span studies at 20°C.

The observation that deletion of *hif-1* and some translation-related longevity interventions negatively impact healthspan, while positively impacting life span represents an important demonstration that longevity and healthspan are not always coupled. Although we have generally chosen not to censor animals that fail to maintain vulval integrity from our longevity analysis, this is common practice among some laboratories, and a discussion of how to appropriately address this phenotype is warranted. It will also be of interest to determine which additional longevity interventions or experimental conditions other than temperature influence the frequency of Vid.

Role of the hypoxic response in mammalian aging

The function of HIF-1 as both a positive and negative modulator of longevity and healthspan in *C. elegans* raises the question as to whether mammalian HIF plays any similar roles. It is unknown whether mammalian FOXO proteins are activated by knockdown of HIF, but if this response is conserved, then it is reasonable to speculate that reduced HIF activity under normoxic conditions may be beneficial against age-associated disease. Interestingly, FOXO4 has been reported to regulate HIF-1α activity (Dimova *et al.*, 2010), suggesting that FOXO and HIF transcription factors are likely to interact in a complex manner in mammals.

HIF-1α has been implicated as an important factor in human healthspan. One study found that a specific HIF-1α allele is associated with athletic skill and fast twitch muscle fiber predominance in weight lifters (Ahmetov *et al.*, 2008), while additional studies have indicated that elite athletes show a higher sensitivity of HIF-1α during an acute hypoxic test (Mounier *et al.*, 2009; Pialoux *et al.*, 2009). Mutation of the VHL-1 ortholog results in

von Hippel Lindau syndrome, an autosomal dominant disorder characterized by a variety of tumors, particularly angiomas and hemangioblastomas (Iliopoulos & Kaelin, 1997). Clearly, therefore, human healthspan can be negatively impacted by constitutive stabilization of HIF, but it may be the case that altered sensitivity of the hypoxic response or partial stabilization, perhaps in a tissue-specific manner, can also prove beneficial. As the downstream targets of HIF-1 involved in promoting longevity and healthspan are identified in *C. elegans*, it may be possible to modulate HIF function in mammals to slow aging and the delay progression of age-related diseases.

Materials and methods

Strains and growth conditions

Standard procedures for *C. elegans* strain maintenance and manipulation were used, as previously described (Kaeberlein *et al.*, 2006; Smith *et al.*, 2008; Sutphin & Kaeberlein, 2008). Except for RNAi experiments, all experimental procedures were performed on animals fed UV-killed *E. coli* OP50 from hatching. Experimental animals were maintained on solid Nematode Growth Medium (NGM) supplemented with 50 μg mL^{-1} ampicillin. Except where stated otherwise, experiments were performed on animals maintained at 20°C. Nematode strains used in this study are described in Table S6. For RNAi experiments, animals were maintained on RNAi feeding strains. RNAi plates consisted of NGM supplemented with 1 mM β-D-isothiogalacto-pyranoside and 25 μg mL^{-1} carbenicillin. Unless otherwise indicated, worms were raised on RNAi bacteria from hatching. RNAi clones were verified by sequencing the region of the RNAi plasmid expressing the double-stranded RNA after purification from the corresponding bacterial clone and by phenotypic analysis of animals maintained on the RNAi bacteria. Sequence verification of the RNAi clones used in this study has been previously published (Mehta *et al.*, 2009).

Life span analysis

Life span analyses were carried out as described (Sutphin & Kaeberlein, 2009). Statistical analysis and replication of life span experiments are provided in Tables S1–5. Ruptured animals were not censored from life span experiments, except where indicated in Fig. 3 and Table S3. Animals that foraged off the surface of the plate during the course of the experiment were not considered.

Quantification of DAF-16::GFP puncta

Fluorescence microscopy was performed using a Zeiss SteREO Lumar.V12 (Thornwood, NY, USA) microscope as previously described (Steinkraus *et al.*, 2008; Mehta *et al.*, 2009). Eggs were prepared from gravid DAF-16::GFP adult worms and placed on NGM with EV or RNAi bacteria. Animals were grown at 20°C for 3 days and then treated with specific RNAi or heat

shock (37°C) for 2 h, before being imaged using a Zeiss SteREO Lumar.V12 microscope as previously described (Steinkraus *et al.*, 2008). Fluorescence microscopy was performed. Animals were paralyzed with 25 mM sodium azide and placed on a Teflon printed 8-well glass slide with 6- mm well diameter (Electron Microscopy Sciences). The GFP filter (470⁄40 excitation band-pass filter and 525⁄50 emission band-pass filter) was employed for imaging the DAF-16::GFP worms at 150× magnification with exposure time of 40 ms for bright field and 500 ms for GFP filter. The Image J 1.341.5.0_07 software was employed for inverting the images and converting them to 32-bit format and then manually counting the worms for the presence or absence of distinct GFP puncta.

Statistical analysis

A Wilcoxon rank-sum test (MATLAB 'ranksum' function) was used to generate *P*-values to determine statistical significance for life span assays. The mean life spans, number of animals, number of replicate experiments, and *P*-values are provided in Tables S1–5. A two-tailed Student's *t*-test was performed using the TTEST function in Microsoft Excel to calculate *P*-values for DAF-16::GFP positive worms.

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Author contributions

MK and SFL conceived and designed the study and wrote the manuscript. Experimental work was performed by SFL and AB.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Fig. S1 Representative images of DAF-16::GFP animals.

Table S1 Life spans for Fig. 1.

Table S2 Vulval integrity defect (Vid) frequency.

Table S3 Life spans censored for vulval integrity defects.

Table S4 Life spans of daf16 and hif1 animals.

Table S5 Life spans of daf16 and hif1 animals censored for vulval integrity defects.

Table S6 List of strains used.

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