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Proteasomal Regulation of the Hypoxic Response Modulates Aging in *C. elegans*

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The *Caenorhabditis elegans* von Hippel–Lindau tumor suppressor homolog VHL-1 is a cullin E3 ubiquitin ligase that negatively regulates the hypoxic response by promoting ubiquitination and degradation of the hypoxic response transcription factor HIF-1. Here, we report that loss of VHL-1 significantly increased life span and enhanced resistance to polyglutamine and β-amyloid toxicity. Deletion of HIF-1 was epistatic to VHL-1, indicating that HIF-1 acts downstream of VHL-1 to modulate aging and proteotoxicity. VHL-1 and HIF-1 control longevity by a mechanism distinct from both dietary restriction and insulin-like signaling. These findings define VHL-1 and the hypoxic response as an alternative longevity and protein homeostasis pathway.

Loss of protein homeostasis is increasingly becoming recognized as an important contributor to several age-associated diseases and may play a causal role in aging (*1*, *2*). A link between aging and protein homeostasis in the nematode *Caenorhabditis elegans* is supported by observations that increasing life span by reducing insulin and insulin-like signaling (ILS) or by dietary restriction (DR) also improves function in transgenic models of proteotoxic disease associated with aberrant protein aggregation (*3*, *4*).

A primary cellular mechanism for degrading damaged proteins is the ubiquitin-proteasomal system, which involves covalent attachment of ubiquitin to target proteins before degradation. RNA interference (RNAi) knockdown of proteasome components reduces resistance to polyglutamine toxicity and shortens life span in *C. elegans* (*5*, *6*), and we noted that proteasome inhibition led to accelerated paralysis in animals expressing a 35-residue polyglutamine repeat fused to yellow fluorescent protein (YFP) in body wall muscle cells (Q35YFP) (fig. S2). To further explore the relations between proteasomal function and protein homeostasis, we initiated an RNAi screen of known or predicted E3 ubiquitin ligases for altered resistance to polyglutamine toxicity (*7*) (table S1). Cullin-RING ubiquitin ligases (CULs) consist of multiple protein subunits that include a cullin protein, a RING finger protein, an adaptor protein, and a substrate recognition subunit (fig. S3) (*8*). Similar to proteasome inhibition, RNAi knockdown of genes encoding CUL1 or CUL2 core components accelerated paralysis in Q35YFP animals (fig. S3).

In contrast to knockdown of CUL core components, we identified an RNAi clone corresponding to a CUL2 substrate recognition subunit, VHL-1, that significantly delayed paralysis in Q35YFP animals (Fig. 1A). A similar increase in resistance to β-amyloid toxicity was also observed in response to *vhl-1(RNAi)* (Fig. 1B). VHL-1 is homologous to the mammalian von Hippel–Lindau tumor suppressor protein, which ubiquitinates the α subunit of the hypoxic response transcription factor, HIF-1 (*9*). Under normoxic conditions, ubiquitination of HIF-1 by VHL-1 represses the hypoxic response by targeting HIF-1 for proteasomal degradation (fig. S4). In order for VHL-1 to ubiquitinate HIF-1, HIF-1 must be hydroxylated by the EGL-9 prolyl hydroxylase (*10*). Similar to *vhl-1(RNAi)*, *egl-9(RNAi)* also enhanced resistance to both polyglutamine (Fig. 1C) and β-amyloid toxicity (Fig. 1D). Noting prior correlation between resistance to proteotoxicity and increased life span, we next determined whether *vhl-1* and *egl-9* also modulate aging by measuring the effect of RNAi knockdown of *vhl-1* or *egl-9* on life span in the RNAi-sensitive *rrf-3(pk1426)* background. Animals maintained on either *vhl-1 (RNAi)* or *egl-9(RNAi)* lived significantly longer than animals maintained on empty vector (EV) bacteria (Fig. 1, E and F).

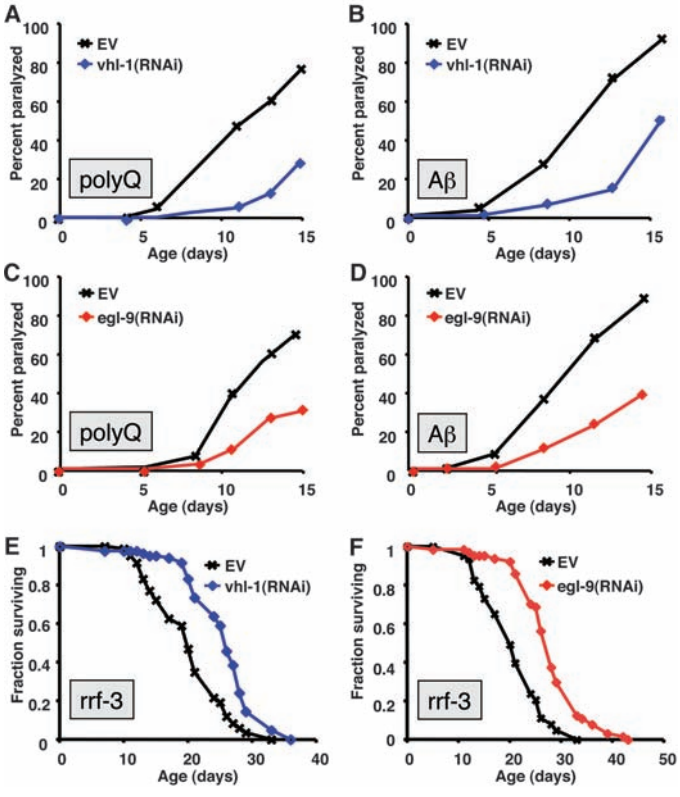

Fig. 1. VHL-1 and EGL-9 modulate proteotoxic stress and life span. RNAi knockdown of *vhl-1* significantly enhances resistance to (**A**) polyglutamine (polyQ) toxicity ($P < 1 \times 10^{-5}$) and (**B**) β-amyloid (Aβ) toxicity ($P < 1 \times 10^{-5}$) relative to animals fed EV bacteria. RNAi knockdown of *egl-9* significantly enhances resistance to (**C**) polyglutamine toxicity ($P < 1 \times 10^{-5}$) and (**D**) β-amyloid toxicity ($P < 1 \times 10^{-5}$) relative to animals fed EV bacteria. RNAi knockdown of (**E**) *vhl-1* ($P < 1 \times 10^{-5}$) or (**F**) *egl-9* ($P < 1 \times 10^{-5}$) significantly increased adult life span relative to the EV-fed control. Paralysis and life-span statistics are in tables S1 and S6.

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To determine whether increased stability of HIF-1 could account for the enhanced longevity associated with *vhl-1* knockdown, we examined the life spans of animals deleted for *vhl-1*, *hif-1*, or both *vhl-1* and *hif-1* (*10*). The *hif-1(ia4)* allele removes exons 2, 3, and 4 of *hif-1*, including the DNA binding domain, and is believed to be a null allele (*11*) (Fig. 2A). The *vhl-1(ok161)* allele removes exons 1 and 2 of *vhl-1* and is also a putative null allele (Fig. 2B). As observed for *vhl-1(RNAi)* animals, deletion of *vhl-1* significantly increased life span (Fig. 2C). Deletion of *hif-1* alone did not substantially influence life span but completely suppressed the life-span extension imparted by deletion of *vhl-1* (Fig. 2C). Consistent with the observed longevity effects, the accumulation of autofluorescent age pigments, which has been proposed as a biomarker of aging and health span in *C. elegans* (*12*), was reduced in *vhl-1(ok161)* animals (Fig. 2D and fig. S5). This reduction was also fully suppressed by deletion of *hif-1*.

Given that deletion of *vhl-1* increased life span and resistance to proteotoxic stress, we speculated that there may be a fitness cost associated with constitutive expression of HIF-1 under normoxic conditions. One cost associated with many long-lived mutants is a decrease in fecundity. We quantified the number of eggs laid during adulthood (brood size) for N2, *vhl-1 (ok161)*, *hif-1(ia4)*, and *vhl-1(ok161); hif-1(ia4)* animals. A significant decrease in brood size was observed for *vhl-1(ok161)* animals but not for *hif-1(ia4)* animals (Fig. 2E). As observed for life span and age-pigment accumulation, deletion of *hif-1* suppressed the brood size defect of *vhl-1(ok161)* animals. Induction of HIF-1 by growth under hypoxic conditions also resulted in a significant decrease in brood size (figs. S6 and S7) and a corresponding increase in life span (fig. S8). These observations support the idea that repression of HIF-1 under normoxic conditions confers a fitness benefit in the form of enhanced fecundity.

We next examined the relations between DR and the hypoxic response. DR can be accomplished in *C. elegans* by reducing the availability of the bacterial food source, with complete removal of bacterial food during adulthood (bacterial deprivation) providing maximal life-span extension (*13*, *14*). If *vhl-1* and DR act in the same pathway to modulate longevity, then life-span extension from bacterial deprivation should require *hif-1* and not further extend the life span of *vhl-1* mutants. In contrast, bacterial deprivation extended the life span of *hif-1(ia4)* animals to an extent similar to that of controls (Fig. 3A) and further extended the long life span of *vhl-1(ok161)* animals (Fig. 3B). Bacterial deprivation also increased the life span of *hif-1(ia4)*; *vhl-1(ok161)* double mutants (Fig. 3C).

A common genetic model of DR in *C. elegans* is mutation of *eat-2*, which results in decreased food consumption because of a defect in pharyngeal pumping (*15*). Unlike *eat-2(ad465)* mutants, *vhl-1(ok161)* animals did not display a

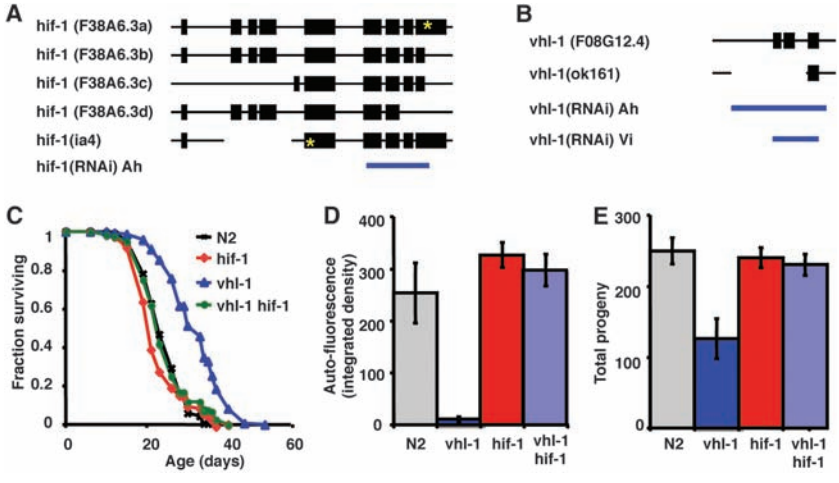

Fig. 2. VHL-1 modulates longevity, age-pigment accumulation, and reproduction in a HIF-1–dependent manner. Gene structures of (**A**) known *hif-1* splice variants, *hif-1 (ia4)* deletion, and *hif-1(RNAi)* and (**B**) known *vhl-1* splice variants, *vhl-1(ok161)* deletion, and *vhl-1(RNAi)*. Black boxes represent exons; yellow asterisk indicates a stop codon. Blue boxes indicate RNAi target sequences from Ahringer (Ah) or Vidal (Vi) library clones. The RNAi clones used to knock down *hif-1* and *vhl-1* target all known splice variants. (**C**) *Vhl-1(ok161)* animals are significantly longer-lived than wild-type (N2) animals ($P < 1 \times 10^{-5}$); *vhl-1(ok161); hif-1(ia4)* double-mutant animals are not longer-lived than N2 ($P = 0.66$). (**D**) Accumulation of autofluorescent age pigment is significantly reduced by deletion of *vhl-1* ($P < 1 \times 10^{-5}$). Autofluorescence is not significantly different in N2 versus *vhl-1(ok161); hif-1(ia4)* double-mutant animals ($P = 0.17$). (**E**) *Vhl-1(ok161)* animals produce significantly fewer progeny than N2 animals do ($P = 3.6 \times 10^{-3}$). No significant difference in brood size was observed for *vhl-1(ok161); hif-1(ia4)* double-mutant animals ($P = 0.69$) or *hif-1(ia4)* animals ($P = 0.43$), relative to N2 animals. Data in (D) and (E) are mean ± SD of at least nine animals per condition. Life-span statistics provided in table S6.

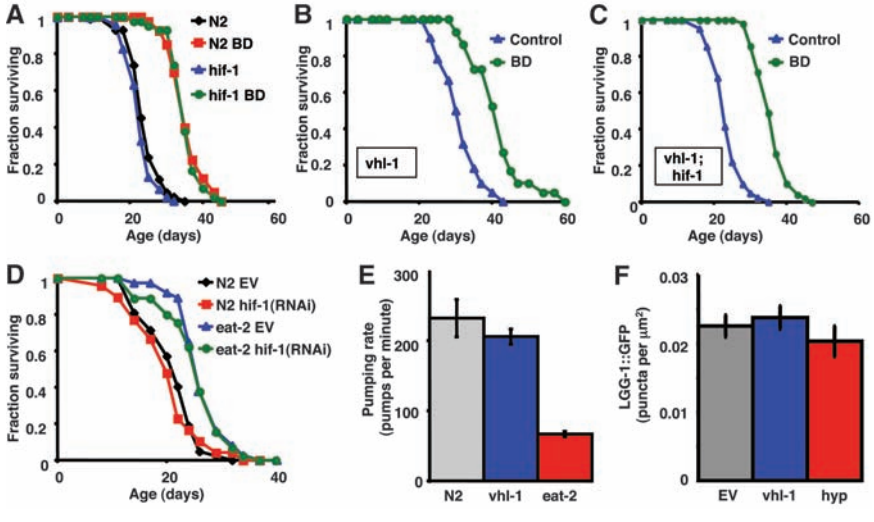

Fig. 3. VHL-1 and HIF-1 modulate longevity by a mechanism distinct from dietary restriction. (**A**) Life-span extension from bacterial deprivation (BD) is not significantly different in N2 and *hif-1(ia4)* animals ($P = 0.97$). (**B**) BD significantly increases the life span of *vhl-1(ok161)* animals ($P < 1 \times 10^{-5}$) and (**C**) *vhl-1(ok161)*; *hif-1(ia4)* double-mutant animals ($P < 1 \times 10^{-5}$). (**D**) *Hif-1(RNAi)* does not significantly alter the life-span extension of *eat-2(ad465)* animals ($P = 0.6$). (**E**) The *eat-2(ad465)* mutation significantly reduces pharyngeal pumping rate relative to rates in N2 ($P < 1 \times 10^{-5}$) or *vhl-1(ok161)* ($P < 1 \times 10^{-5}$) animals. Pharyngeal pumping rate is not significantly different in N2 and *vhl-1(ok161)* animals ($P = 0.06$). Data are mean ± SD. (**F**) Relative to animals fed EV bacteria under normoxic conditions, *vhl-1(RNAi)* under normoxia or growth on EV bacteria under hypoxia (hyp, 0.5% oxygen) failed to significantly increase autophagy, as indicated by the presence of LGG-1::GFP puncta ($P = 0.6$ and 0.5, respectively). Thirty-five animals per condition were imaged for EV and *vhl-1(RNAi)*. Seven animals were imaged for hypoxia. Data are mean ± SEM. Life-span statistics provided in table S6.

significant reduction in pumping rate (Fig. 3E), and, similar to the case for bacterial deprivation, knockdown of *hif-1* had no detectable effect on life-span extension from mutation of *eat-2* (Fig. 3D). Knockdown of *vhl-1* or growth under hypoxic conditions also failed to cause a significant increase in the abundance of autophagic vesicles (Fig. 3F and fig. S9), a phenotype reported to be required for life-span extension associated with DR (*16*, *17*). Thus, DR and the hypoxic response are likely to modulate longevity via distinct genetic pathways.

Decreased activity of the insulin-like receptor DAF-2 has been shown to increase life span (*18*, *19*) and promote resistance to hypoxia (*20*), leading us to consider whether *vhl-1* and *daf-2* act in the same genetic pathway to limit longevity. Like DR, however, *daf-2(RNAi)* further extended the already long life span of *vhl-1(ok161)* animals (Fig. 4A), and deletion of *hif-1* (Fig. 4B) or both *hif-1* and *vhl-1* (Fig. 4C) did not prevent life-span extension from *daf-2(RNAi)*. Life-span extension of animals with reduced ILS activity, including *daf-2* mutants, is dependent on the FOXO family transcription factor DAF-16, which acts downstream of DAF-2 to regulate gene expression (*21*, *22*). In order for DAF-16 to regulate target genes, it must be localized to the nucleus, a process that can be monitored by visualization of a DAF-16::GFP (green fluorescent protein) reporter (*23*). Transient heat shock or *daf-2(RNAi)* increased nuclear localization of DAF-16, whereas *vhl-1(RNAi)* had no detectable effect (Fig. 4D and fig. S10), suggesting that DAF-16 is not activated by loss of *vhl-1*. Consistent with this, *daf-16(RNAi)* did not fully suppress the increase in life span (Fig. 4E) or the reduced abundance of age pigment (Fig. 4F and fig. S11) associated with deletion of *vhl-1*, and *vhl-1(RNAi)* increased the life span of *daf-16* null animals (fig. S12). In contrast, *daf-16(RNAi)* fully suppressed the enhanced longevity of *daf-2(e1370)* animals (fig. S12), further phenotypically differentiating deletion of *vhl-1* from mutation of *daf-2*.

Our data support a model in which *vhl-1* and *daf-2* modulate longevity by different mechanisms, but it remains possible that ILS and the hypoxic response act through an overlapping set of target genes (fig. S1). Multiple DAF-16 target genes appear to be important for life-span extension in response to reduced ILS (*24*), and we speculate that multiple HIF-1 target genes may contribute to life-span extension in *vhl-1(ok161)* animals, some of which may be shared with DAF-16. Microarray studies have indicated that HIF-1 and DAF-16 have shared target genes (*25*, *26*), and mutation of *daf-2* can lead to increased resistance to hypoxic stress (*20*). In addition, reduced ILS and hypoxic response both induce resistance to heat stress (*27*), a phenotype often correlated with longevity. Like DAF-2, VHL-1 acts postdevelopmentally to modulate life span by a mechanism distinct from DR; however, unlike the case for *daf-2(e1370)* animals, *vhl-1(ok161)* animals did not show an enhanced frequency of dauer formation (table S2), suggesting that, if shared downstream effectors modulate aging and protein homeostasis, they are separable from the DAF-16 target genes involved in dauer formation.

Several features of the hypoxic response are highly conserved from nematodes to mammals, including regulation of mammalian HIF-1 by VHL-1 and the identity of many HIF-1 target genes. This high level of conservation suggests that induction of the hypoxic response is likely to have many similar physiological effects in nematodes and humans. Although inappropriate activation of the hypoxic response can promote tumorigenesis, therapeutically targeting specific components of this pathway may prove useful for treating age-associated diseases in people, particularly disorders associated with proteotoxicity in postmitotic cells, such as Huntington's disease, Alzheimer's disease, and other neurological disorders.

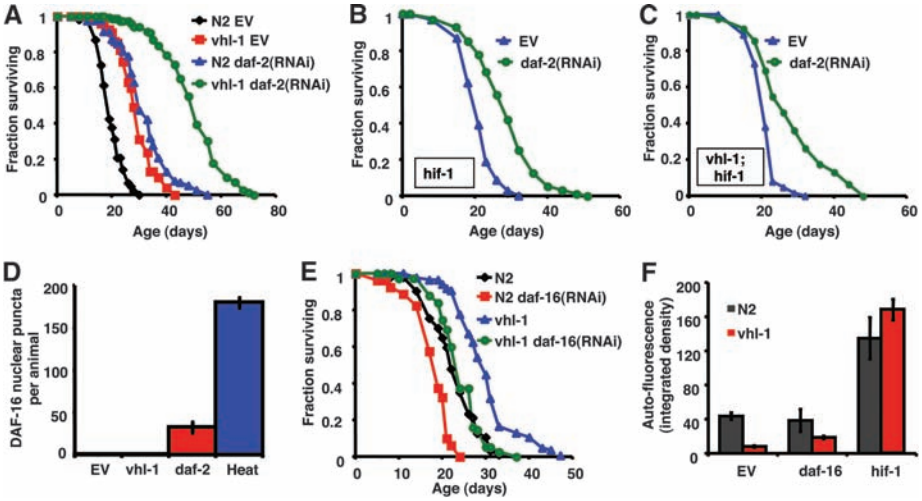

Fig. 4. ILS and VHL-1 modulate longevity by distinct mechanisms. *Daf-2(RNAi)* significantly increases the life span of (**A**) *vhl-1(ok161)* ($P < 1 \times 10^{-5}$), (**B**) *hif-1(ia4)* ($P < 1 \times 10^{-5}$), and (**C**) *hif-1(ia4); vhl-1(ok161)* animals ($P < 1 \times 10^{-5}$). (**D**) *Vhl-1(RNAi)* does not induce nuclear localization of DAF-16. *Daf-2(RNAi)* or heat shock significantly increases DAF-16 nuclear foci ($P < 1 \times 10^{-5}$ in each case). DAF-16 nuclear foci per animal was quantified for 10 animals per group. (**E**) *Daf-16(RNAi)* does not prevent life-span extension from deletion of *vhl-1* ($P < 1 \times 10^{-5}$). (**F**) Deletion of *vhl-1* significantly reduces autofluorescence in animals fed EV bacteria ($P < 1 \times 10^{-5}$) or *daf-16(RNAi)* ($P = 0.004$) but does not reduce autofluorescence in animals fed *hif-1(RNAi)* ($P = 0.9$). Median integrated pixel density shown for at least 10 randomly chosen animals per condition. Life-span statistics provided in table S6. Data in (D) and (F) are mean ± SEM.

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