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

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Abstract

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 lifespan and enhanced resistance to polyglutamine and amyloid beta 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/IGF-1-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 *C. elegans* is supported by observations that increasing lifespan by reducing insulin/IGF-1-like signaling (IIS) 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 ubiquitinproteasomal system, which involves covalent attachment of ubiquitin to target proteins prior to degradation. RNAi knock-down of proteasome components reduces resistance to polyglutamine toxicity 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 YFP in body wall muscle cells (Q35YFP) (Fig. S2). To further explore the relationship between proteasomal function and protein homeostasis, we initiated an RNAi screen of known or predicted E3 ubiquitin ligases for altered resistance to polyglutamine toxicity (Table S1). Cullin-RING ubiquitin ligases (CULs) consist of multiple protein subunits including a cullin protein, a RING-finger protein, an adaptor protein, and a substrate recognition subunit (Fig. S3) (7). Similar to proteasome inhibition, RNAi knock-down of genes encoding CUL1 or CUL2 core components accelerated paralysis in Q35YFP animals (Fig. S3).

In contrast to knock-down of CUL core components, we identified an RNAi clone corresponding to a CUL2 substrate recognition subunit, VHL-1, that significantly delayed

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paralysis in Q35YFP animals (Fig. 1A). A similar increase in resistance to amyloid beta 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 (8). 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 (9). Similar to *vhl-1(RNAi)*, *egl-9(RNAi)* also enhanced resistance to both polyglutamine (Fig. 1C) and amyloid beta toxicity (Fig. 1D). Noting prior correlation between resistance to proteotoxicity and increased lifespan, we next determined whether *vhl-1* and *egl-9* also modulate aging by measuring the effect of RNAi knock-down of *vhl-1* or *egl-9* on lifespan 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. 1E, F).

To determine whether increased stability of HIF-1 could account for the enhanced longevity associated with *vhl-1* knock-down, we examined the lifespans of animals deleted for *vhl-1*, *hif-1*, or both *vhl-1* and *hif-1*(9). 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 (10) (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 lifespan (Fig. 2C). Deletion of *hif-1* alone did not substantially influence lifespan, but completely suppressed the lifespan extension imparted by deletion of *vhl-1* (Fig. 2C). Consistent with the observed longevity effects, the accumulation of auto-fluorescent age-pigments, which has been proposed as a biomarker of aging and health span in *C. elegans* (11), was reduced in *vhl-1(ok161)* animals (Fig. 2D, Fig. S5). This reduction was also fully suppressed by deletion of *hif-1*.

Given that deletion of *vhl-1* increased lifespan 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 lifespan 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 (Fig. S6, S7) and a corresponding increase in lifespan (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 relationship 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 lifespan extension (12,13). If *vhl-1* and DR act in the same pathway to modulate longevity, then lifespan extension from bacterial deprivation should require *hif-1* and not further extend the lifespan of *vhl-1* mutants. In contrast, bacterial deprivation extended the lifespan of *hif- 1(ia4)* animals to an extent similar to that of controls (Fig. 3A) and further extended the long lifespan of *vhl-1(ok161)* animals (Fig. 3B). Bacterial deprivation also increased the lifespan 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 due to a defect in pharyngeal pumping (14). Unlike *eat-2(ad465)* mutants, *vhl-1(ok161)* animals did not display a significant reduction in pumping rate (Fig 3E), and, similar to the case for bacterial deprivation, knock-down of *hif-1* had no detectable effect on lifespan extension from mutation of *eat-2* (Fig 3D). Knock-down of *vhl-1* or growth under

hypoxic conditions also failed to cause a significant increase in the abundance of autophagic vesicles (Fig. 3F,Fig. S9), a phenotype reported to be required for lifespan extension associated with DR (15,16). Thus, DR and the hypoxic response are likely to modulate longevity via distinct genetic pathways.

Decreased activity of the insulin/IGF-1-like receptor DAF-2 has been shown to increase lifespan (17,18) and promote resistance to hypoxia (19), 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 lifespan 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 lifespan extension from *daf-2*(*RNAi*). Lifespan extension of animals with reduced IIS 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 (20,21). 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 reporter (22). Transient heat shock or *daf-2(RNAi)* increased nuclear localization of DAF-16, while *vhl-1(RNAi)* had no detectable effect (Fig. 4D, 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 lifespan (Fig. 4E) or reduced abundance of age-pigment (Fig. 4F, Fig. S11) associated with deletion of *vhl-1*, and *vhl-1(RNAi)* increased the lifespan 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 IIS and the hypoxic response act through an overlapping set of target genes (Fig. S1). Multiple DAF-16 target genes appear to be important for lifespan extension in response to reduced IIS (23), and we speculate that multiple HIF-1 target genes may contribute to lifespan 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 (24,25), and mutation of *daf-2* can lead to increased resistance to hypoxic stress (19). In addition, reduced IIS and hypoxic response both induce resistance to heat stress (26), a phenotype often correlated with longevity. Like DAF-2, VHL-1 acts post-developmentally to modulate lifespan 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 HIF1 by VHL1 and the identity of many HIF1 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 post-mitotic cells, such as Huntington's disease, Alzheimer's diseases, and other neurological disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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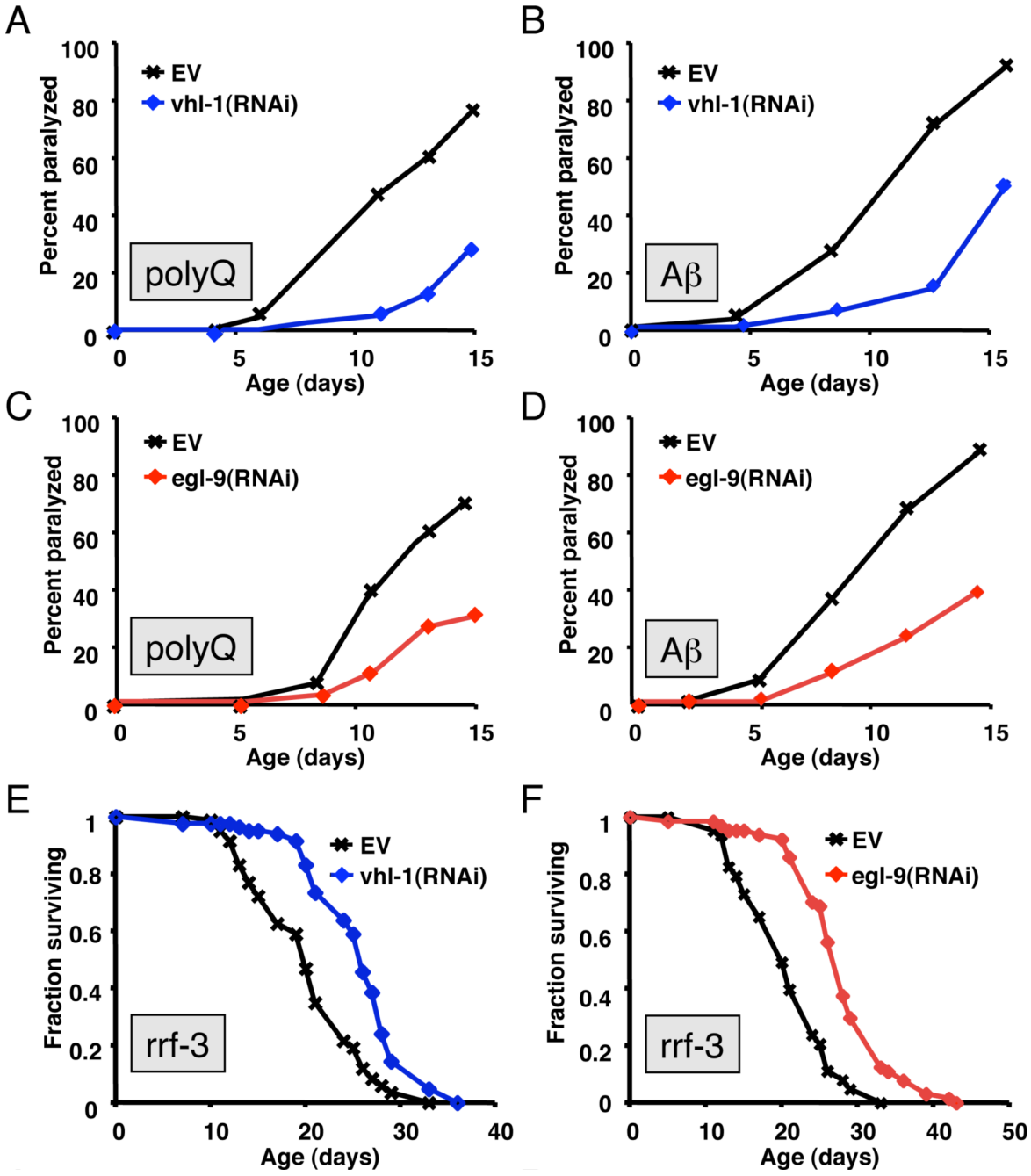

Fig. 1.

VHL-1 and EGL-9 modulate proteotoxic stress and lifespan. RNAi knock-down of *vhl-1* significantly enhances resistance to (**A**) polyglutamine toxicity ($p<1\times10_{-5}$) and (**B**) amyloid beta toxicity ($p<1\times10_{-5}$), relative to animals fed empty vector (EV) bacteria. RNAi knock-down of *egl-9* significantly enhances resistance to (**C**) polyglutamine toxicity ($p<1\times10_{-5}$) and (**D**) amyloid beta toxicity ($p<1\times10_{-5}$), relative to animals fed EV bacteria. RNAi knock-down of (**E**) *vhl-1* ($p<1\times10_{-5}$) or (**F**) *egl-9* ($p<1\times10_{-5}$) significantly increased adult lifespan relative to the EV-fed control. Paralysis and lifespan statistics in Table S1 and S6.

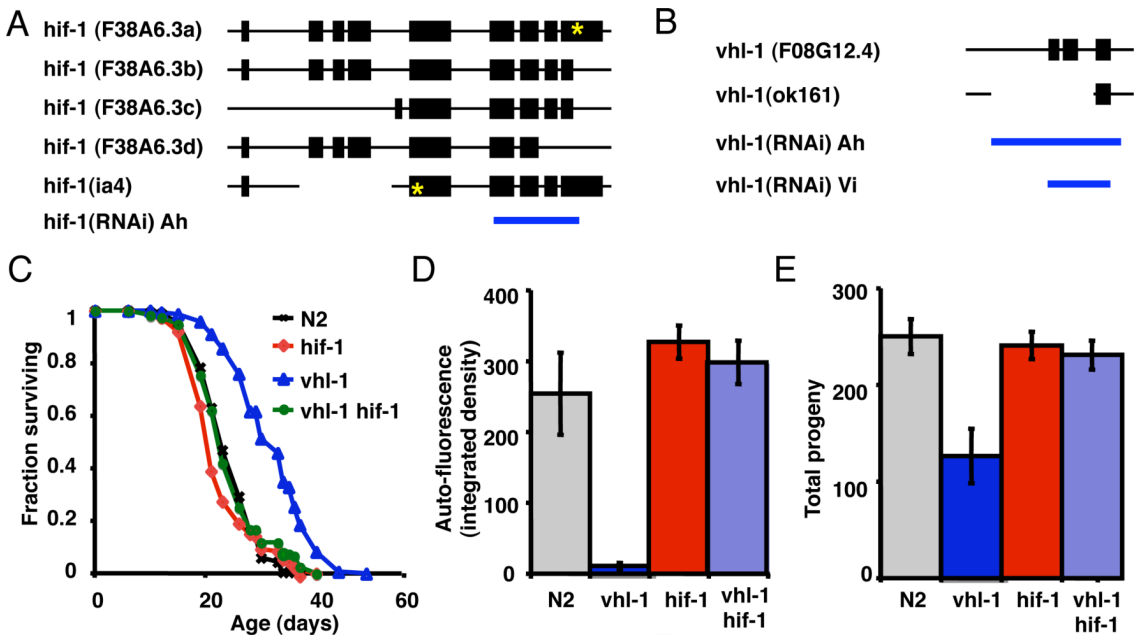

Fig. 2.

VHL-1 modulates longevity, age-pigment accumulation, and reproduction in a HIF-1-dependent manner. Gene structure 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 * indicates a stop codon. Blue boxes indicate RNAi target sequences from Anringer (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 ($p<1\times10_{-5}$); *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* ($p < 1\times10_{-5}$). 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 ($p=3.6\times10_{-3}$). 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, E are mean ± SD of at least 9 animals per condition. Lifespan statistics provided in Table S6.

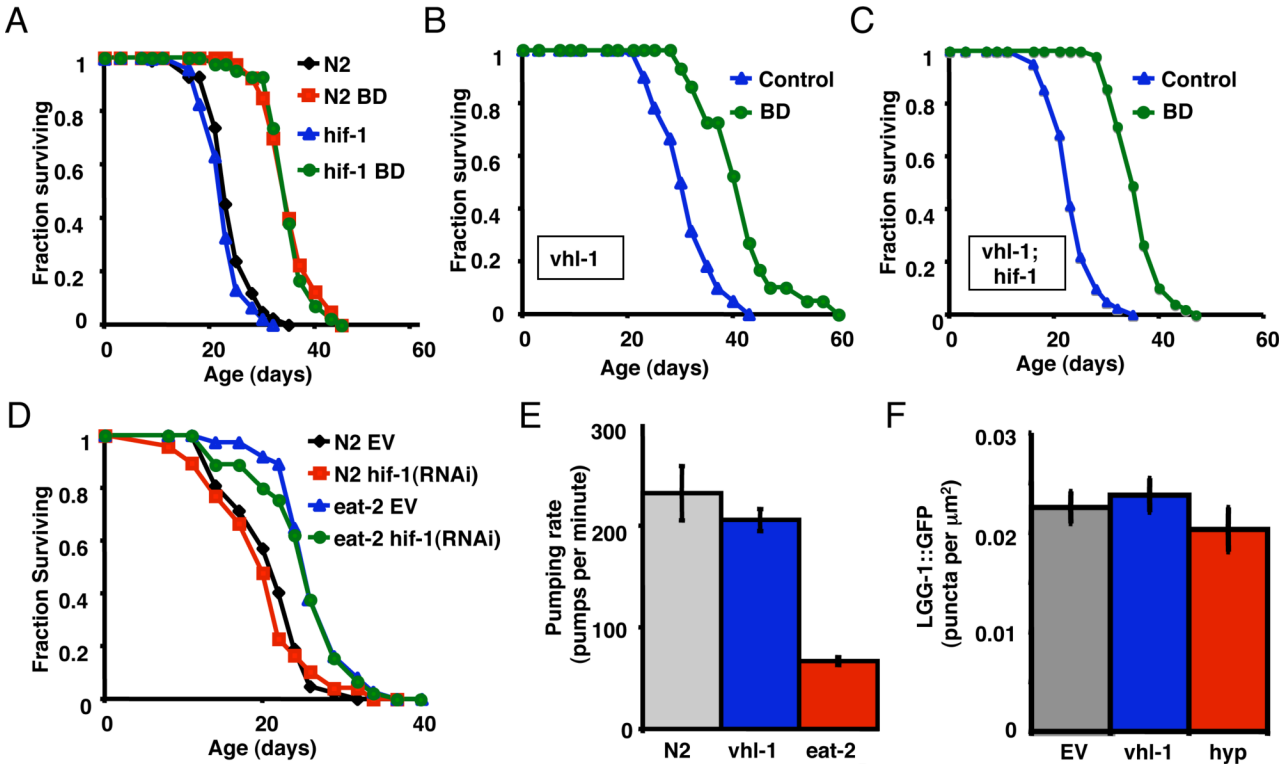

Fig. 3.

VHL-1 and HIF-1 modulate longevity by a mechanism distinct from dietary restriction. (**A**) Lifespan extension from bacterial deprivation (BD) is not significantly different in N2 and *hif-1(ia4)* animals (p=0.97). (**B**) BD significantly increases the lifespan of *vhl-1(ok161)* animals ($p<1\times10_{-5}$) or (**C**) *vhl-1(ok161)*; *hif-1(ia4)* double mutant animals ($p<1\times10_{-5}$). (**D**) *Hif-1(RNAi)* does not significantly alter the lifespan extension of *eat-2(ad465)* animals (p=0.6). (**E**) The *eat-2(ad465)* mutation significantly reduces pharyngeal pumping rate relative to N2 ($p<1\times10_{-5}$) or *vhl-1(ok161)* animals ($p<1\times10_{-5}$). 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 empty vector (EV) bacteria under normoxic conditions, *vhl-1(RNAi)* under normoxia or growth on EV 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). 35 animals per condition were imaged for EV and *vhl-1(RNAi)*. 7 animals were imaged for hypoxia. Data are mean ± SEM. Lifespan statistics provided in Table S6.

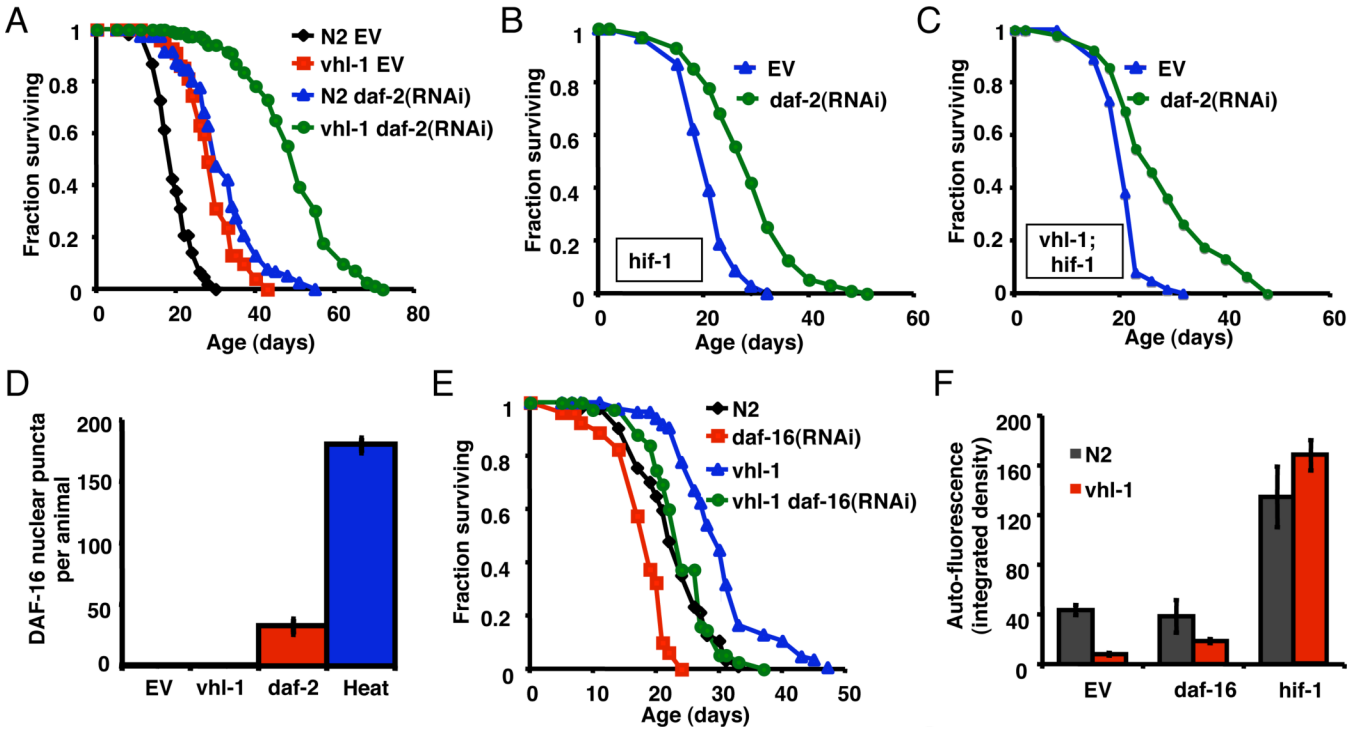

Fig. 4.

Insulin/IGF-1-like signaling and VHL-1 modulate longevity by distinct mechanisms. *Daf-2 (RNAi)* significantly increases the lifespan of (**A**) *vhl-1(ok161)* ($p<1\times10_{-5}$), (**B**) *hif-1(ia4)* ($p<1\times10_{-5}$), or (**C**) *hif-1(ia4); vhl- 1(ok161)* animals ($p<1\times10_{-5}$). (**D**) *Vhl-1(RNAi)* does not induce nuclear localization of DAF-16. *Daf-2(RNAi)* or heat shock significantly increases DAF-16 nuclear foci ($p<1\times10_{-5}$ in each case). DAF-16 nuclear foci per animal was quantified for 10 animals per group. (**E**) *Daf-16(RNAi)* does not prevent lifespan extension from deletion of *vhl-1* ($p<1\times10_{-5}$). **(F)** Deletion of *vhl-1* significantly reduces auto-fluorescence in animals fed empty vector bacteria ($p<1\times10_{-5}$) 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. Lifespan statistics provided in Table S6. Data in D, F are mean ± SEM.