1   **Hypoxic response regulators RHY-1 and EGL-9/PHD promote longevity**
2   **through a VHL-1 independent transcriptional response.**
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19 **Abstract:**

20 HIF-1-mediated adaptation to changes in oxygen availability is a critical aspect of healthy physiology.
21 HIF is regulated by a conserved mechanism whereby EGLN/PHD family members hydroxylate HIF in an
22 oxygen-dependent manner, targeting it for ubiquitination by Von-Hippel-Lindau (VHL) family members,
23 leading to its proteasomal degradation.  The activity of the only *C. elegans* PHD family member, EGL-9, is
24 also regulated by a hydrogen sulfide sensing cysteine-synthetase-like protein, CYSL-1, which is, in turn,
25 regulated by RHY-1/acyltransferase. Over the last decade multiple seminal studies have established a
26 role for the hypoxic response in regulating longevity, with mutations in *vhl-1* substantially extending *C.*
27 *elegans* lifespan through a HIF-1-dependent mechanism. However, studies on other components of the
28 hypoxic signaling pathway that similarly stabilize HIF-1 have shown more mixed results, suggesting that
29 mutations in *egl-9* and *rhy-1* frequently fail to extend lifespan.  Here, we show that *egl-9* and *rhy-1*
30 mutants suppress the long-lived phenotype of *vhl-1* mutants.  We also show that RNAi of *rhy-1* extends
31 lifespan of wild-type worms while decreasing lifespan of *vhl-1* mutant worms.  We further identify VHL-
32 1-independent gene expression changes mediated by EGL-9 and RHY-1 and find that a subset of these
33 genes contributes to longevity regulation.  The resulting data suggest that changes in HIF-1 activity
34 derived by interactions with EGL-9 likely contribute greatly to its role in regulation of longevity.

**Introduction.**

Adaptation to changes in oxygen availability is a central requirement for aerobic life.  In response to hypoxia, reduced oxygen-dependent hydroxylation of Hypoxia Inducible Factor α (HIFα) transcription factors by members of the EGLN/Proline-Hydroxylase (PHD) family triggers stabilization of HIFα proteins and activation of a transcriptional stress response that promotes survival (Epstein et al., 2001). This hypoxic response plays critical roles in a variety of pathological conditions including inflammation and cancer (Balamurugan, 2016; Pezzuto & Carico, 2018; Ramakrishnan & Shah, 2016).  Constitutive stabilization of the sole *C. elegans* HIFα family member, HIF-1,  by deletion of the Von-Hippel-Lindau ubiquitin ligase, VHL-1, which ubiquitinates HIF-1 and targets it for degradation, results in HIF-1-dependent increases in stress response and longevity (Cockman et al., 2000; Jiang et al., 2001; Leiser & Kaeberlein, 2010; Mehta et al., 2009; Müller et al., 2009; Treinin et al., 2003; Zhang et al., 2009).

Genetic studies in *C. elegans* have identified additional players in the hypoxic signaling pathway. Activity of EGL-9, the only known *C. elegans* PHD family member, is inhibited by direct interaction with the $H_2S$ sensing cysteine-synthetase family member CYSL-1 (Ma et al., 2012).   CYSL-1 protein levels are in turn reduced through an unknown mechanism by Regulator of Hypoxia-inducible factor-1 (RHY-1), an ER transmembrane protein with predicted acyltransferase activity (Ma et al., 2012; Shen et al., 2006). Predicted loss-of-function mutations in *rhy-1* stabilize HIF-1 and produce expression patterns of HIF-1 target genes that are consistent with reduced EGL-9 activity (Shen et al., 2006).

Interestingly, while *vhl-1* mutation extends *C. elegans* lifespan across culture conditions, the role of EGL-9/PHD is more context dependent.  While *egl-9(RNAi)* extends lifespan at 20°C, the lifespan phenotypes of partial loss-of-function mutations in *egl-9* are temperature-dependent, extending lifespan in a HIF-1-dependent manner at low temperatures (15°C) but not at higher temperatures (20°C and 25°C) (Leiser et al., 2011; Leiser & Kaeberlein, 2010; Miller et al., 2017; Zhang et al., 2009).  The loss-of-function mutant *egl-9(sa307)* also reduces the lifespan of dietary restricted animals and long-lived *rsks-1* mutants when animals are cultured at 25°C, suggesting that EGL-9 activity may promote lifespan in multiple contexts (Di Chen & Kapahi, 2009).  Furthermore, recent work from our lab showed that *rhy-1* putative knockout mutants were not long-lived at any temperature, despite their reported robust activation of hypoxic response genes (Miller et al., 2017; Shen et al., 2006).

Previous studies on the roles of EGL-9, RHY-1, and VHL-1 show that 1) HIF-1 stabilization when *vhl-1* is mutated leads to robust induction of *egl-9* and *rhy-1*, 2) EGL-9 and RHY-1 have VHL-1-independent effects on transcription of some hypoxic response genes, and 3) EGL-9 and RHY-1 play a VHL-1-independent role in pathogen and hydrogen sulfide resistance (Horsman et al., 2019; Luhachack et al., 2012; Shao et al., 2009; Shao et al., 2010; Shen et al., 2005; Shen et al., 2006).  However, the possibility that EGL-9 and RHY-1 modulate longevity through a downstream, VHL-1-independent transcriptional response has not been addressed.  Here we present a genetic study demonstrating that EGL-9 and RHY-1 are necessary for lifespan extension when HIF-1 is stabilized by *vhl-1* mutation.  We show that, like EGL-9, RHY-1 has both longevity promoting and inhibiting activities.  We further identify genes that are oppositely regulated in *vhl-1* and *egl-9* or *rhy-1* mutants, suggesting that RHY-1 and EGL-9 promote a VHL-1-independent transcriptional response when HIF-1 is stabilized by *vhl-1* mutation.  Lastly, we find that RNAi knockdown of four genes downregulated in *vhl-1(ok161)* mutants and upregulated in *egl-9(sa307)* mutants, with likely functions in innate immunity, each partially rescues lifespan extension in

76    *egl-9(sa307);vhl-1(ok161)* mutants.  Together, our results suggest that EGL-9 modulates lifespan by

77    regulating a VHL-1-independent transcriptional program.

78 **Results.**

79 **RHY-1 and EGL-9 promote longevity in *vhl-1* mutants.**

80 We initially hypothesized that the presence of EGL-9 and RHY-1 is necessary to promote longevity
81 downstream of HIF-1 stabilization. This hypothesis would suggest that reduced activity of EGL-9 or RHY-
82 1 would prevent or mitigate lifespan extension when HIF-1 is stabilized by reducing VHL-1 activity.
83 Previous studies show that *vhl-1* and *egl-9* mutant strains show similar lifespan phenotypes at low
84 temperatures (15°C), making epistasis experiments difficult to interpret (Miller et al., 2017).  However,
85 at high temperatures, *egl-9(sa307)* mutants have short to wild-type lifespans while *vhl-1(ok161)*
86 mutants are long-lived (Di Chen & Kapahi, 2009; Leiser et al., 2011; Miller et al., 2017).  Thus, we tested
87 whether *egl-9(sa307)* and/or *rhy-1(ok1402)* mutants suppress the extended longevity phenotype of *vhl-*
88 *1(RNAi)*-treated or *vhl-1(ok161)* mutant animals at high temperature (25°C).  Our results (**Figure 1**) show
89 that *rhy-1(ok1402)* **(Fig. 1A)** and *egl-9(sa307)* **(Fig. 1B)** mutants abrogate the extended longevity
90 phenotype caused by *vhl-1(RNAi)* at 25°C.  Furthermore, *rhy-1(ok1402)* fully abrogated the extended
91 longevity phenotype of *vhl-1(ok161)* mutants **(Fig. 1C)**, while *egl-9(sa307)* mutants partially suppressed
92 the extended longevity phenotype *vhl-1(ok161)* mutants at 25°C **(Fig. 1D)**.  We also observed that *rhy-*
93 *1(RNAi)* reduces the lifespan of *vhl-1* mutants at low temperatures (15°C, **(Fig. 1E)**) and that the lifespan
94 of *rhy-1(ok1402)* mutants are not extended by *vhl-1(RNAi)* at low temperatures (15°C, **(Fig. S1)**),
95 suggesting that these interactions are not fully temperature-dependent.  These results are consistent
96 with a model where RHY-1 and EGL-9 act in the same pathway to promote lifespan downstream of HIF-1
97 stabilization.

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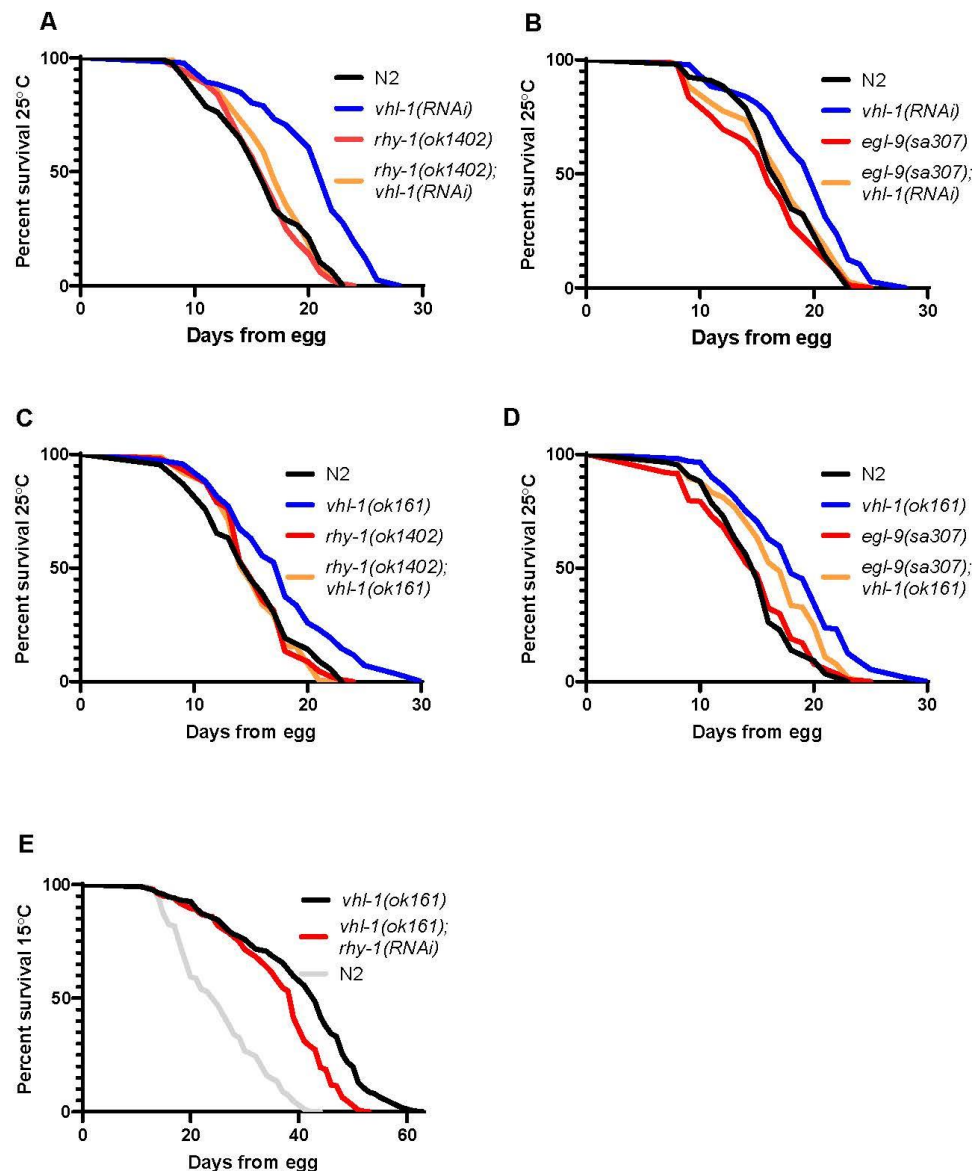
**Figure 1. Epistasis of hypoxic response regulators.** A) Lifespans of N2 (wild-type) or *rhy-1(ok1402)* mutants treated with empty vector or *vhl-1(RNAi)* at 25°C. B) Lifespans of N2 and *egl-9(sa307)* animals treated with empty vector and *vhl-1(RNAi)* at 25°C. C) Lifespans of N2, *vhl-1(ok161)*, *rhy-1(ok1402)* and *rhy-1(ok1402);vhl-1(ok161)* animals at 25°C. D) Lifespans of N2, *egl-9(sa307)*, *vhl-1(ok161)* and *egl-9(sa307);vhl-1(ok161)* animals at 25°C. E) Lifespans of vhl-1(ok161) animals treated with empty vector and *rhy-1(RNAi)* at 15°C, along with N2 controls, (p<.0001 by log-rank). Interactions of *egl-9(sa307)* and *rhy-1(ok161)* with *vhl-1(ok161)* and *vhl-1(RNAi)* were statistically different than interactions of N2 animals with *vhl-1(ok161)* and *vhl-1(RNAi)* by non-overlapping 95% confidence intervals of Mantel-Haenzel hazard ratios (**Table S1**). Data are aggregated from at least three independent experiments (**Table S2**).

109

110 **RHY-1 has longevity promoting and inhibiting activities.**

111 In the course of testing the effects of *rhy-1(RNAi)* on *vhl-1(ok161)* mutants at 15°C, we observed that
112 *rhy-1(RNAi)* substantially extended the lifespan of wild-type control animals **(Fig. 2A)**. This result was
113 surprising since we recently reported a study of the interaction between temperature and longevity and
114 found that *rhy-1(ok1402)* mutants did not extend lifespan at any of the three temperatures (15°C, 20°C,
115 or 25°C) that are commonly used for longevity studies (Miller et al., 2017). We outcrossed *rhy-*
116 *1(ok1402)* five additional times to wild-type to further eliminate any background mutations and
117 repeated lifespan experiments. Consistent with our published data, the outcrossed *rhy-1(ok1402)* strain
118 was not long-lived: observed lifespans were modestly longer than wild-type in two of four trials,
119 identical to wild-type in one trial, and shorter than wild-type in one trial **(Fig. 2B, S1)**. Lifespan extension
120 by *rhy-1(RNAi)* was abrogated by *rhy-1(ok1402)*, consistent with the longevity extension phenotype
121 caused by *rhy-1(RNAi)* being dependent on modulation of the RHY-1 gene product **(Fig. 2C)**.
122 Furthermore, *rhy-1(RNAi)* treated *cysl-1(ok762)* and *hif-1(ia4)* mutants are short lived relative to *rhy-*
123 *1(RNAi)* treated wild-type animals, consistent with a model where HIF-1 and CYSL-1 activity are required
124 for full lifespan extension by *rhy-1(RNAi)* **(Fig. 2D-E)**. Importantly, *rhy-1(RNAi)* treatment does extend
125 the lifespans of *cysl-1(ok762)* and *hif-1(ia4)* mutants relative to vector treated controls (**Figure 2 D,E**)
126 suggesting that it may modulate lifespan through a mechanism that is partially independent of the CYSL-
127 1, HIF-1 pathway. The distinct phenotypes observed in *rhy-1(ok1402)* and *rhy-1(RNAi)* are similar to the
128 reported differences between *egl-9(RNAi),* which extends lifespan at 20°C, and the *egl-9(sa307)* mutant,
129 which extends lifespan at 15°C but not 20°C or 25°C (Di Chen & Kapahi, 2009; Mehta et al., 2009; Miller
130 et al., 2017; Zhang et al., 2009). These results are consistent with a model where partial reductions in
131 activity of *rhy-1* or *egl-9* increase longevity by stabilizing HIF-1, while stronger reduction of *rhy-1* or *egl-9*
132 causes a secondary effect that limits lifespan downstream of HIF-1 stabilization.

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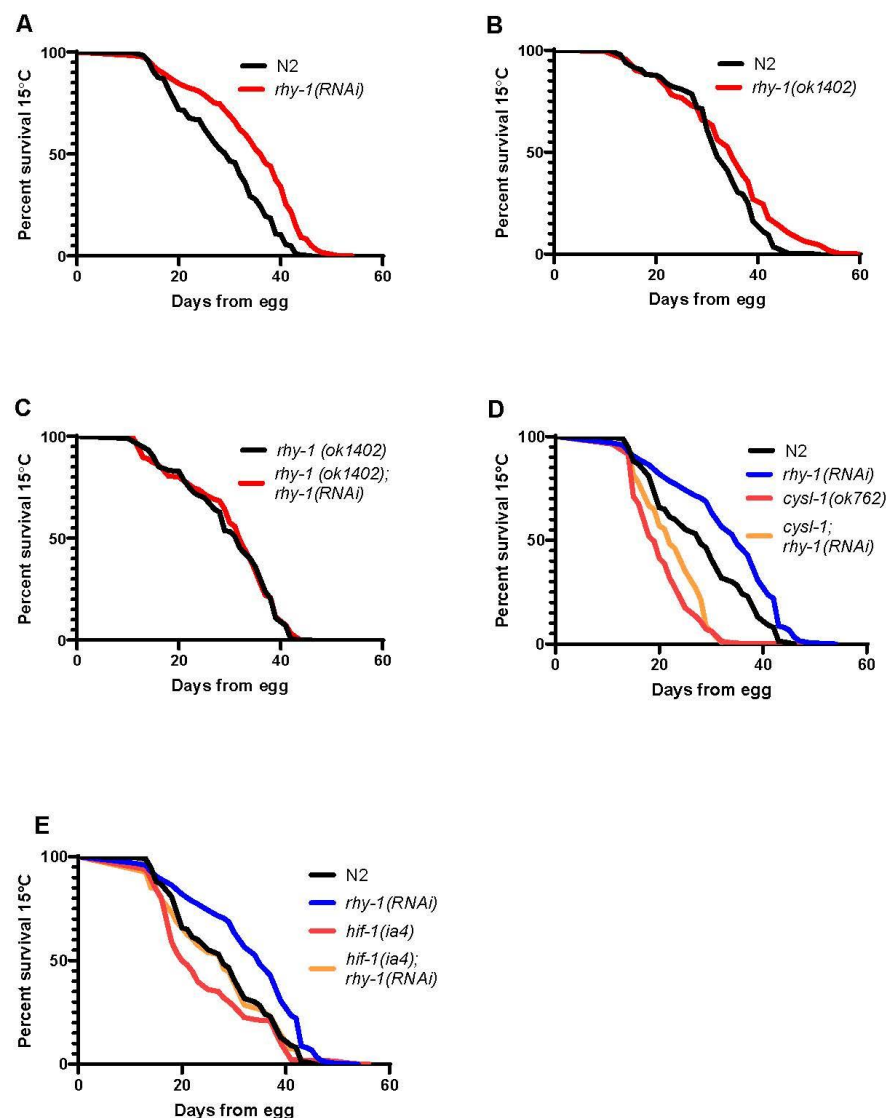
**Figure 2. *rhy-1*-mediated regulation of longevity.** A) Lifespans of N2 (wild-type) animals treated with empty vector or *rhy-1(RNAi)* (p < 0.0001 by log-rank). B) Lifespans of N2 and *rhy-1(ok1402)* animals (p < 0.0001 by log-rank). C) Lifespans of *rhy-1(ok1402)* animals treated with empty vector or *vhl-1(RNAi)*. D) Lifespans of N2 and *cysl-1(ok762)* animals treated with vector or rhy-1(RNAi) (p < 0.001 for *cysl-1(ok762);rhy-1(RNAi)* vs *rhy-1(RNAi)* by log rank) E) Lifespans of *hif-1(ia4)* and N2 animals treated with empty vector or *rhy-1(RNAi)* (p < 0.0001 for *hif-1;rhy-1(RNAi)* vs *rhy-1(RNAi)*). Interaction with *rhy-1(RNAi)* treatment was statistically different between N2 and *hif-1(ia4)* as determined by non-overlapping 95% confidence intervals of Mantel-Haenzel hazard ratios. Hazard ratios for *cysl-1(ok762);rhy-1(RNAi)/cysl-1(ok762)* and *rhy-1(RNAi)*/N2 were (0.58-0.82) and (0.44-0.64) respectively. Lifespan data are aggregated from at least three experiments (Table S1,S2).

145 **RHY-1 and EGL-9 control a VHL-1-independent transcriptional response.**

146 Previous studies reported that *egl-9* causes *vhl-1*-independent changes in expression of some transcripts
147 (Shao et al., 2009; Shen et al., 2005; Shen et al., 2006).  We hypothesized that the dominance of the *egl-*
148 *9(sa307)* and *rhy-1(ok1402)* lifespan phenotypes over the *vhl-1(ok161)* lifespan phenotype might be
149 caused by genes whose transcription is regulated by EGL-9 or RHY-1.  Concurrently with our work,
150 Angeles et al. published an analysis of transcriptome profiles from *rhy-1(ok1402)*, *egl-9(sa307)*, *hif-*
151 *1(ia4)*, *egl-9;hif-1*, and *egl-9;vhl-1* mutants.  They identified a class of genes whose transcription is
152 regulated by EGL-9 in a way that is distinct from, and dominant over, their regulation by VHL-1. We will
153 refer to this class as EGL-9/VHL-1 antagonistic genes.

154 To identify genes that might modulate lifespan downstream of the hypoxic response, we had previously
155 profiled the transcriptomes of *egl-9(sa307)*, *vhl-1(ok161), rhy-1(ok402),* and *hif-1(ia4)* mutants.  We
156 reanalyzed our data using the methodology that Angeles et al. reported and an up-to-date bioinformatic
157 pipeline (Angeles-Albores et al., 2018; Bray et al., 2016; Pimentel et al., 2017). Our datasets identified a
158 subset of genes that were differentially expressed in the HIF-1 negative regulator mutants relative to
159 wild-type, further confirming that these changes are implicated in the hypoxic response, **(Fig. 3A, Table**
160 **1, Table S3,S4**).  Conversely, we observed low overlap between the datasets for genes differentially
161 expressed in the *hif-1(ia4)* background, suggesting that differences between *hif-1(ia4)* and wild-type in
162 individual datasets may largely reflect strain-specific effects rather than HIF-1-dependent transcription
163 under normoxia **(Fig 3B)**.

164 We next plotted β coefficients of differentially expressed genes shared between *vhl-1(ok161)* and *egl-*
165 *9(sa307)* in Leiser and Sternberg datasets **(Fig 3C)**.  Both datasets produce a similar pattern, with *egl-*
166 *9(sa307)*, and *vhl-1(ok161)* causing highly correlated changes in expression for most shared differentially
167 expressed genes. Both datasets also contained EGL-9/VHL-1 antagonistic genes, which were either
168 upregulated in *vhl-1(ok161)* and downregulated in *egl-9(sa307)* (**Fig. 3C**, quadrant II), or upregulated in
169 *egl-9(sa307)* or *rhy-1(ok1402)* and downregulated in *vhl-1(ok161)* (**Fig. 3C**, quadrant IV).  The genes in
170 quadrants II and IV, who we hypothesized could play a role in different outcomes between *vhl-1(ok161)*
171 and *egl-9(sa307)* or *rhy-1(ok1402)* strains, are listed in **Table S5**.  Together, our and the Sternberg lab's
172 results show that HIF-1 stabilization through loss of its negative regulators has both many common
173 effects and a smaller number of opposing effects depending on which negative regulator is mutated.

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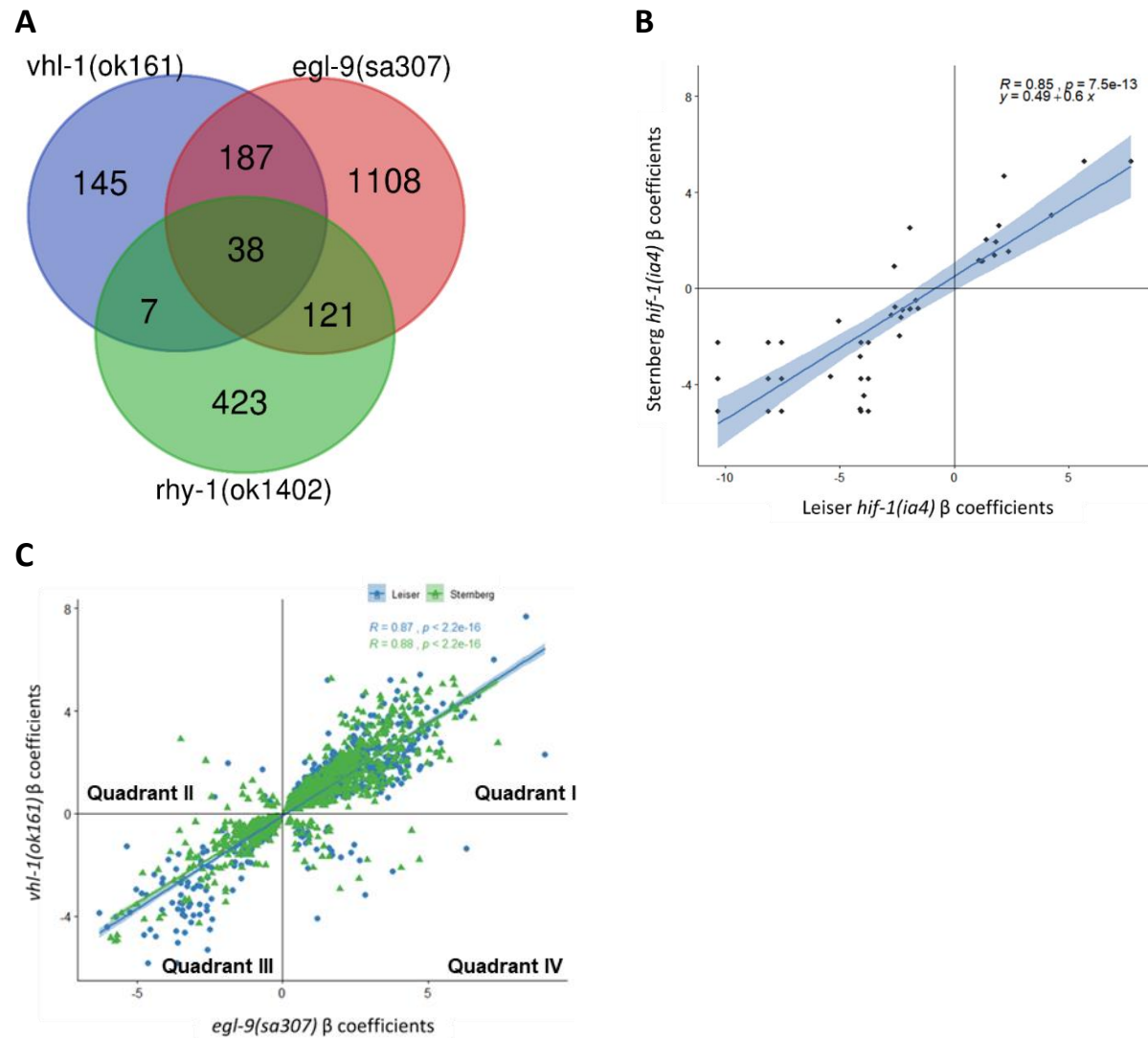
**Figure 3. A subset of genes are antagonistically regulated by *egl-9* and *vhl-1*.** A) Overlap between genes that are differentially expressed in both Leiser and Sternberg datasets for *vhl-1(ok161)* and *egl-9(sa307)* along with differentially expressed genes from Sternberg *rhy-1(ok1402)*. B) Overlap between differentially expressed genes in *hif-1(ia4)* in Leiser and Sternberg datasets. C) Correlation of expression levels of differentially expressed genes in *egl-9(sa307)* and *vhl-1(ok161)* in Leiser and Sternberg datasets.

| ORF | Gene | Description |
|---|---|---|
| K08C7.5 | fmo-2 | FMO5 (flavin containing dimethylaniline monoxygenase 5) ortholog |
| T05B4.2 | nhr-57 | nuclear hormone receptor family transcription factor |
| C14C6.5 | C14C6.5 | predicted role in innate immune response |
| F22D6.10 | col-60 | collagen structural protein in cuticle |
| F26H9.5 | F26H9.5 | PSAT1 (phosphoserine aminotransferase 1) ortholog |
| C08E8.3 | C08E8.3 | unknown function |
| K06A4.7 | K06A4.7 | unknown function |
| F15B9.1 | far-3 | fatty acid binding protein |
| F38B2.4 | F38B2.4 | AK1 (adenylate kinase 1) ortholog |
| Y37A1B.5 | Y37A1B.5 | SELENBP1 (selenium binding protein 1) ortholog |
| F38A3.2 | ram-2 | structural protein in cuticle |
| F28F8.2 | acs-2 | ACSF2 (acyl-CoA synthetase family member 2) ortholog |
| F08G5.4 | col-130 | collagen structural protein in cuticle |
| T10D4.13 | ins-19 | predicted insulin protein |
| C31C9.2 | C31C9.2 | PHGDH (phosphoglycerate dehydrogenase) ortholog |
| F22B5.4 | F22B5.4 | unknown function |
| F10D2.9 | fat-7 | SCD5 (stearoyl-CoA desaturase 5) ortholog |
| R08E5.3 | R08E5.3 | methyltransferase orthologs |
| Y22F5A.4 | lys-1 | lysozyme activity, predicted role in innate immune response |
| C05E7.1 | C05E7.1 | unknown function |
| Y58A7A.1 | Y58A7A.1 | SLC31A1 (solute carrier family 31 member 1) ortholog |
| M05D6.6 | taap-1 | FAM162A/B (family with sequence similarity 162 member A/B) ortholog |
| W07A12.7 | rhy-1 | Negative regulator of HIF-1 |
| M199.4 | clec-190 | C-type lectin predicted to have carbohydrate binding activity |
| B0222.6 | col-144 | collagen structural protein in cuticle |
| M05D6.5 | M05D6.5 | HIGD2A/B (HIG1 hypoxia inducible domain family member 2A/B) ortholog |
| F09A5.9 | ttr-34 | predicted transthyretin-like protein |
| W07A12.6 | oac-54 | predicted amino-acyl transferase protein |
| F02H6.5 | sqrd-1 | SQOR (sulfide quinone oxidoreductase) ortholog |
| ZK637.13 | glb-1 | predicted role in heme/oxygen binding and carrying |
| F54C9.4 | col-38 | collagen structural protein |
| Y22F5A.5 | lys-2 | lysozyme activity, predicted role in innate immune response |
| F37B1.8 | gst-19 | HPGDS (hematopoietic prostaglandin D synthase) ortholog |
| T03F1.11 | T03F1.11 | predicted for CIBs (calcium and integrin binding family member) ortholog |
| VF13D12L.3 | VF13D12L.3 | predicted oxidoreductase protein |
| C04G6.2 | C04G6.2 | unknown function |
| K10B3.7 | gpd-3 | GAPDH (glyceraldehyde-3-phosphate dehydrogenase) ortholog |
| R02E4.3 | R02E4.3 | unknown function |

182

183 **Table 1. Core Hypoxic response genes.** List of 38 genes that were differentially regulated in *rhy-1(ok1402)* (Sternberg only), *egl-9(sa307)* and *vhl-1(ok161)* mutants in both Leiser and Sternberg RNA-seq datasets.

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188 **Knockdown of egl-9 target genes rescues lifespan *egl-9(sa307);vhl-1(ok161)* mutants**.

189 We next tested the hypothesis that EGL-9/VHL-1 antagonistic genes regulate longevity.  Previous results
190 suggest that individual longevity-pathway-target-genes often have small effects on lifespan, and
191 longevity increases are more likely than longevity decreases to reflect modulation of the aging process
192 as a whole (Murphy et al., 2003).  Thus, we identified candidate EGL-9/VHL-1 antagonistic genes whose
193 downregulation in *vhl-1(ok161)* mutants was reversed in *egl-9(sa307);vhl-1(ok161)* mutants and
194 determined whether RNAi targeting them could extend life lifespan of *egl-9;vhl-1(ok161)* mutants
195 **(Figure 4 A,C,E,G, Table S6)**.

196 EGL-9 and VHL-1 have distinct roles in pathogen resistance, with *egl-9(sa307)* mutants exhibiting HIF-1-
197 dependent resistance to fast killing by *pseudomonas aeruginosa* while *vhl-1(ok161)* mutants do not
198 (Luhachack et al., 2012; Shao et al., 2010).  We noticed that our list of EGL-9/VHL-1 antagonistic genes
199 contained several genes with reported or predicted roles in pathogen response, including *nlp-31*, which
200 encodes five neuropeptide-like proteins with functions in defense against fungal pathogens and gram-
201 negative pathogenic bacteria, *lys-7*, which encodes a lysozyme with a reported role in defense against
202 gram-negative bacteria, *lys-10*, which encodes another lysozyme, and *lips-10*, which encodes an enzyme
203 that, like lysozymes, has hydrolase activity  (Couillault et al., 2004; Harris et al., 2010; Marsh et al., 2011;
204 Nathoo et al., 2001).  We found that treatment with *lys-10(RNAi), lips-10(RNAi)*, *lys-7(RNAi),* and *nlp-*
205 *31(RNAi)* each extended longevity of *egl-9;vhl-1* mutants **(Fig. 4 B,D,F,H)**.

206 We also tested treatment with RNAi targeting the ferritin genes, *ftn-1* and *ftn-2*, predicted oxidative and
207 heavy metal response genes that have been identified as EGL-9/VHL-1 antagonistic genes in multiple
208 studies (Ackerman & Gems, 2012; Romero-Afrima et al., 2020).  We found slight but significant increases
209 in lifespan in *egl-9(sa307);vhl-1(ok161)* animals treated with *ftn-1(RNAi)* and *ftn-2(RNAi)*, however the
210 magnitude of these changes was small and they were not consistent across individual replicates
211 (significant in 2 of 4 trials) (**Figure S2** and **Table S2**).  These data are consistent with ferritins playing a
212 minor role in modulation of lifespan by the hypoxic response.

213 Taken together, these results are consistent with a model in which EGL-9 activity promotes longevity
214 during HIF-1 stabilization through VHL-1-independent inhibition of target genes, including multiple
215 genes with predicted functions in defense against pathogens.

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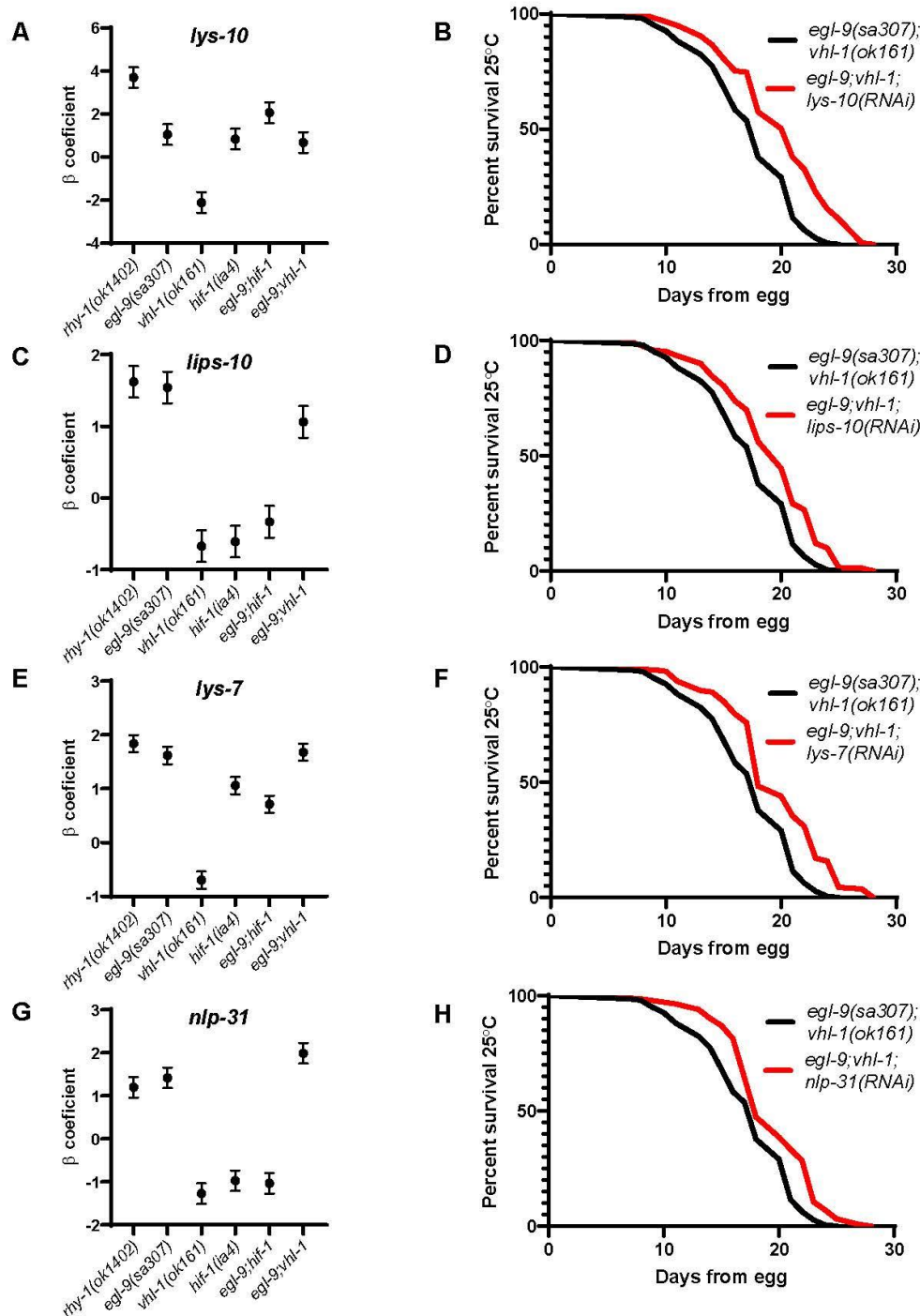
**Figure 4. VHL-1/EGL-9 antagonistic HIF-1 targets rescue lifespan in *egl-9;vhl-1* mutants.** A, C, E, G) expression levels of selected transcripts from RNA-seq analyses. B-D-F-H) Treatment with *nlp-31(RNAi)*, *lys-7(RNAi)*, *lys-10(RNAi)* and *lips-10(RNAi)* increases lifespan of *egl-9(sa307);vhl-1(ok161)* mutants (p<.05 by log-rank). Lifespan data are aggregated from at least three experiments, and are significant (p<.05 by log-rank with Bonferroni correction) in at least 3 of 5 individual trials (Table S2).

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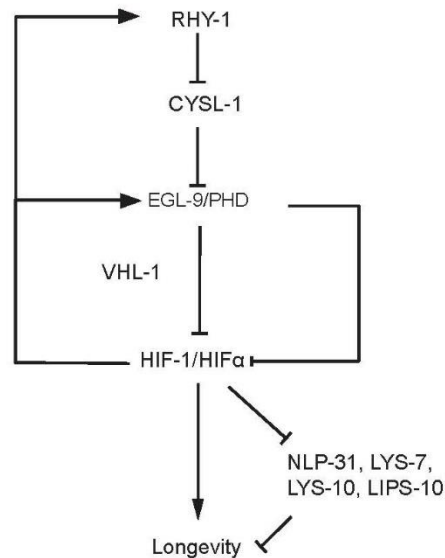
**Figure 5**. **Epistatic model of lifespan regulation by VHL-1, RHY-1, and EGL-9.**  RHY-1 and EGL-9 act in the same pathway to inhibit HIF-1 activity through both VHL-1-dependent and VHL-1-independent mechanisms. When HIF-1 is stabilized through loss of VHL-1, expression of RHY-1 and EGL-9 is increased, driving reductions in expression of longevity reducing target genes including, NLP-31, LYS-7, LYS-10 and LIPS-10 through a HIF-dependent and VHL-1-independent mechanism.  Inhibition of EGL-9 activity causes upregulation of genes including NLP-31, LYS-7, LYS-10 and LIPS-10, inhibiting longevity.

231

232 **Discussion.**

233 Collectively, our results show that, while reduction in RHY-1 and EGL-9 activity can increase lifespan via
234 the hypoxic response, RHY-1 and EGL-9 activity also promote longevity downstream of HIF-1
235 stabilization by *vhl-1* mutation. We further demonstrate that RHY-1 and EGL-9 activity are required to
236 control the direction of differential expression of numerous transcripts in *vhl-1* mutants and that EGL-9-
237 dependent suppression of *nlp-31*, *lys-7*, *lys-10*, and *lips-10* promotes longevity in *vhl-1* mutants.

238 **Genetic interactions of *vhl-1*, *egl-9*, and *rhy-1*.**

239 While HIF has emerged as a key regulator of longevity, it was previously unknown how hypoxic signaling
240 pathway components interact to influence longevity.  HIF-1 hydroxylation is required for its interaction
241 with VHL-1, so in theory we might expect *egl-9* mutant phenotypes to be epistatic to *vhl-1* mutant
242 phenotypes. However, reports that 1) EGL-9 and RHY-1 have VHL-1-independent roles in the expression
243 and tissue distribution of hypoxic response genes, 2) EGL-9 interacts with other proteins through
244 proline-hydroxylase-activity-dependent and -independent mechanisms to influence phenotype, and 3)
245 EGL-9 and RHY-1 are transcriptionally upregulated when HIF-1 is stabilized, make these phenotypic
246 interactions difficult to predict (Luhachack et al., 2012; Shao et al., 2009; Shao et al., 2010; Shen et al.,
247 2005; Shen et al., 2006).

248 We found that *rhy-1(ok1402)* blocked lifespan extension by *vhl-1(RNAi)* and *vhl-1(ok161)*, while *egl-*
249 *9(sa307)* blocked lifespan extension by *vhl-1(RNAi)* and partially blocked lifespan extension by *vhl-*
250 *1(ok161)*.  While these results are broadly consistent with a model where RHY-1 regulates lifespan
251 through its known EGL-9 modulating activity, it is interesting that *rhy-1* mutation has a stronger effect
252 on the longevity of *vhl-1(ok161)* mutants than the *egl-9(sa307)* mutation.  This could be explained by the
253 reportedly more robust HIF-1 stabilization and upregulation of pro-longevity HIF-1 target genes in *egl-*
254 *9(sa307)* relative to *rhy-1(ok1402)*, or by an additional, HIF-1-independent role for RHY-1 in longevity
255 determination (Shen et al., 2006).

256 We confirmed the surprising, previously reported, result that the *rhy-1(ok1402)* mutation does not
257 extend lifespan, despite stabilizing HIF-1.  However, interestingly, *rhy-1(RNAi)* extends lifespan through a
258 mechanism that is partially dependent on its established interactions with CYSL-1 and HIF-1.  While off-
259 target effects of RNAi are a concern when mutant and RNAi phenotypes differ, the observation that the
260 *rhy-1(RNAi)*-mediated lifespan increase is fully abrogated in a *rhy-1(ok1402)* mutant background
261 strongly suggests that modulation of RHY-1 is the primary factor influencing lifespan in this context. It is
262 worth noting that, while *cysl-1(ok762)* and *hif-1(ia4)* mutations reduce the longevity promoting effect of
263 *rhy-1(RNAi)*, neither completely abrogates it.  This suggests that RHY-1 may have secondary, HIF-1-
264 independent roles that influence longevity.  Published reports showing that *rhy-1;hif-1* compound
265 mutants have a synthetic deleterious effect on fertility and that RHY-1 modulates hydrogen-sulfide
266 resistance in a HIF-1-independent manner also suggest interesting HIF-1-independent roles for RHY-1
267 (Horsman et al., 2019; Shen et al., 2006).

268 A published mechanism explaining the interaction between RHY-1 and HIF-1 suggests that *rhy-1* mutants
269 should largely phenocopy *egl-9* mutants (Ma et al., 2012).  While interpretation of *egl-9* phenotypes is
270 complicated by the lack of a viable *egl-9* null mutant, published results do suggest that *egl-9(n571)*, a
271 point mutant that is predicted to affect splicing, and *egl-9(RNAi)* have more robust longevity promoting
272 phenotypes than the strong loss-of-function mutant, *egl-9(sa307)*, a deletion in the EGL-9 catalytic

273    domain (Darby et al., 1999; Miller et al., 2017; Shao et al., 2009; Trent et al., 1983). These results are
274    consistent with EGL-9 also having longevity promoting and limiting functions, with the mutant
275    phenotype depending on the severity of the loss in activity.  These data are consistent with a model in
276    which EGl-9 and RHY-1 act in the same pathway to both limit wild-type longevity by destabilizing HIF-1
277    and increase longevity when HIF-1 is stabilized.

278    **VHL-1-independent EGL-9 targets modulate lifespan.**

279    Along with other groups, we identified a substantial subset of targets that are transcriptionally regulated
280    in opposite directions by EGL-9 and VHL-1 (Ackerman & Gems, 2012; Angeles-Albores et al., 2018). This
281    suggests that upregulation of EGL-9 and RHY-1 when HIF-1 is stabilized has substantial effects on the
282    hypoxic transcriptome in addition to possible feedback regulation of HIF-1. Previous publications have
283    established that EGL-9 has VHL-1-independent activities that can increase or reduce resistance to
284    various pathogens (Luhachack et al., 2012; Shao et al., 2010).  Here, we find that several RNAi clones
285    targeting genes with reported or likely functions in innate immunity, *nlp-31(RNAi) lys-7(RNAi)*, *lys-*
286    *10(RNAi)*, and *lips-10(RNAi)*, extend lifespan in *egl-9(sa307);vhl-1(ok161)* mutants, suggesting that EGL-
287    9-dependent downregulation of these genes promotes longevity in *vhl-1(ok161)* mutants.

288    The mechanisms underlying VHL-1-independent transcriptional regulation by EGL-9 have not been fully
289    established. Previous studies report that *egl-9(sa307)* and *hif-1(ia4)* loss of function mutants cause
290    transcriptional upregulation of the direct HIF-1 transcriptional target *ftn-1*, while *vhl-1* loss of function
291    mutants and overexpression of non-hydroxylatable HIF-1 (P621A) suppress *ftn-1* expression (Ackerman
292    & Gems, 2012; Romero-Afrima et al., 2020). These results are consistent with a model in which binding
293    of hydroxylated and non-hydroxylated HIF-1 may have opposite effects on the *ftn-1* promoter region,
294    with *vhl-1(ok161)* mutation causing increases in hydroxylated HIF-1 while *hif-1(ia4)* and *egl-9(sa307)*
295    mutation both eliminate hydroxylated HIF-1 (Ackerman & Gems, 2012; Romero-Afrima et al., 2020). A
296    recent analysis from the Sternberg group showed expression patterns consistent with a role for
297    hydroxylated HIF-1 in expression of a larger set of transcripts that are oppositely affected by VHL-1 and
298    EGL-9 (Angeles-Albores et al., 2018).

299    Other reports suggest that EGL-9 may trigger VHL-1-independent transcriptional responses through
300    more complex mechanisms. Multiple labs report that EGL-9 affects gene expression through
301    mechanisms that are independent of its hydroxylation activity (Luhachack et al., 2012; Shao et al., 2009).
302    One study suggests that EGL-9 represses HIF-1 activity through a mechanism that requires physical
303    interaction between EGL-9/PHD and the WD repeat containing protein SWAN-1 (Shao et al., 2010).  EGL-
304    9 might also affect gene expression via hydroxylation of substrates other than HIF-1, with one study
305    indicating that LIN-10 is a target of EGL-9 hydroxylation (Park et al., 2012).  Mechanistic biochemical
306    studies to 1) identify EGL-9 hydroxylation targets and protein-protein interaction partners, and 2)
307    establish whether hydroxylated and non-hydroxylated HIF-1 interact with distinct transcriptional
308    complexes, will be needed to fully understand the complex biological function of EGL-9/PHD.

309    Constitutive sterile activation of the innate immune response increases during mammalian aging and is a
310    key driver of many age-related pathologies (Franceschi & Campisi, 2014).  A trade-off between
311    constitutive immune activation and longevity has also been established in *Drosophila* (Libert et al.,
312    2006).   In *C. elegans*, immune-related signaling genes contribute to the longevity phenotypes of long-
313    lived insulin signaling mutants; however, to our knowledge, this is the first evidence of antagonism
314    between immune activation and non-pathogen exposed survival in *C. elegans* (Murphy et al., 2003).

315  Further mechanistic studies of the connection between pathogen resistance genes and accelerated
316  aging-related phenotypes in tractable model systems may yield insights and interventions that can be
317  translated to mammalian inflammatory aging pathologies.

318  **Conclusion**.

319  Drugs that inhibit PHD activity are currently in clinical trials for anemia, and animal studies have
320  suggested that they may have efficacy in models of neurodegenerative conditions, indicating that
321  modulation of the hypoxic response to treat age-related disorders in humans may be on the horizon
322  (Ashok et al., 2017; Haase, 2017; Li et al., 2018; Mehta et al., 2009). However, the role of HIF-1 activity in
323  modulating aging and age-related pathologies is highly complex.  The finding that RHY-1 and EGL-9/PHD
324  modulate aging by a VHL-1-independent mechanism indicates that further studies to illuminate the
325  mechanisms underlying non-canonical roles of proline-hydroxylases in influencing transcription, aging,
326  and disease will be critical to support effective translation of compounds that modulate the hypoxic
327  response.

328

329 **Methods.**

330 Worm culture and RNAi:

331 Standard procedures for *C. elegans* strain maintenance and handling were used (Ahringer, 2006;
332 Stiernagle, 2019). Experiments were performed on animals grown on *Escherichia coli* HT115 expressing
333 empty vector or target RNAi from the Ahringer library.  Nematode strains N2, *rhy-1(ok1402)*, *egl-*
334 *9(sa307)*; *vhl-1(ok161)*, *hif-1(ia4)*, *cysl-1(ok762)* and *egl-9(sa307);vhl-1(ok161)* were obtained from the
335 Caenorhabditis Genetics Center.  *rhy-1(ok1402)* was outcrossed 5 times to a recently unfrozen CGC N2
336 strain, and *rhy-1(ok1402);vhl-1(ok161)* was made from outcrossed *rhy-1(ok1402)* using standard
337 methods.

338 Lifespans:

339 Lifespans were performed as described previously with minor modifications (Amrit et al., 2014).
340 Animals were cultured on standard RNAi plates (NGM + 4ml 1M IPTG/L) inoculated with empty vector
341 RNAi or the clone being tested, at the temperature of the assay, for at least two generations prior to
342 measuring lifespans.  Synchronized populations were transferred to lifespan plates (NGM+100mg
343 Carbenecillin 4ml1M IPTG/l+660ul 150mM FUDR/L) at adulthood. Animals with age-related vulval
344 integrity defects were included, animals that left the plate were not considered (Leiser et al., 2016).
345 Replicates and statistics are included in supplemental table 1.

346 RNA isolation, sequencing and analysis:

347 Worm strains were synchronized by treating gravid adult worms with sodium hypochlorite and
348 collecting ~1000 offspring per genetic condition. Once the offspring reached young adulthood, they
349 were collected in M9 buffer and immediately flash-frozen in liquid nitrogen. RNA was extracted
350 following Invitrogen's TRIZOL RNA extraction protocol. Before library preparation the samples were
351 analyzed on an Agilent 2100 BioAnalyzer. Only samples with an RNA integrity numbers (RIN) equal to or
352 greater than 9.0 were used in downstream analyses. Single-strand reverse transcription, library
353 preparation, and sequencing were performed on an Illumina machine. Read alignments were mapped
354 using Kallisto and differential expression analyses was performed using Sleuth. We used a q-value cutoff
355 of <.05 for downstream comparisons across conditions as it's adjusted for multiple hypothesis testing
356 (Bray et al., 2016; Pimentel et al., 2017).

357

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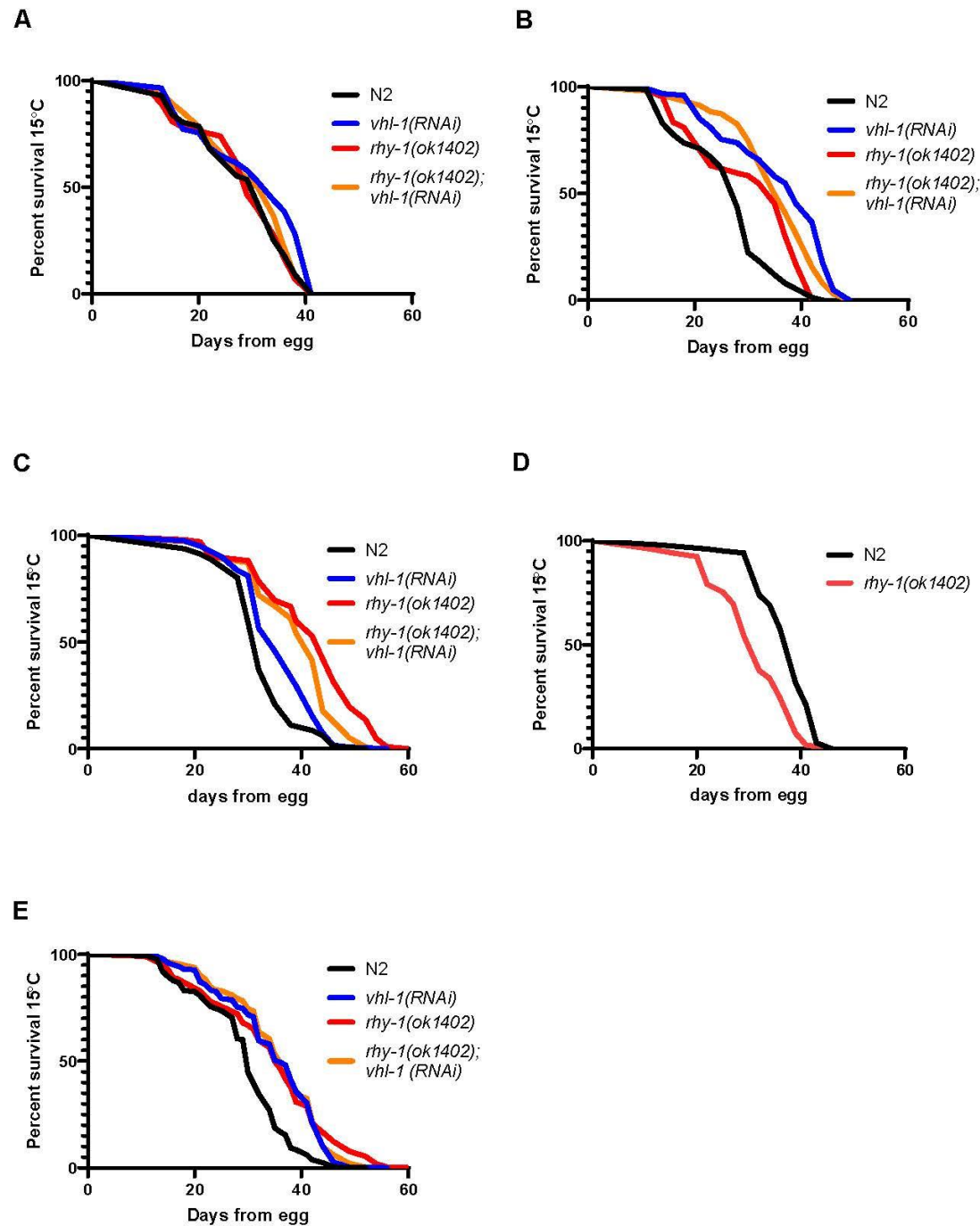
**Figure S1. *rhy-1-mediated effects of lifespan.*** A-C) Individual lifespan experiments with N2 and rhy-1(ok1402) animals treated with empty vector or *vhl-1(RNAi)*. D). Additional lifespan trial comparing N2 and rhy-1(ok1402) lifespans on empty vector RNAi.  E. Aggregated data from A-C.

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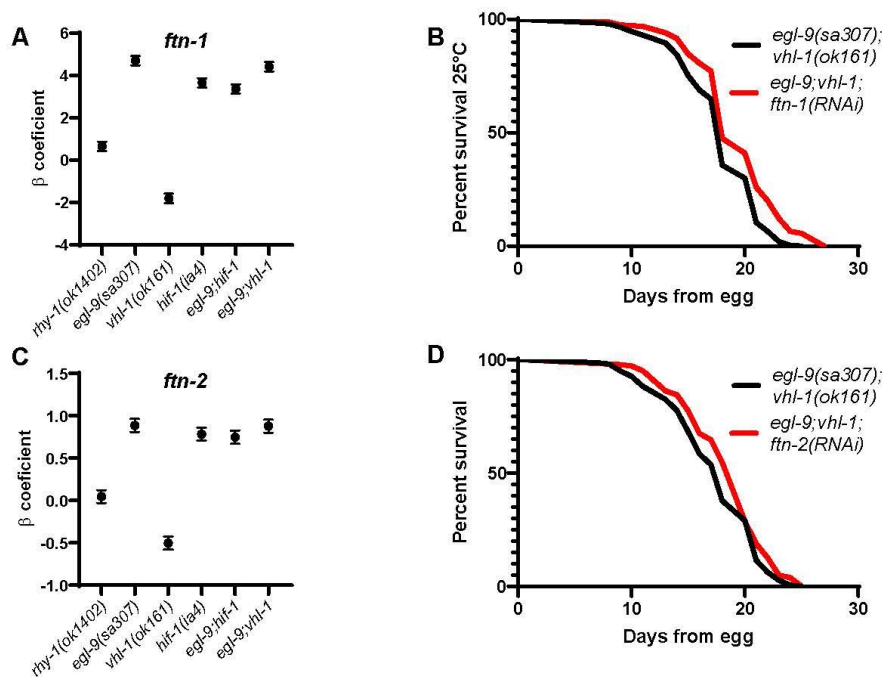


479

480 **Figure S2.** *Effects of additional* **VHL-1/EGL-9 antagonistic HIF-1 targets on lifespan.** A, C) expression
481 levels of selected transcripts from RNA-seq analyses. B-D) Treatment with *ftn-1(RNAi),* and *ftn-2(RNAi)*
482 increases lifespan of *egl-9(sa307);vhl-1(ok161)* mutants (p<.05 by log-rank). Lifespan data are
483 aggregated from at least three experiments, and are significant (p<.05 by log-rank with Bonferroni
484 correction) in 2 out of 4 individual trials (Table S2).