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Two Distinct Roles for EGL-9 in the Regulation of HIF-1-Mediated Gene Expression in *Caenorhabditis elegans*

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ABSTRACT

Oxygen is critically important to metazoan life, and the EGL-9/PHD enzymes are key regulators of hypoxia (low oxygen) response. When oxygen levels are high, the EGL-9/PHD proteins hydroxylate hypoxia-inducible factor (HIF) transcription factors. Once hydroxylated, HIFα subunits bind to von Hippel-Lindau (VHL) E3 ligases and are degraded. Prior genetic analyses in *Caenorhabditis elegans* had shown that EGL-9 also acted through a *vhl-1*-independent pathway to inhibit HIF-1 transcriptional activity. Here, we characterize this novel EGL-9 function. We employ an array of complementary methods to inhibit EGL-9 hydroxylase activity *in vivo*. These include hypoxia, hydroxylase inhibitors, mutation of the proline in HIF-1 that is normally modified by EGL-9, and mutation of the EGL-9 catalytic core. Remarkably, we find that each of these treatments or mutations eliminates oxygen-dependent degradation of HIF-1 protein, but none of them abolishes EGL-9-mediated repression of HIF-1 transcriptional activity. Further, analyses of new *egl-9* alleles reveal that the evolutionarily conserved EGL-9 MYND zinc finger domain does not have a major role in HIF-1 regulation. We conclude that *C. elegans* EGL-9 is a bifunctional protein. In addition to its well-established role as the oxygen sensor that regulates HIF-1 protein levels, EGL-9 inhibits HIF-1 transcriptional activity via a pathway that has little or no requirement for hydroxylase activity or for the EGL-9 MYND domain.

CELLS and tissues are often deprived of oxygen during normal development and during disease. Examples include animals that encounter hypoxic soil or aqueous microenvironments, mammalian tissues that receive insufficient oxygen when the cardiovascular system is taxed or disabled, and cells at the center of a poorly vascularized tumor. Most metazoans rely on aerobic respiration as a primary source of energy, and adaptation to hypoxia is of central importance. The hypoxia-inducible factor (HIF) transcription complexes have been termed master regulators of hypoxia response, because they regulate most hypoxia-induced changes in gene expression in animals as diverse as humans and the nematode *Caenorhabditis elegans* (Kaelin and Ratcliffe 2008). In mammals, these HIF targets include genes that regulate growth, energy metabolism, cellular differentiation, apoptosis, inflammation, and angiogenesis (Siddiq *et al.* 2007; Rankin and Giaccia 2008; Weidemann and Johnson 2008).

The EGL-9/PHD proteins act as cellular oxygen sensors, and they are at the core of HIF regulatory networks. When oxygen levels are sufficiently high, PHD/EGL-9 proteins hydroxylate conserved proline residues in the HIFα subunits. Once hydroxylated, HIFα proteins bind to the von Hippel-Lindau tumor suppressor protein (VHL) (Bruick and McKnight 2001; Ivan *et al.* 2001; Jaakkola *et al.* 2001; Min *et al.* 2002). VHL targets HIFα for polyubiquitination and proteasomal degradation (Maxwell *et al.* 1999; Ohh *et al.* 2000).

The nematode *C. elegans* has provided important insights into hypoxia signaling. The *egl-9* gene was first identified in genetic screens for mutations that disrupted egg laying (Trent *et al.* 1983) and for mutations that conferred resistance to the bacterial pathogen *Pseudomonas aeruginosa* (Darby *et al.* 1999). Subsequent studies identified *C. elegans* EGL-9 as the oxygen-sensitive enzyme that controlled oxygen-dependent degradation of HIF-1, and EGL-9 was shown to be orthologous to mammalian PHD1, PHD2, and PHD3 (Epstein *et al.* 2001). *C. elegans* that carry a deletion in *hif-1* are not able to survive development in hypoxia (Jiang *et al.* 2001; Padilla *et al.* 2002). *hif-1* and *egl-9* have been shown to have roles in other important processes, including heat acclimation, neural development, behavioral responses to oxygen or carbon dioxide, cyanide resistance, and aging (Gallagher and Manoil 2001; Jiang *et al.* 2001; Treinin *et al.* 2003; Bretscher *et al.* 2008; Chang and Bargmann 2008; Pocock and Hobert 2008; Chen *et al.* 2009; Mehta *et al.* 2009; Miller and Roth 2009; Zhang *et al.* 2009).

Genetic analyses in *C. elegans* have shown that EGL-9 regulates HIF-1 via two distinct pathways: oxygen-dependent degradation of HIF-1 and an uncharacter-

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ized *vhl-1*-independent pathway in which EGL-9 represses HIF-1 transcriptional activity (illustrated in Figure 1A). In previous studies, we had discovered that the mRNA transcripts for HIF-1 target genes were expressed at much higher levels in *egl-9* mutants, compared to *vhl-1* mutants (Shen *et al.* 2006). Other studies had suggested that mammalian PHD proteins might also regulate HIF activity in some VHL-independent contexts (Ozer *et al.* 2005; To and Huang 2005). These findings supported the intriguing hypothesis that EGL-9/PHD proteins had VHL-independent roles that might not involve HIF hydroxylation.

In this study, we investigate the *vhl-1*-independent mechanism by which *C. elegans* EGL-9 represses HIF-1 activity. We find that while hydroxylation of HIF-1 at proline residue 621 by EGL-9 is required for HIF-1 destabilization, it is not essential for the *vhl-1*-independent functions of EGL-9. Further, we show that the two EGL-9 pathways have differing sensitivities to mutations or pharmacological treatments that impair hydroxylase activity. Collectively, these data show that EGL-9 represses HIF-1 transcriptional activity via a pathway that has little or no requirement for EGL-9 hydroxylase activity.

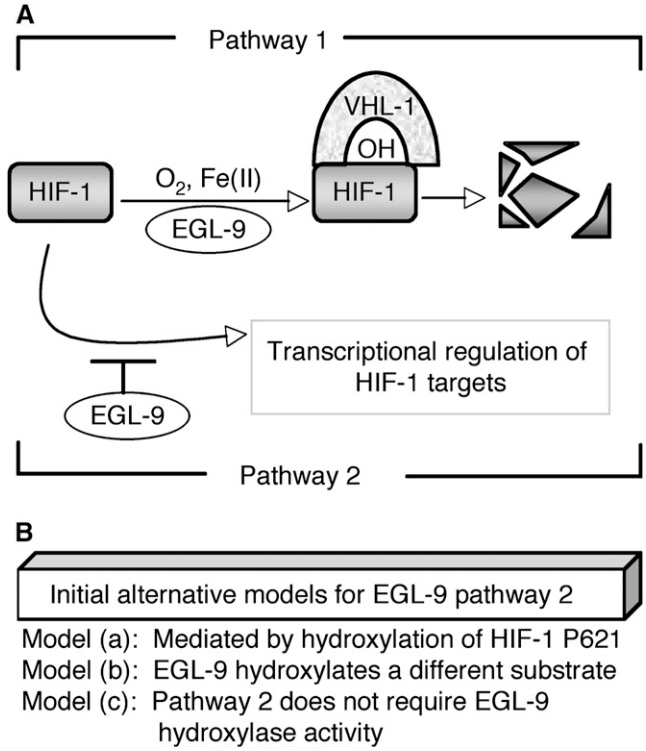


Figure 1.—EGL-9 functions and models tested in this study. (A) EGL-9 regulates HIF-1 by two pathways, and they are illustrated here. First, EGL-9 controls oxygen-dependent degradation of HIF-1 (labeled pathway 1). EGL-9 hydroxlates HIF-1 on a conserved proline residue (P621), and this enables binding of HIF-1 to the VHL-1 E3 ligase. HIF-1 is then degraded. Molecular oxygen, Fe(II), and 2-oxoglutarate are required for the hydroxylation reaction. EGL-9 also suppresses expression of HIF-1 targets by a second pathway that does not require VHL-1 (labeled pathway 2 here). (B) Initial alternative models for the VHL-1-independent functions of EGL-9 (pathway 2). Each model predicts a different combination of experimental outcomes. Model a postulates that pathway 2 (like pathway 1) requires hydroxylation of HIF-1 proline 621. Model b is that EGL-9 hydroxylates a different target to inhibit HIF-1 transcriptional activity. This model predicts that all EGL-9 functions would be abrogated by mutations or treatments that eliminated EGL-9 hydroxylase activity. Model c is that EGL-9 represses HIF-1-mediated transcription by a mechanism that does not require EGL-9 hydroxylase activity.

MATERIALS AND METHODS

Alleles and worm culture: *C. elegans* were grown at 20° using standard methods (Brenner 1974). The loss-of-function alleles, transgenes, and strains described in this study are listed in supporting information (Table S1, Table S2, and Table S3). All new mutations and integration events were outcrossed to wild-type animals at least four times.

Constructs and worm transformation: The *Pegl-9::egl-9::tag* expression construct includes 1.6 kb of *egl-9* 5′ regulatory sequence, genomic sequence for the first three *egl-9* exons and the remaining exons from the cDNA for the predominant *egl-9* mRNA isoform (*egl-9a*, illustrated in Figure S1). The *egl-9* coding sequences are fused in frame to green fluorescent protein (GFP). Further details of plasmid construction are in supplemental methods. To create the *Pegl-9::egl-9(H487A)::tag* construct, the *egl-9* codon for histidine 487 was changed to encode alanine.

The *Phif-1::hif-1::tag* construct contains 5.2 kb of *hif-1* 5′ regulatory sequence, the genomic sequence for the first exon and first intron of *hif-1*, cDNA sequence for *hif-1* exons 2–9, and an epitope tag. To create the *Phif-1::hif-1(P621G)::tag* construct, the codon for proline 621 was modified to encode glycine. The *hif-1* transgenes are further characterized in Figure S2 and in Zhang *et al.* (2009).

The *egl-9* constructs were introduced to the strain ZG305 [*egl-9(sa307)*; *unc-119(ed3)*], and the *hif-1* constructs were introduced to ZG228 [*hif-1(ia04), unc-119(ed3)*], by microparticle bombardment with the *unc-119* rescue plasmid (pPD#MMO16b) as a cotransformation marker (Praitis *et al.* 2001). The resulting transgenic strains were each backcrossed at least four times.

MOS1 mediated mutagenesis: We generated the *egl-9* loss-of-function alleles *ia58*, *ia60*, and *ia61* in a screen for Mos1 transposon-mediated mutations that dramatically increased the expression of the *Pnhr-57::*GFP reporter (Shen *et al.* 2006). The methods for Mos1 mobilization have been described previously (Granger *et al.* 2004). We screened ~164,600 genomes, as diagrammed in Figure S3.

Protein blots: Protein blots were probed with monoclonal antibodies recognizing the following epitopes: GFP (antibody from Roche at 1:1000 dilution); HA (antibody from Cell Signaling Technology clone 6E2 at 1:1000 dilution); myc (mouse ascites, clone 9E10, from the Developmental Studies Hybridoma Bank at 1:1000 dilution), or AHA-1 (Jiang *et al.* 2001) (1:100 dilution). The secondary antibody (goat anti-mouse IgG+IgM from Bio-Rad) was diluted 1:2000. Further information about experimental and statistical analyses are in supplemental methods (File S1).

Real-time PCR: We used Trizol reagent (Invitrogen) to isolate total RNA from developmentally synchronized populations of L4 or young adult stage worms. Total RNA from each sample was treated by RNase free DNase (Promega) and

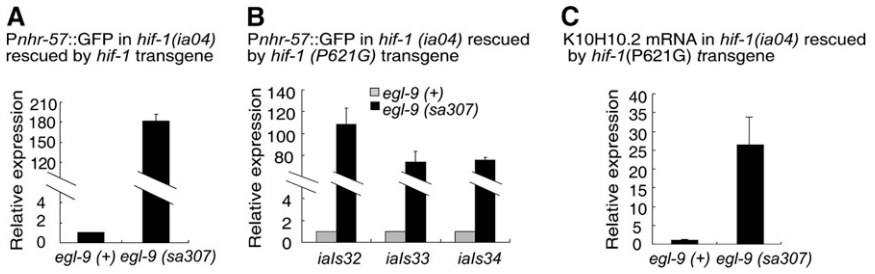


Figure 2.—The HIF-1(P621G) mutation does not prevent *egl-9*-mediated repression of HIF-1 activity. (A) In control experiments, the transgene encoding wild-type *hif-1*, rescued expression of *Pnhr-57*::GFP in *hif-1(ia04)* mutant animals, as measured by protein blots. The *egl-9(sa307)* loss-of-function mutation dramatically increased expression of the *Pnhr-57*::GFP reporter. +, the wild-type allele. (B and C) To determine whether the HIF-1(P621G) mutation abrogated all regulation by *egl-9*, the expression of two HIF-1 targets, *Pnhr-57*::GFP and K10H10.2, were compared in *egl-9(+)* and *egl-9(sa307)* animals. These experiments were conducted in *hif-1(ia04)* mutants rescued by the *hif-1(P621G)*::*tag* transgene. (B) The HIF-1(P621G) mutation did not prevent repression of *Pnhr-57*::GFP by *egl-9*. *Pnhr-57*::GFP expression was assayed by protein blots. This result was consistent across three independently isolated *hif-1(P621G)*::*tag* transgenic lines (*ialS32*, *ialS33*, and *ialS34*) ($P < 0.01$ in each case). (C) The *hif-1(P621G) ialS32* mutation does not abolish regulation of K10H10.2 expression by *egl-9*. Quantitative RT–PCR experiments established a significant difference in K10H10.2 mRNA levels between *egl-9(+)* and *egl-9(sa307)* ($P < 0.05$).

reverse transcribed to complementary DNA using Oligo(dT_{18}) primers and AffinityScript reverse transcriptase (Stratagene). Quantitative RT–PCR was performed using the iQ SYBR GREEN supermix (Bio-Rad) real-time PCR system, and each reaction included cDNA from 50–100ng total RNA. The primers for K10H10.2 and *inf-1* have been published previously (Shen *et al.* 2006). *inf-1* is not regulated by hypoxia and was used as an input control (Shen *et al.* 2005, 2006). At least three biological replicates were analyzed for each experiment, and each PCR reaction was performed in duplicate. The standard curve method was used to analyze the expression levels. Two-sample paired *t*-tests were used to assess statistical significance of differences.

RESULTS

Prior studies had shown that *egl-9* inhibited HIF-1 by two genetic pathways: (1) VHL-1-mediated oxygen-dependent degradation (Epstein *et al.* 2001) and (2) repression of HIF-1 transcriptional activity via a pathway that did not require *vhl-1* (Shen *et al.* 2006) (illustrated in Figure 1A). While oxygen-dependent degradation is well characterized, the mechanisms by which EGL-9 inhibits HIF-1 activity are not understood. In the studies described here, we have conducted a series of experiments to distinguish between alternative models for how EGL-9 represses expression of HIF-1 targets independent of VHL-1 (pathway 2 in Figure 1A). The simplest model was that EGL-9-mediated hydroxylation of HIF-1 at proline 621 resulted in both degradation of HIF-1 protein and inhibition of HIF-1 transcriptional activity (model a in Figure 1B). An alternative model was that EGL-9 hydroxylated another region of HIF-1 or a different protein to repress HIF-1-mediated transcription (model b in Figure 1B). Finally, we considered the possibility that EGL-9 inhibited HIF-1 transcriptional activity via a novel mechanism that did not require EGL-9 prolyl hydroxylase activity (model c in Figure 1B). The experiments described herein are designed to distinguish between these three models.

Effects of the HIF-1(P621G) mutation: EGL-9 hydroxylates proline 621 of HIF-1 (Epstein *et al.* 2001). This is the only known EGL-9 target in the HIF-1 protein, and HIF-1 transgenes that carry the P621G mutation are not degraded through the EGL-9/VHL-1 pathway (Zhang *et al.* 2009) (Figure S2C). To determine whether the HIF-1 P621G mutation eliminated all regulation of HIF-1 by EGL-9, we examined the effects of *egl-9* mutations in animals expressing HIF-1(P621G). In these experiments, the endogenous *hif-1* gene was knocked out, and *hif-1* function was restored by an integrated transgene expressing either epitope-tagged wild-type HIF-1 or stabilized HIF-1(P621G). To assess the effects of the mutations on HIF-1 activity, we assayed the expression of two HIF-1 target genes: K10H10.2 and the *Pnhr-57*::GFP reporter (Shen *et al.* 2006). In control experiments, we confirmed that in animals expressing the wild-type HIF-1 transgene, a strong loss-of-function mutation in *egl-9* resulted in dramatic overexpression of *Pnhr-57*::GFP (Figure 2A). We then assayed the expression of HIF-1 targets in three independently isolated lines expressing HIF-1(P621G), and we found that the *egl-9(sa307)* mutation caused markedly higher expression of *Pnhr-57*::GFP in these animals (Figure 2B). K10H10.2 mRNA levels were also significantly increased by the *egl-9(sa307)* loss-of-function mutation in animals expressing the stabilized HIF-1 (Figure 2C). These data demonstrated that the HIF-1(P621G) mutation did not abolish all regulation of HIF-1 by EGL-9, and this effectively disproved "model a" as described in Figure 1B.

Hypoxia or iron chelator treatments that inhibit oxygen-dependent degradation of HIF-1 do not eliminate all EGL-9 functions: Having established that EGL-9 had functions other than hydroxylation of HIF-1 proline 621, it was important to determine whether those other functions required EGL-9 hydroxylase activity. We reasoned that if EGL-9 hydroxylated other substrates to repress HIF-1 activity (model b in Figure 1B), then all EGL-9 functions would be repressed by treatments that inhibited EGL-9 hydroxylase activity. The HIF-1 hydroxylation reaction requires oxygen, 2-oxoglutarate, and Fe^{+2} (Kaelin and Ratcliffe 2008). Oxygen deprivation or the iron chelator 2, 2′-dipyridyl (DIP) inhibits this

reaction (Bishop *et al.* 2004). In control experiments, we confirmed that HIF-1 protein was stabilized by hypoxia (0.5% oxygen) or DIP treatments (Figure 3A). We next asked whether inhibitors of EGL-9 hydroxylation activity could fully phenocopy a strong loss-of-function mutation in *egl-9*. We found that hypoxia, DIP, or a deletion mutation in *vhl-1* increased *Pnhr-57*::GFP to similar levels (Figure 3B). Although the hypoxia and DIP treatments prevented oxygen-dependent degradation of HIF-1, they did not increase expression of *Pnhr-57*::GFP to the levels caused by strong loss-of-function mutations in *egl-9* (compare the last bar in Figure 3B to the other conditions). Hypoxia or DIP had no effect on *Pnhr-57*::GFP levels in *vhl-1* mutant animals. Taken together, these data demonstrated that the *vhl-1*-independent functions of EGL-9 were relatively insensitive to inhibitors of hydroxylase activity.

Mutation in the EGL-9 catalytic core has differential effects on the two EGL-9 pathways: The findings that hypoxia or DIP treatments did not increase expression of *Pnhr-57*::GFP in *vhl-1* mutants provided support for a model in which EGL-9 had functions that did not require hydroxylase activity (model c in Figure 1B). To test this hypothesis further, we analyzed the consequences of the EGL-9(H487A) mutation. The histidine 487 residue is in the catalytic core of EGL-9. This region of the protein contributes to the Fe(II)-binding pocket and is highly conserved (Figure 4A) (McDonough *et al.* 2006). Mutation of the analogous residue in the mammalian PHD2 protein (His313) has been shown to eliminate hydroxylase activity (Pan *et al.* 2007).

To compare the functions of wild-type EGL-9 with EGL-9(H487A), we first constructed and assayed an epitope-tagged wild-type *egl-9* minigene, in which genomic sequence including 5′ regulatory sequences, three exons, and two introns were fused to cDNA for the remaining exons in the predominant *egl-9* mRNA isoform (Figure 4B). The minigene was able to restore *egl-9* function in an *egl-9(sa307)* mutant, as assayed by destabilization of HIF-1 (compare lanes 1 and 2 in Figure 4C), repression of *Pnhr-57*::GFP expression (compare bars 1 and 2 in Figure 4D), or repression of K10H10.2 mRNA levels (compare bars 1 and 2 in Figure 4E). We then introduced the H487A point mutation to the wild-type *egl-9* minigene and analyzed the ability of the *egl-9(H487A)* minigene to rescue *egl-9(sa307)*. As expected, EGL-9(H487A) did not destabilize HIF-1 (lanes 3, 4, and 5 in Figure 4C). The *iaIs38*, *iaEx101*, and *iaEx110* transgenes all expressed similar levels of EGL-9 wild-type or mutant protein, while expression from *iaEx104* was slightly lower (Figure S4).

Having confirmed that EGL-9(H487A) could not rescue oxygen-dependent degradation of HIF-1 in *egl-9 (sa307)* mutants (pathway 1 in Figure 1A), we next asked whether this catalytically deficient EGL-9 was able to repress expression of HIF-1 target genes (pathway 2 in Figure 1A). To address this question, we compared the

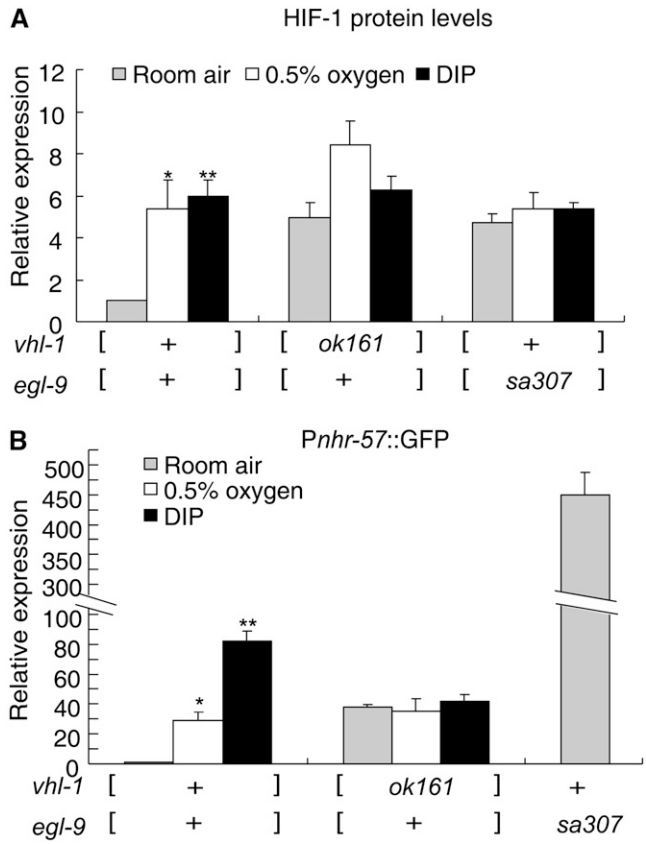


Figure 3.—Differential effects of hypoxia or iron chelators on the two EGL9 pathways. (A) In control experiments, hypoxia (0.5% oxygen, 1 hr) or the iron chelator 2, 2′-dipyridyl (DIP, 200 μM, 4 hr) increased HIF-1 protein to levels similar to those caused by loss-of-function mutations in *vhl-1* or *egl-9*. The bar graph shows HIF-1 protein levels relative to that in untreated *vhl-1(+)* and *egl-9(+)* animals. In these strains, the only functional copy of *hif-1* is the epitope-tagged transgene. The error bars indicate the standard errors from three independent biological replicates. (B) To determine whether hydroxylase inhibitors had similar effects on HIF-1 target genes as a mutation in *egl-9*, expression of the reporter was assayed by protein blots. Hypoxia or DIP treatment increased expression of the reporter to levels found in *vhl-1* loss-of-function mutants, but the treatments did not completely phenocopy the effects of a loss-of-function mutation in *egl-9*. In the bar graph, *Pnhr-57*::GFP levels are shown relative to that in untreated *vhl-1(+)* and *egl-9(+)* animals. The *vhl-1 (ok161)* and *egl-9(sa307)* mutations are strong loss-of-function alleles. +, the wild-type allele. The bars represent average values from three independent replicates, and the error bars reflect standard error. The statistics were comparing the hypoxia or DIP treated data to untreated data with the same genotype. Asterisks represent statistically significant differences between treatment and room air for animals of the same genotype. $*P < 0.05$; $**P < 0.01$.

ability of wild-type *egl-9* and *egl-9(H487A)* minigenes to inhibit expression of *Pnhr-57*::GFP in animals that carried a strong loss-of-function mutation in the endogenous *egl-9* gene. The wild-type *egl-9* minigene (*iaIs38*) destabilized HIF-1 (see lane 2 of Figure 4C) and repressed *Pnhr-57*::GFP (compare first and second bars of Figure 4D). Stabilization of HIF-1 by DIP resulted in

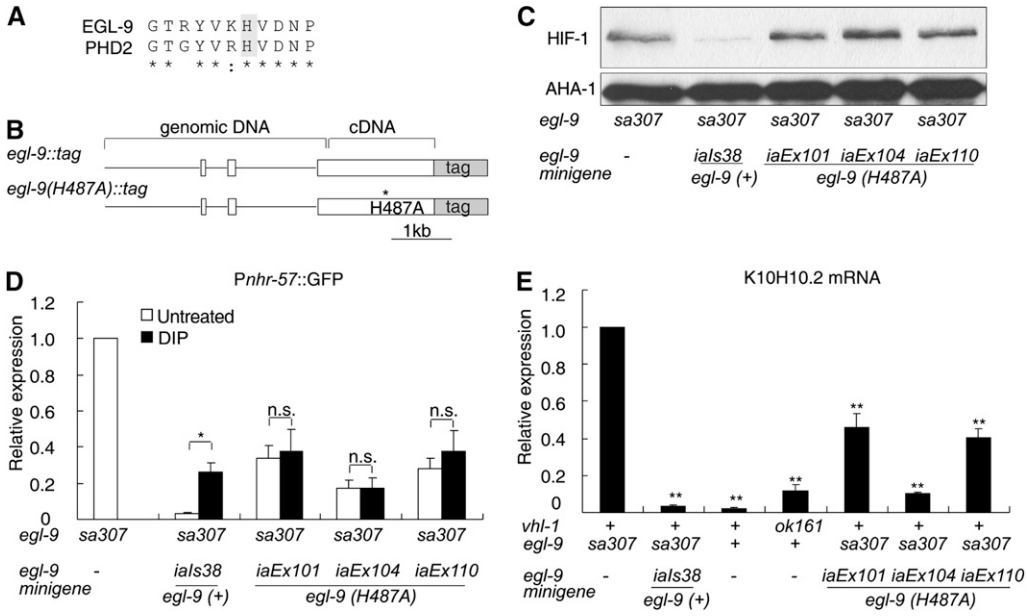


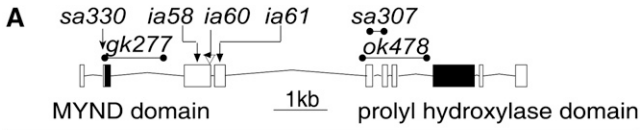
FIGURE 4.—The *egl-9* (H487A) mutation does not abolish *egl-9*-mediated repression of HIF-1 activity. (A) Alignment of human PHD2 and *C. elegans* EGL-9 at the region around EGL-9 His487. (B) Diagrams of *egl-9* minigenes. The minigenes are fusions of genomic and cDNA sequences. Boxes represent coding regions. GFP is fused in frame to *egl-9*. The H487A mutation disrupts the Fe(II) binding pocket in EGL-9 and impairs EGL-9 catalytic activity. (C) In *egl-9 (sa307)* mutants, HIF-1 destabilization was rescued by the transgene coding for wild-type *egl-9(iaIs38)*, but not by the *egl-9 (H487A)* transgenes (*iaEx101*, *iaEx104*, or *iaEx110*). AHA-1 protein levels have been shown to be unaffected by severe loss-of-function mutations in *egl-9* or *vhl-1* (SHEN *et al.* 2006), and AHA-1 serves as a loading control. (D and E) Transgenes expressing either wild-type *egl-9* or *egl-9(H487A)* can suppress expression of HIF-1 targets in an *egl-9(sa307)* background. (D) In strains carrying the wild-type *egl-9* transgene, repression of *Pnhr-57*::GFP is more effective and is inhibited by DIP. In strains expressing *egl-9(H487A)*, DIP does not have a significant effect. The bar graph shows averages of *Pnhr-57*::GFP protein levels from three biological replicates, normalized to expression levels in an *egl-9(sa307)* mutant. The asterisk indicates DIP causes a statistically significant difference in the expression of the reporter ($*P < 0.05$) and NS is no significant difference. (E) The wild-type *egl-9* transgene and the *egl-9(H487)* mutant transgene were able to repress K10H10.2 mRNA levels in an *egl-9(sa307)* mutant. K10H10.2 mRNA levels were also assayed in wild-type N2 and in *vhl-1*-deficient animals for comparison. The bar graph shows the relative K10H10.2 mRNA levels in each strain, compared to *egl-9(sa307)* and the error bars reflect standard error. NS, no significant difference. $*P < 0.05$; $**P < 0.01$.

increased *Pnhr-57*::GFP expression, but not to the levels seen in animals that lack *egl-9* function (compare the third bar and the first bar in Figure 4D). In these assays, the HIF-1 reporter gene was similarly regulated in animals containing the wild-type *egl-9* locus or in animals expressing *egl-9* from the *iaIs38* transgene.

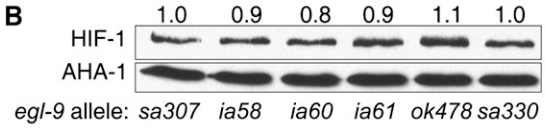
Each of the three *egl-9(H487A)* transgenic arrays were also able to repress expression of *Pnhr-57*::GFP, although not to the level achieved by the wild-type *egl-9* transgene (Figure 4D). As expected, DIP had no significant effect when the EGL-9 hydroxylase domain was disabled by the H487A mutation (the solid bars in sets 3, 4, and 5 of Figure 4D). These data demonstrated that while disruption of the EGL-9 iron-binding pocket was sufficient to abolish oxygen-dependent degradation of HIF-1 (Figure 4C), this mutation did not eliminate all EGL-9-mediated inhibition of the HIF-1 reporter (Figure 4D).

These findings supported a model in which EGL-9 had two functions: (i) hydroxylation of HIF-1 in the HIF-1 oxygen-dependent degradation pathway and (ii) repression of HIF-1 transcriptional activity by a pathway that had little or no requirement for EGL-9 hydroxylation activity. To further compare the ability of wild-type and catalytically deficient *egl-9* transgenes to repress expression of HIF-1 targets, we analyzed K10H10.2 mRNA by RT–PCR. As shown in Figure 4E, when the wild-type *egl-9* transgene was introduced to an *egl-9 (sa307)* mutant, it reduced expression of K10H10.2 >10-fold (compare first and second bars of Figure 4E) to levels similar to those in wild-type animals (third bar in Figure 4E). The *egl-9(H487A)* transgenes did not destabilize HIF-1 (as shown in Figure 4C), so we expected that if they retained the ability to repress HIF-1 transcriptional activity via *vhl-1*-independent pathways, then the *egl-9(H487A)* transgenic animals would express K10H10.2 at levels lower than *egl-9(sa307)* controls and similar to the levels seen in *vhl-1(ok161)* mutants. As shown in the last three bars of Figure 4E, K10H10.2 mRNA levels were significantly reduced in each of the three *egl-9(H487A)* lines, relative to the *egl-9(sa307)* control. In sum, the data in Figure 4 show that the catalytically deficient EGL-9 protein was unable to destabilize HIF-1, but retained the ability to repress expression of HIF-1 targets.

***egl-9* mutations and their effects on HIF-1:** We sought to understand the relationships between EGL-9 functions and EGL-9 structure further by characterizing a series of mutations in the endogenous *egl-9* gene. Like its mammalian cognates, EGL-9 includes both an MYND zinc finger domain and a prolyl hydroxylase domain (Figure 5A). The *egl-9* locus encodes at least nine mRNA isoforms. We sequenced 19 *egl-9* cDNAs and have submitted these sequences to WormBase (ROGERS *et al.*



Alleles	Description	Mutation and flanking sequences
ia58	1bp deletion (frame shift)	tccgccaacatttt(c)gatcaca
ia60	MOS1 insertion	agtgatatcagta (MOS1)gactcg
ia61	5bp duplication (frame shift) and a point mutation	cacagcaactat(actat)tcca(c ▸ t) ctccagcg



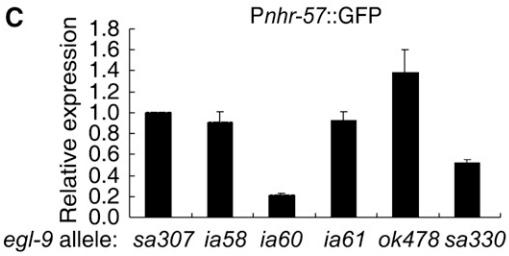


FIGURE 5.—Characterization of *egl-9* loss-of-function alleles. (A) Diagram of *egl-9* exons and introns and description of new mutations. Boxes represent exons for the predominant *egl-9* mRNA isoform, and the exons encoding the MYND or hydroxylase domains are filled. Lines represent deleted sequences in the *gk277*, *sa307*, or *ok478* alleles; arrows indicate the positions of the *ia58* and *ia61* mutations; and the position of the *ia60* transposon insertion is shown. The alleles isolated in this study are described in the table. (B) Relative effects of *egl-9* mutations on HIF-1 protein levels. These animals carry the *hif-1(ia04)* deletion mutation, and *hif-1* function is restored by the *hif-1::tag* transgene. The numbers above the lanes reflect HIF-1 levels relative to those detected in *egl-9 (sa307)* mutants, as determined from three replicate experiments. (C) Expression of the *Pnhr-57*::GFP reporter in *egl-9* mutants, relative to animals homozygous for the *egl-9 (sa307)*, a strong loss-of-function *egl-9* allele. The bars represent average values relative to those in *egl-9(sa307)*, from three independent replicates, and the error bars reflect standard error.

2008). The structures and relative frequencies of the alternatively spliced *egl-9* transcripts are diagrammed in Figure S1. Prior chemical mutagenesis screens had isolated loss-of-function mutations in *egl-9* that caused defects in egg laying or resistance to cyanide produced by the bacterial pathogen *P. aeruginosa* (DARBY *et al.* 1999; GALLAGHER and MANOIL 2001). More recently, the *C. elegans* gene knockout consortium isolated two *egl-9* deletion alleles: *gk277* and *ok478* (diagrammed in Figure 5A). We isolated three additional loss-of-function mutations in *egl-9* in a MOS1 transposon-mediated screen to identify mutations that caused overexpression of *Pnhr-57*::GFP. The screen is illustrated in Figure S3, and the new *egl-9* mutations are described in Figure 5A.

Initially, we characterized six *egl-9* loss-of-function mutations. These included a mutation previously shown to be a strong loss-of-function allele (*sa307*), the three new alleles from the MOS1-mediated screen (*ia58*, *ia60*, and *ia61*), a deletion allele that removed exons common to all known *egl-9* isoforms (*ok478*), and a mutation that had been shown to confer resistance to *P. aeruginosa*, but did not cause egg-laying defects (*sa330*; DARBY *et al.* 1999). For each allele, we characterized its effects on HIF-1 stability and on expression of a HIF-1 target gene, *Pnhr-57*::GFP. When comparing the relative effects of these mutations, we used *egl-9(sa307)* as a reference allele, because prior studies had shown that *egl-9(sa307)* was a severe mutation that abolished degradation of HIF-1 through the EGL-9/VHL-1 pathway (EPSTEIN *et al.* 2001; SHEN *et al.* 2006). All six of the *egl-9* mutations resulted in HIF-1 overexpression phenotypes very similar to that seen in *egl-9(sa307)*, indicating that each of these alleles severely disabled oxygen-dependent degradation of HIF-1 (Figure 5B).

We next asked how each of these mutations affected expression of a HIF-1 target gene. We found that while the *Pnhr-57*::GFP reporter was overexpressed in all of these strains, two of the alleles, *ia60* and *sa330*, caused slightly less severe phenotypes (Figure 5C). The *ia60* allele is a MOS1 transposon insertion in exon 3, and the *sa330* mutation causes an early translational stop in exon 2 of the predominant *egl-9* transcript (Figure 5A) (DARBY *et al.* 1999). The egg-laying defects caused by the *egl-9(ia60)* or *egl-9(sa330)* alleles were also less severe, when compared to other *egl-9* mutations (DARBY *et al.* 1999 and data not shown). These findings suggest that the proteins encoded by the *egl-9 ia60* and *sa330* alleles cannot destabilize HIF-1, but they retain some ability to repress expression of HIF-1 target genes.

Characterization of *egl-9(gk277)*, an allele that deletes the MYND zinc finger domain: The *egl-9 (gk277)* mutation provided a unique opportunity to investigate the role of the MYND motif in EGL-9 function. This deletion allele removes most of *egl-9* exon 2 and intron 2 (Figure 5A). We generated cDNA from *egl-9(gk277)* animals and determined that in these mutants *egl-9* exon 1 was spliced to exon 3, resulting in an in-frame deletion of the evolutionarily conserved MYND zinc finger domain. We determined that HIF-1 protein levels were 1.8-fold higher in *egl-9(gk277)*, relative to animals containing the wild-type *egl-9* gene, and the statistical significance of this difference is marginal (lanes 1 and 2 in Figure 6A). By comparison, strong loss-of-function mutations in *egl-9* or *vhl-1* caused 3- to 4-fold increases in HIF-1 levels. In a *vhl-1(ok161)* background, the *egl-9(gk277)* mutation had no effect on HIF-1 protein levels (lanes 3 and 4 in Figure 6A).

We next investigated the effects of the *gk277* mutation on the expression of two genes regulated by HIF-1. While the *Pnhr-57*::GFP reporter and the endogenous K10H10.2 gene were overexpressed in *egl-9(sa307)*

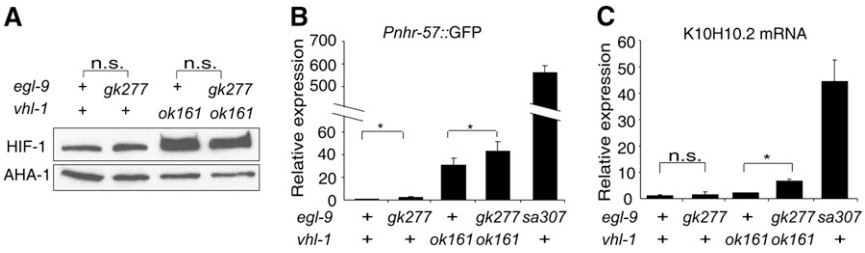


FIGURE 6.—The *egl-9(gk277)* mutation removes the MYND zinc finger domain, but has little effect on HIF-1 protein levels or on expression of HIF-1 targets. (A) The *egl-9(gk277)* mutation does not significantly increase expression of HIF-1 protein in *vhl-1(+)* or *vhl-1(ok161)*. (B and C) The *egl-9(gk277)* mutation has a significant effect on the expression of HIF-1 target genes, but these effects are much smaller than the severe *egl-9(sa307)* mutation. The bar graphs show the expression of the *Pnhr-57*::GFP reporter (B) and the endogenous HIF-1 target gene K10H10.2 (C) in each strain, relative to the values for animals carrying wild-type alleles of *vhl-1* and *egl-9*. The bars represent average values from three independent replicates, and the error bars reflect standard error. NS, no significant difference. $*P < 0.05$.

animals by ~400 and 40-fold, respectively, the *gk277* mutation caused only 2- to 3-fold increases in the expression of these HIF-1 targets (Figure 6, B and C) In animals in which HIF-1 was stabilized by the *vhl-1 (ok161)* mutation, the *gk277* mutation had relatively small effects on the expression of either gene (third and fourth bars in Figure 6, B and C). Thus, deletion of the MYND domain in *egl-9(gk277)* animals had little effect on the ability of EGL-9 to inhibit HIF-1 transcriptional activity.

DISCUSSION

EGL-9 is the key regulator of HIF-1 protein stability and HIF-1 activity. In *C. elegans* and in mammals, HIF induces the expression of EGL-9/PHD, and this establishes a negative feedback loop that attenuates HIF activity (BISHOP *et al.* 2004; SHEN *et al.* 2005; BERRA *et al.* 2006). The catalytic functions of EGL-9/PHD enzymes are of central importance in normal development, homeostasis, and disease states and have been studied intensively (KAELIN and RATCLIFFE 2008). In humans, mutations in the PHD2 active site and other mutations that stabilize HIF have been linked to familial erythrocytosis (PERCY *et al.* 2006, 2007; AL-SHEIKH *et al.* 2008; MARTINI *et al.* 2008). Efforts are underway to develop small molecule inhibitors of PHD activity for the treatment of anemia and other diseases that may be mitigated by increased HIF protein stability (HEWITSON *et al.* 2004). Here, we have shown that EGL-9 has a second function: it represses HIF-1 activity through a pathway that has little or no requirement for EGL-9 hydroxylase activity.

Hydroxylase-deficient EGL-9 still represses HIF-1 activity: The evidence that *C. elegans* EGL-9 regulates HIF-1 stability and HIF-1 activity through two distinct mechanisms is multifold: (i) Loss-of-function mutations in *egl-9* or *vhl-1* stabilize HIF-1 protein, but mutations in *egl-9* cause much higher levels of HIF-1 target gene expression (SHEN *et al.* 2006) (Figure S2B; Figure 3, A and B); (ii) mutation of the proline normally hydroxylated by EGL-9 [in HIF-1(P621G)] releases HIF-1 from oxygen-dependent degradation, but it does not prevent EGL-9-mediated inhibition of HIF-1 target gene expression (Figure S2C; Figure 2, B and C); (iii) treatments that inhibit EGL-9 catalytic activity do not fully phenocopy the *egl-9* phenotype, and hypoxia or DIP have little effect on HIF-1 activity in *vhl-1* mutants (BISHOP *et al.* 2004) (Figure 3B); and (iv) the H487A mutation in the iron-binding pocket of the EGL-9 catalytic domain stabilizes HIF-1, but EGL-9(H487A) still inhibits transcriptional activity (Figure 4, C, D, and E).

We conclude that EGL-9 is a bifunctional protein, with some functions that require hydroxylase activity and others that do not. We have considered the formal possibility that the EGL-9(H487A) mutant protein retains some catalytic function. However, it is clear that the mutation in the EGL-9 iron-binding pocket abrogates hydroxylase activity, as assayed by destabilization of HIF-1 (Figure 4C). Further, some EGL-9 functions are insensitive to inhibitors of hydroxylase activity. In EGL-9 (H487A) animals or in *vhl-1* mutants, DIP treatments do not increase HIF-1 activity (Figures 3B and 4D). We conclude that the two EGL-9 pathways have dramatically different requirements for EGL-9 prolyl hydroxylase activity.

It has been proposed that mammalian PHD proteins may also have functions that do not require hydroxylase activity. PHD2 overexpression was shown to reduce expression of HIF targets in a cell line lacking VHL and in a manner independent of HIF destabilization. Further, PHD2 exerted this inhibitory effect on the HIF-1α transcriptional activation region fused to a heterologous DNA binding domain (TO and HUANG 2005). These data are consistent with a model in which PHD2 could recruit repressors to the HIF transcriptional complex, independent of its role in VHL-mediated degradation of HIF. In an independent study of hypoxia-treated HeLa cells, PHD2 was shown to be associated with HIF DNA binding sites. PHD2 was also shown to bind the ING4 tumor suppressor, which has been proposed to act as a transcriptional repressor (OZER *et al.* 2005). In a third study, expression of a catalytically deficient form of PHD2 was shown to inhibit endothelial cell proliferation (TAKEDA and

Fong 2007). Most recently, PHD2 has been shown to have functions in angiogenesis that do not require hydroxylase activity (Chan *et al.* 2009). These findings do not take away from the central importance of PHDs as oxygen sensors, but they provide strong evidence that mammalian PHD proteins have additional functions that do not require hydroxylase activity.

Characterization of *egl-9* loss-of-function alleles: Several *egl-9* loss-of-function alleles have been isolated in screens for mutations that result in egg-laying deficiencies (Trent *et al.* 1983), resistance to *P. aeruginosa* infection (Darby *et al.* 1999) or overexpression of HIF-1 target genes (this study). In all cases that have been tested, depletion of *hif-1* by RNAi or mutation has been shown to suppress *egl-9* mutant phenotypes (Shen *et al.* 2006; Chang and Bargmann 2008; Gort *et al.* 2008; Pocock and Hobert 2008). We assayed several of these alleles to determine whether they had differential effects on HIF-1 stabilization or VHL-1-independent suppression of HIF-1 activity. With the exception of the *gk277* deletion of the MYND domain, all of the mutations impaired both pathways. Two findings are of particular interest. First, there is a correlation between the degree of *Pnhr-57*::GFP overexpression and the egl phenotypes caused by the *egl-9* mutations. *egl-9(sa330)* and *egl-9(ia60)* mutants are not egg-laying defective. Second, the *egl-9(sa330)* mutation causes a translational stop in exon 2 of the predominant *egl-9* transcript (Darby *et al.* 1999) (Figure 5C), but it does not abolish all *egl-9* function. Six of the nine *egl-9* mRNA isoforms do not include exon 2 or the MYND domain. It is possible that these other transcripts provide some function in *egl-9(sa330)* mutants.

The EGL-9 MYND domain: The MYND zinc finger domain is an evolutionarily conserved feature of PHD proteins. Since the divergence of nematode and mammalian lineages, the PHD gene amplified in mammals. The PHD1, PHD2, and PHD3 genes have diverged and have acquired different tissue distributions, subcellular localization patterns, and functions (Fong and Takeda 2008; Kaelin and Ratcliffe 2008). PHD3 lacks an MYND domain, and, although it has different substrate specificities than PHD1 and PHD2, it can hydroxylate some substrates (Epstein *et al.* 2001). Deletion of the MYND domain in PHD2 resulted in increased HIF-1 destabilization in HeLa cells (Choi *et al.* 2005), suggesting that the MYND domain might modulate PHD activity in some contexts. The *gk277* mutation presents a unique opportunity to examine the *in vivo* effects of deleting the MYND domain, and *C. elegans egl-9(gk277)* mutants had been reported to have defects in neuronal pathfinding (Pocock and Hobert 2008). It was, therefore, of particular interest to determine whether the *egl-9(gk277)* mutation impaired one or both of the EGL-9 pathways for HIF-1 regulation. In our assays, the *egl-9(gk277)* mutation did not change total HIF-1 levels and had only marginal effects on the expression of HIF-1 targets.

This study provides a foundation for further experiments to understand the mechanism by which EGL-9 inhibits expression of HIF-1 transcriptional targets. We have established that mutations or treatments that inhibit hydroxylase activity do not eliminate all regulation of HIF-1 by EGL-9. The next important steps will be to identify EGL-9/PHD interaction partners and to characterize their respective roles in regulating HIF-1 stabilization or activity.

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GENETICS

Supporting Information
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Two Distinct Roles for EGL-9 in the Regulation of HIF-1-Mediated Gene Expression in *Caenorhabditis elegans*

Zhiyong Shao, Yi Zhang and Jo Anne Powell-Coffman

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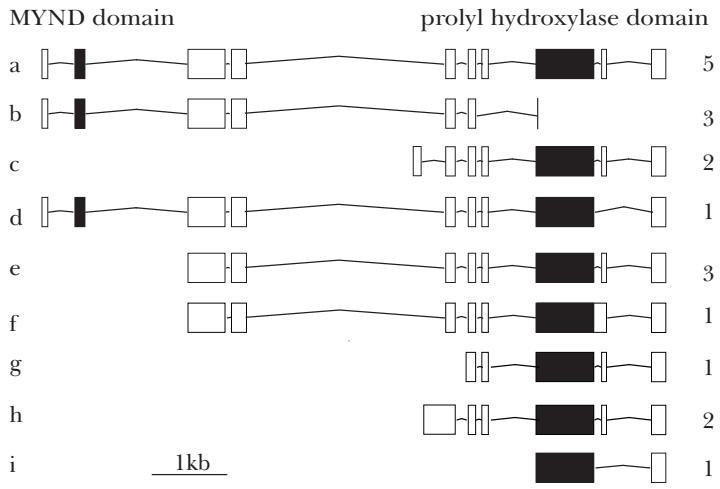

FIGURE S1.—Sequencing of existing cDNAs reveals multiple *egl-9* mRNA isoforms. Boxes represent exons and lines represent introns. MYND and proly1 hydroxylase domains are filled with black. Nineteen cDNAs from the National Institute of Genetics, Mishima, Japan were sequenced in full, an the number cDNAs of each *egl-9* isoform in that collection is shown in the righthand column. The cDNA sequences have been submitted to www.wormbase.org.

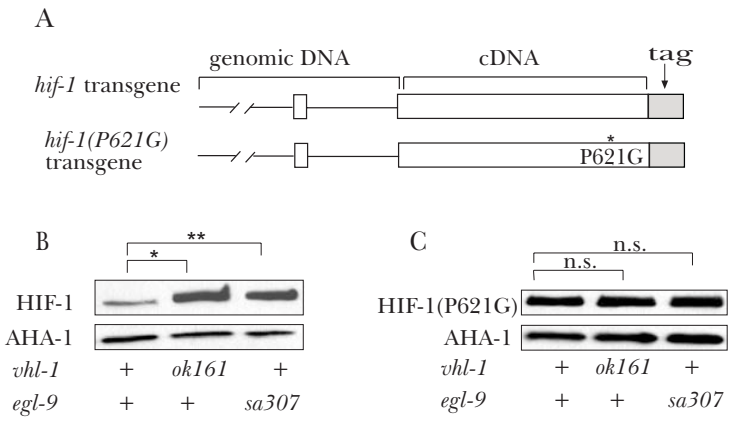

FIGURE S2.—The HIF-1 (P621G) mutation stabilizes HIF-1 protein. A) Diagrams of minigenes that direct expression of epitope-tagged HIF-1 or HIF-1 (P621G). These transgenes are described as *Phif-1::hif-1::tag* and *Phif-1::hif-1 (p621G)::tag* Boxes represent translated regions. The asterisk indicates sequence encoding proline 621, which was mutated to code for glycine in the P621G construct. B,C) Representative protein blots to analyze the expression of epitope-tagged HIF-1 or HIF-1 (P621G) in transgenic animals. Each strain shown carried the *hif-1 (ia04)* deletion allele, and HIF-1 function was restored by an integrated copy of one of the *hif-1* transgenes. Blots were probed with antibodies that recognize the myc epitope. B) Regulation of epitope-tagged HIF-1 by *egl-9* and *vhl-1* in transgenic *C. elegans*. *egl-9 (sa307)* and *vhl-1 (ok161)* are strong loss-of-function alleles, and □+□ represents the allele present in wild-type *C. elegans* (Bristol N2). AHA-1 protein levels are unchanged in these mutant backgrounds. C) The P621G mutation prevented *vhl-1* and *egl-9*-mediated destabilization of HIF-1 protein. Data from at least three independent biological replicates were analyzed to determine whether loss-of-function mutations in *vhl-1* or *egl-9* resulted in statistically significant differences in HIF-1 or HIF-1 (P621G) protein levels. n.s.: no significant difference; *: $p<0.05$; **:$p<0.01$

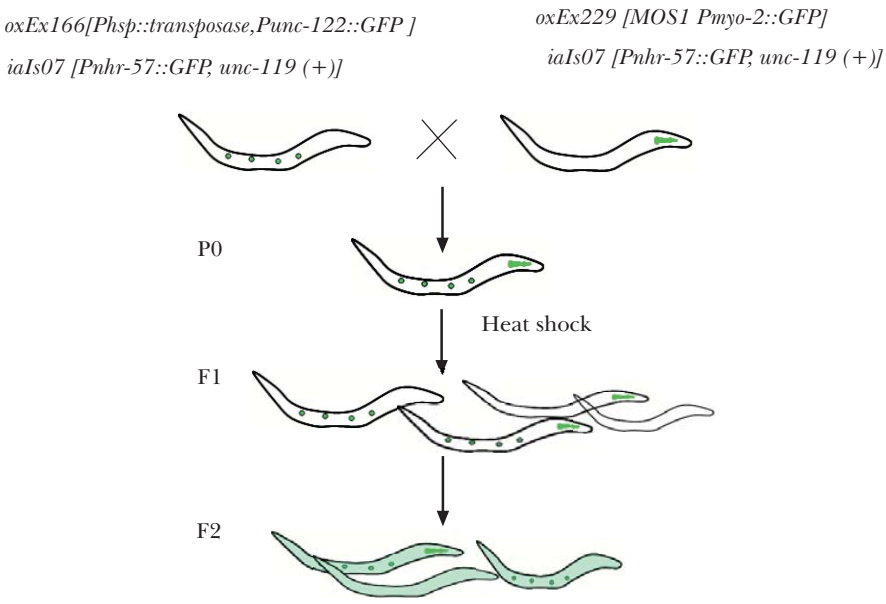

FIGURE S3.—Diagram describing the MOS1-mediated screen to identify alleles that caused over-expression of *Pnhr-57*::GFP. The extrachromosomal array that included the MOS1 transposon also carried the *Pmyo-2*::GFP pharyngeal marker. The array containing *Phsp::transposase* included the *Punc-122*::GFP gene, which labels coelomocytes. Both starting strains carried the *Pnhr-57*::GFP reporter. F1 eggs that were laid between 18-40 hours after heatshock treatment were collected. Animals over-expressing the HIF-1 reporter were isolated. Inverse PCR and complementation tests with existing *egl-9* alleles were used to map the mutation site.

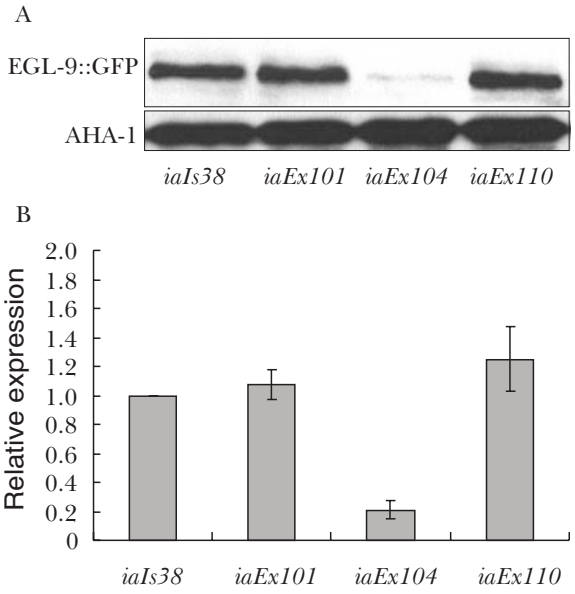

FIGURE S4.—Relative expression levels of epitope-tagged EGL-9 protein in transgenic lines. Protein blots were probed with primary antibodies recognizing the GFP tag on the *egl-9::*GFP and *egl-9 (H487A)::*GFP transgenes and AHA-1, which was assayed as a loading control. While the expression of EGL-9 protein I iaEx104 is about 3 folds lower than in *iaIs38, iaEx101,* and *iaEx110* transgenes, *iaEx104* suppresses *hif-1* targets as well (Figure 4). This suggests that there may be a threshold level o fEGL-9 required for rescue, and that this threshold is achieved by all 4 strains. These data are shown as a representative blot (A) and a bar chart summarizing three biological replicates (B).

FILE S1

Supporting Methods

Construction of egl-9 and hif-1 plasmids: To create *Pegl-9::egl-9::tag*, 1.6 kb of sequence 5' to the *egl-9* translational start was amplified and cloned into the pPD95.75 vector (from Andrew Fire) (primers: CGCGCATGCGTGTATGTGTGTGAAAGAG and GCGGTCGACGCAACTTTTTTCTGTCACATTCAG). The remaining coding sequence for *egl-9* (exons 3 to end) was amplified from cDNA, using primers 5'GCGGTCGACCACATGACATGAGCAGTGCCCCAAATG and 5'GCGGGATCCGAGATGTAATACTCTGGGTTTGTGGAAGG. The resulting fragment was digested with *Sal*I and *Bam*HI and inserted into the construct containing the *egl-9* promoter. Finally, the genomic sequence including the first three *egl-9* exons was amplified (primers: 5'GCGGTCGACCACATGACATGAGCAGTGCCCCAAATG and 5'GGATTGGAATCGATGGCTCTGG) and added to the construct using *Sal*I and *Cla*I restriction sites. *Pegl-9::egl-9(H487A)::tag* was made by mutating codon His487 to encode alanine.

The *Phif-1::hif-1::tag* construct contains 5.2 kb of *hif-1* 5' regulatory sequence, the genomic sequence for the first exon and first intron of *hif-1*, and cDNA sequence for exons 2 - 9. The cDNA sequence is from the predominant *hif-1* mRNA isoform *(hif-1a)*. This construct also includes an epitope tag that consists of one copy of HA and five copies of c-myc (from clone CD3-128, Arabidopsis Biology Resource Center), inserted in frame after the *hif-1* coding sequences. The coding sequences are followed by a stop codon and 400 bp of genomic sequence 3' to the *hif-1* coding region. To create the *Phif-1::hif-1 (P621G)::tag* construct, the codon for proline 621 was modified to encode glycine, and the HA sequence was removed from the epitope tag.

Hypoxia treatment and protein blots: To achieve 0.5% oxygen, nitrogen was mixed with room air. To assess the levels of HIF-1 or *Pnhr-57*::GFP, 40 – 100 synchronized L4-stage animals were boiled in 10ul M9 and 10ul 2x SDS buffer [50mM Tris-HCl PH6.8, 10%(v/v) glycerol, 2% (w/v) SDS, 100mM DTT, 0.1% (w/v) bromphenol blue] for 10 minutes before loading to 10% or 12% polyacrylamide gels. P*nhr-57*::GFP is expressed at much higher levels in animals carrying strong loss-of-function mutations in *egl-9*, and a smaller fraction of the lysate was loaded in each lane of the gel (the equivalent of 4 animals). After separation, proteins were transferred to nitrocellulose membranes. The blots were probed with monoclonal antibodies recognizing the following epitopes: GFP (antibody from Roche at 1:1000 dilution); HA (antibody from Cell Signaling Technology clone 6E2 at 1:1000 dilution); myc (mouse ascites, clone 9E10, from the Developmental Studies Hybridoma Bank at 1:1000 dilution), or AHA-1 (JIANG *et al.* 2001) (1:100 dilution). The secondary antibody (goat anti-mouse IgG+IgM from Biorad) was diluted 1:2000. The relative intensity of protein bands were quantified by Image J software.

TABLE S1

Descriptions of mutant alleles used in this study

Gene name	Allele name	Description	References
hif-1	*ia04*	deletion, loss of function	(JIANG et al. 2001)
vhl-1	*ok161*	deletion, loss of function	(EPSTEIN et al. 2001)
egl-9	*sa307*	deletion, loss of function	(DARBY et al. 1999)
	ok478	deletion, loss of function	*
	gk277	deletion, weak loss of function	*
	sa330	loss of function	(DARBY et al. 1999)
	ia58	loss of function	This study
	ia60	loss of function	This study
	ia61	loss of function	This study
unc-119	*ed3*	loss of function	(MADURO and PILGRIM 1995)

* These alleles were generated by the *C. elegans* knock-out consortium.

References for Table S1

DARBY, C., C. L. COSMA, J. H. THOMAS and C. MANOIL, 1999 Lethal paralysis of *Caenorhabditis elegans* by *Pseudomonas aeruginosa*. Proc Natl Acad Sci U S A **96:** 15202-15207.

EPSTEIN, A. C., J. M. GLEADLE, L. A. MCNEILL, K. S. HEWITSON, J. O'ROURKE *et al.*, 2001 *C. elegans* EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell **107:** 43-54.

JIANG, H., R. GUO and J. A. POWELL-COFFMAN, 2001 The *Caenorhabditis elegans hif-1* gene encodes a bHLH-PAS protein that is required for adaptation to hypoxia. Proc Natl Acad Sci U S A **98:** 7916-7921.

MADURO, M., and D. PILGRIM, 1995 Identification and cloning of *unc-119*, a gene expressed in the *Caenorhabditis elegans* nervous system. Genetics **141:** 977-988.

TABLE S2

Transgenes used in this study

Construct assayed *	Integration event or array	Description and co-transformation marker **
Pnhr-57::GFP	*iaIs07*	Integrated (SHEN et al. 2006)
Phif-1::hif-1::tag	*iaIs28*	Integrated with *unc-119* (+)
Phif-1::hif-1	*iaIs32*	Integrated with *unc-119* (+)
(P621G)::tag	*iaIs33*	Integrated with *unc-119* (+)
	iaIs34	Integrated with *unc-119* (+)
Pegl-9::egl-9::tag	*iaIs38*	Integrated with *unc-119* (+)
Pegl-9::egl-9	*iaEx101*	Extrachromosomal array with *unc-119* (+)
(H487)::tag	*iaEx104*	Extrachromosomal array with *unc-119* (+)
	iaEx110	Extrachromosomal array with *unc-119* (+)

***** The promoter is listed first, followed by the coding sequences.

****** Integration events were achieved by microparticle bombardment (PRAITIS *et al.* 2001) and were backcrossed at least four times prior to further characterization.

References for Table S2

PRAITIS, V., E. CASEY, D. COLLAR and J. AUSTIN, 2001 Creation of low-copy integrated transgenic lines in *Caenorhabditis elegans*. Genetics **157:** 1217-1226.

SHEN, C., Z. SHAO and J. A. POWELL-COFFMAN, 2006 The *Caenorhabditis elegans rhy-1* gene inhibits HIF-1 hypoxia-inducible factor activity in a negative feedback loop that does not include *vhl-1*. Genetics **174:** 1205-1214.

TABLE S3

Strains described in this study

Strain	Genotype
ZG228[a]	*unc-119 (ed3)* III; *hif-1 (ia04)* V
ZG430[a]	*iaIs07*[*Pnhr-57::gfp unc-119 (+)*] IV; *egl-9 (sa307), hif-1 (ia04)* V; *iaIs28*[*Phif-1::hif-1a::tag unc-119 (+)*]
ZG432[a]	*iaIs07*[*Pnhr-57::gfp unc-119 (+)*] IV; *hif-1 (ia04)* V; *vhl-1 (ok161)* X; *iaIs28*[*Phif-1::hif-1a::tag unc-119 (+)*]
ZG433[a]	*iaIs07*[*Pnhr-57::gfp unc-119 (+)*] IV; *hif-1 (ia04)* V; *iaIs28* [*Phif-1::hif-1a::tag unc-119 (+)*]
ZG551[a]	*iaIs07*[*Pnhr-57::gfp unc-119 (+)*] IV; *egl-9 (sa307), hif-1 (ia04)* V; *iaIs32*[*Phif-1::hif-1a(P621G)::tag unc-119 (+)*]
ZG552[a]	*iaIs07*[*Pnhr-57::gfp unc-119 (+)*] IV; *hif-1 (ia04)* V; *iaIs32*[*Phif-1::hif-1a(P621G)::tag unc-119 (+)*]
ZG553[a]	*iaIs07*[*Pnhr-57::gfp unc-119 (+)*] IV; *hif-1 (ia04)* V; *vhl-1 (ok161)* X; *iaIs32* [*Phif-1::hif-1a(P621G)::tag unc-119 (+)*]
ZG555[a]	*iaIs07*[*Pnhr-57::gfp unc-119 (+)*] IV; *egl-9 (sa307), hif-1 (ia04)* V; *iaIs33* [*Phif-1::hif-1a(P621G)::tag unc-119 (+)*]
ZG557[a]	*iaIs07*[*Pnhr-57::gfp unc-119 (+)*] IV; *egl-9 (sa307), hif-1 (ia04)* V; *iaIs34* [*Phif-1::hif-1a(P621G)::tag unc-119 (+)*]
ZG677[a]	*iaIs07*[*Pnhr-57::gfp unc-119 (+)*] IV; *hif-1 (ia04)* V; *iaIs33* [*Phif-1::hif-1a(P621G)::tag unc-119 (+)*]
ZG678[a]	*iaIs07*[*Pnhr-57::gfp unc-119 (+)*] IV; *hif-1 (ia04)* V; *vhl-1 (ok161)* X; *iaIs33* [*Phif-1::hif-1a(P621G)::tag unc-119 (+)*]
ZG679[a]	*iaIs07*[*Pnhr-57::gfp unc-119 (+)*] IV; *hif-1 (ia04)* V; *iaIs34* [*Phif-1::hif-1a(P621G)::tag unc-119 (+)*]
ZG680[a]	*iaIs07*[*Pnhr-57::gfp unc-119 (+)*] IV; *hif-1 (ia04)* V; *vhl-1 (ok161)* X; *iaIs34* [*Phif-1::hif-1a(P621G)::tag unc-119 (+)*]
ZG119[b]	*iaIs07*[*Pnhr-57::gfp unc-119 (+)*] IV; *vhl-1 (ok161)* X
ZG120[b]	*iaIs07*[*Pnhr-57::gfp unc-119 (+)*] IV
ZG302[b]	*iaIs07*[*Pnhr-57::gfp unc-119 (+)*] IV; *egl-9 (sa307)* V
ZG430[b]	*iaIs07*[*Pnhr-57::gfp unc-119 (+)*] IV; *egl-9 (sa307), hif-1 (ia04)* V; *iaIs28*[*Phif-1::hif-1a::tag unc-119 (+)*]
ZG432[b]	*iaIs07*[*Pnhr-57::gfp unc-119 (+)*] IV; *hif-1 (ia04)* V; *vhl-1 (ok161)* X; *iaIs28*[*Phif-1::hif-1a::tag*, *unc-119 (+)*]
ZG433[b]	*iaIs07*[*Pnhr-57::gfp unc-119 (+)*] IV; *hif-1 (ia04)* V; *iaIs28* [*Phif-1::hif-1a::tag unc-119 (+)*]
ZG399[c]	*iaIs07*[*Pnhr-57::gfp unc-119 (+)*] IV; *oxEx166*[*hspp::Mostransposase lin-15 (+) Punc-122::gfp*]
ZG405[c]	*iaIs07*[*Pnhr-57::gfp unc-119 (+)*] IV; *oxEx229*[*Mos1 myo-2::gfp*]
ZG305[d]	*unc-119 (ed3)* III; *egl-9 (sa307)* V
ZG494[d]	*unc-119 (ed3)* III; *egl-9 (sa307)* V; *iaIs38*[*Pegl-9::egl-9::tag unc-119 (+)*]
ZG676[d]	*iaIs07*[*Pnhr-57::gfp unc-119 (+)*] IV; *egl-9 (sa307), hif-1 (ia04)* V; *iaIs38*[*Pegl-9::egl-9::tag unc-119 (+)*]
ZG681[d]	*iaIs07*[*Pnhr-57::gfp unc-119 (+)*] IV; *egl-9 (sa307)* V; *vhl-1 (ok161)* X; *iaIs38*[*Pegl-9::egl-9::tag unc-119 (+)*]
ZG682[d]	*iaIs07*[*Pnhr-57::gfp unc-119 (+)*] IV; *egl-9 (sa307), hif-1 (ia04)* V; *iaIs38*[*Pegl-9::egl-9::tag unc-119 (+)*], *iaIs28* [*Phif-1::hif-1a::tag unc-119 (+)*]
ZG686[d]	*unc-119 (ed3)* III; *egl-9 (sa307)* V; *iaEx101* [*Pegl-9::egl-9 (H487A)::tag unc-119 (+)*]
ZG691[d]	*unc-119 (ed3)* III; *egl-9 (sa307)* V; *iaEx104* [*Pegl-9::egl-9 (H487A)::tag unc-119 (+)*]
ZG696[d]	*iaIs07*[*Pnhr-57::gfp unc-119 (+)*] IV; *egl-9 (sa307)* V; *iaIs38*[*Pegl-9::egl-9::tag unc-119 (+)*]
ZG700[d]	*iaIs07*[*Pnhr-57::gfp unc-119 (+)*] IV; *egl-9 (sa307), hif-1 (ia04)* V; *iaEx104*[*Pegl-9::egl-9 (H487A)::tag unc-119 (+)*]
ZG701[d]	*iaIs07*[*Pnhr-57::gfp unc-119 (+)*] IV; *egl-9 (sa307), hif-1 (ia04)* V; *iaIs28* [*Phif-1::hif-1a::tag unc-119 (+)*] *iaEx110*[*Pegl-9::egl-9 (H487A)::tag unc-119 (+)*]
ZG702[d]	*unc-119 (ed3)* III; *egl-9 (sa307)* V; *iaEx110*[*Pegl-9::egl-9 (H487A)::tag unc-119 (+)*]
ZG704[d]	*iaIs07*[*Pnhr-57::gfp unc-119 (+)*] IV; *egl-9 (sa307)* V; *iaEx110*[*Pegl-9::egl-9 (H487A)::tag unc-119 (+)*]
ZG707[d]	*iaIs07*[*Pnhr-57::gfp unc-119 (+)*] IV; *egl-9 (sa307)* V; *iaEx104* [*Pegl-9::egl-9 (H487A)::tag unc-119 (+)*]
ZG708[d]	*iaIs07*[*Pnhr-57::gfp unc-119 (+)*] IV; *egl-9 (sa307), hif-1 (ia04)* V; *iaIs28* [*Phif-1::hif-1a::tag unc-119 (+)*] *iaEx101*[*Pegl-9::egl-9 (H487A)::tag unc-119 (+)*]

ZG443[e]	*iaIs07*[*Pnhr-57::gfp unc-119 (+)*] IV; *egl-9 (ia58)* V
ZG444[e]	*iaIs07*[*Pnhr-57::gfp unc-119 (+)*] IV; *egl-9 (gk277)* V
ZG447[e]	*iaIs07*[*Pnhr-57::gfp unc-119 (+)*] IV; *egl-9 (gk277)* V; *vhl-1 (ok161)* X
ZG448[e]	*iaIs07*[*Pnhr-57::gfp unc-119 (+)*] IV; *egl-9 (ia60)* V
ZG449[e]	*iaIs07*[*Pnhr-57::gfp unc-119 (+)*] IV; *egl-9 (ia61)* V
ZG457[e]	*iaIs07*[*Pnhr-57::gfp unc-119 (+)*] IV; *egl-9 (gk277), hif-1 (ia04)* V; *iaIs28* [*Phif-1::hif-1a::tag unc-119 (+)*]
ZG458[e]	*iaIs07*[*Pnhr-57::gfp unc-119 (+)*] IV; *egl-9 (gk277), hif-1 (ia04)* V; *vhl-1 (ok161)* X; *iaIs28* [*Phif-1::hif-1a::tag unc-119 (+)*]
ZG492[e]	*iaIs07*[*Pnhr-57::gfp unc-119 (+)*] IV; *egl-9 (ok478)* V
ZG493[e]	*iaIs07*[*Pnhr-57::gfp unc-119 (+)*] IV; *egl-9 (sa330)* V
ZG671[e]	*iaIs07*[*Pnhr-57::gfp unc-119 (+)*] IV; *egl-9 (ia58), hif-1 (ia04)* V; *iaIs28* [*Phif-1::hif-1a::tag unc-119 (+)*]
ZG672[e]	*iaIs07*[*Pnhr-57::gfp unc-119 (+)*] IV; *egl-9 (ia60), hif-1 (ia04)* V; *iaIs28* [*Phif-1::hif-1a::tag unc-119 (+)*]
ZG673[e]	*iaIs07*[*Pnhr-57::gfp unc-119 (+)*] IV; *egl-9 (ia61), hif-1 (ia04)* V; *iaIs28* [*Phif-1::hif-1a::tag unc-119 (+)*]
ZG674[e]	*iaIs07*[*Pnhr-57::gfp unc-119 (+)*] IV; *egl-9 (ok478), hif-1 (ia04)* V; *iaIs28* [*Phif-1::hif-1a::tag unc-119 (+)*]
ZG675[e]	*iaIs07*[*Pnhr-57::gfp unc-119 (+)*] IV; *egl-9 (sa330), hif-1 (ia04)* V; *iaIs28* [*Phif-1::hif-1a::tag unc-119 (+)*]

[a] Strains used to compare the regulation and function of HIF-1 and HIF-1 (P621G)
[b] Strains used to analyze the effects of hypoxia and DIP
[c] Strains used in Mos1 mutagenesis
[d] Strains to compare the regulation and functions of EGL-9 and EGL-9 (H487A)
[e] Additional strains generated to characterize new and existing *egl-9* loss-of-function alleles