
Figures and figure supplements

Hypoxia-inducible factor induces cysteine dioxygenase and promotes cysteine homeostasis in *Caenorhabditis elegans*

Kurt Warnhoff et al.

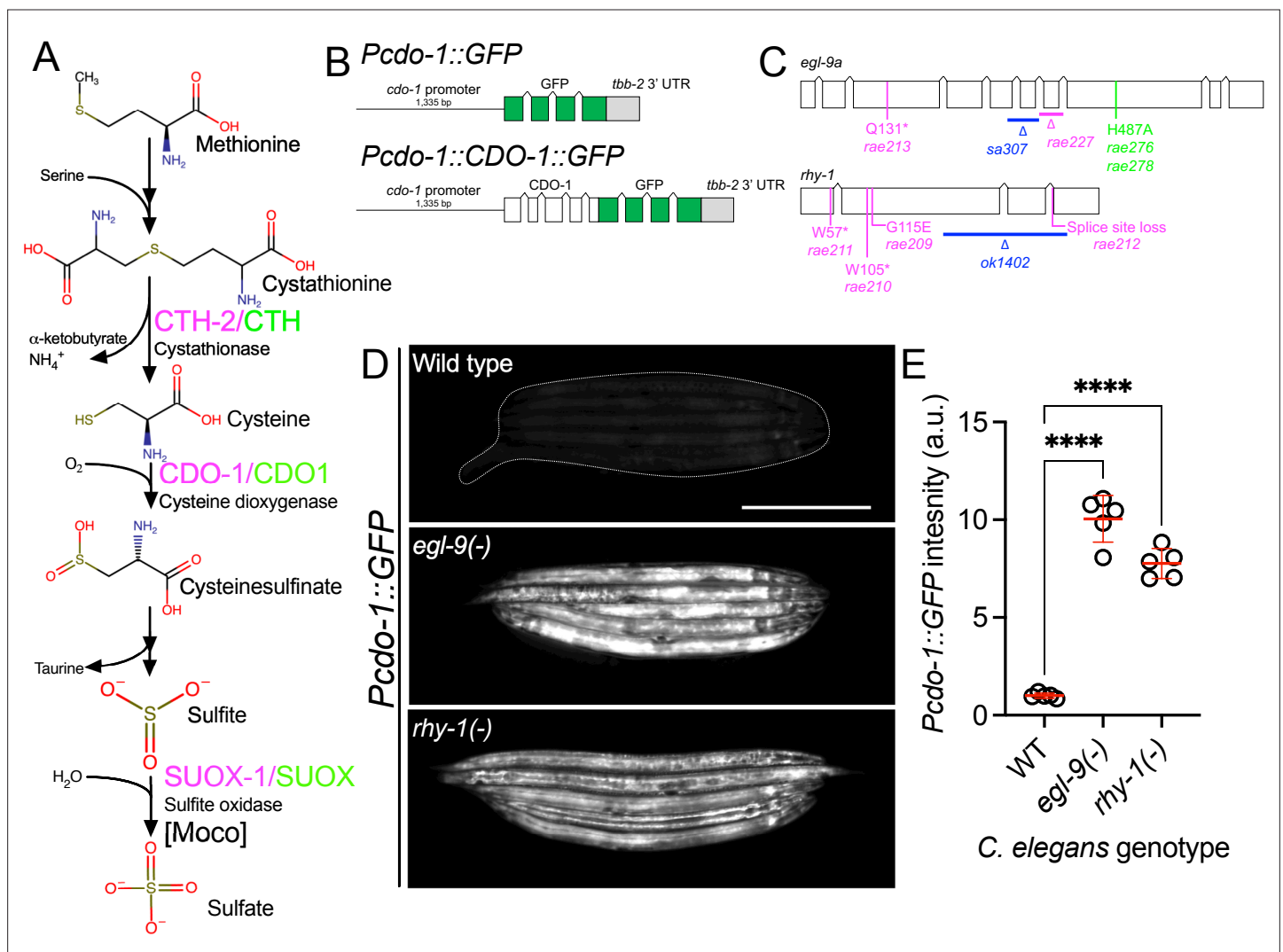


Figure 1. *egl-9* and *rhy-1* inhibit *cdo-1* transcription. (A) Pathway for sulfur amino acid metabolism beginning with methionine. We highlight the roles of cystathionase (CTH-2/CTH), cysteine dioxygenase (CDO-1/CDO1), and the Moco-requiring sulfite oxidase enzyme (SUOX-1/SUOX). *C. elegans* enzymes (magenta) and their human homologs (green) are displayed. (B) *Pcd-1::GFP* promoter fusion (upper) and *Pcd-1::CDO-1::GFP* C-terminal protein fusion (lower) transgenes used in this work are displayed. Boxes indicate exons, connecting lines indicate introns. The *cdo-1* promoter is shown as a straight line. (C) *egl-9a* and *rhy-1* gene structures. Boxes indicate exons and connecting lines are introns. Colored annotations indicate mutations generated or used in our work. Magenta; chemically-induced mutations that activated *Pcd-1::CDO-1::GFP* fusion protein. Blue; reference null alleles isolated independent of our work. Green; CRISPR/Cas9-generated mutation that inactivates the prolyl hydroxylase domain of EGL-9. (D) Expression of *Pcd-1::GFP* transgene is displayed for wild-type, *egl-9(sa307)*, and *rhy-1(ok1402)* *C. elegans* animals at the L4 stage. Scale bar is 250 μ m. White dotted line outlines animals with basal GFP expression. For GFP imaging, exposure time was 100ms. (E) Quantification of GFP expression displayed in (D). Individual datapoints are shown (circles) as are the mean and standard deviation (red lines). *n* is 5 individuals per genotype. Data are normalized so that wild-type expression of *Pcd-1::GFP* is 1 arbitrary unit (a.u.). ****, $p < 0.0001$, ordinary one-way ANOVA with Dunnett's post hoc analysis.

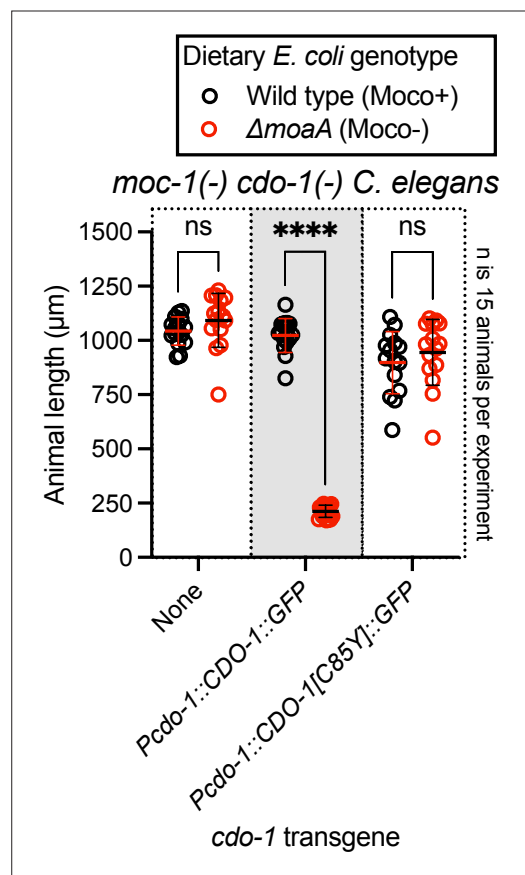


Figure 1—figure supplement 1. The *Pcdo-1::CDO-1::GFP* transgene encodes a functional cysteine dioxygenase enzyme. *moc-1(ok366) cdo-1(mg622)* double mutant animals expressing *Pcdo-1::CDO-1::GFP* or *Pcdo-1::CDO-1[C85Y]::GFP* transgenes were cultured from synchronized L1 larvae for 72 hr on wild-type (black, Moco+) or $\Delta moaA$ mutant (red, Moco-) *E. coli*. Animal lengths were determined for each condition. Individual datapoints are shown (circles) as are the mean and standard deviation. Sample size (n) is displayed for each experiment. ****, $p < 0.0001$, multiple unpaired t test with Welch's correction. ns indicates no significant difference was identified.

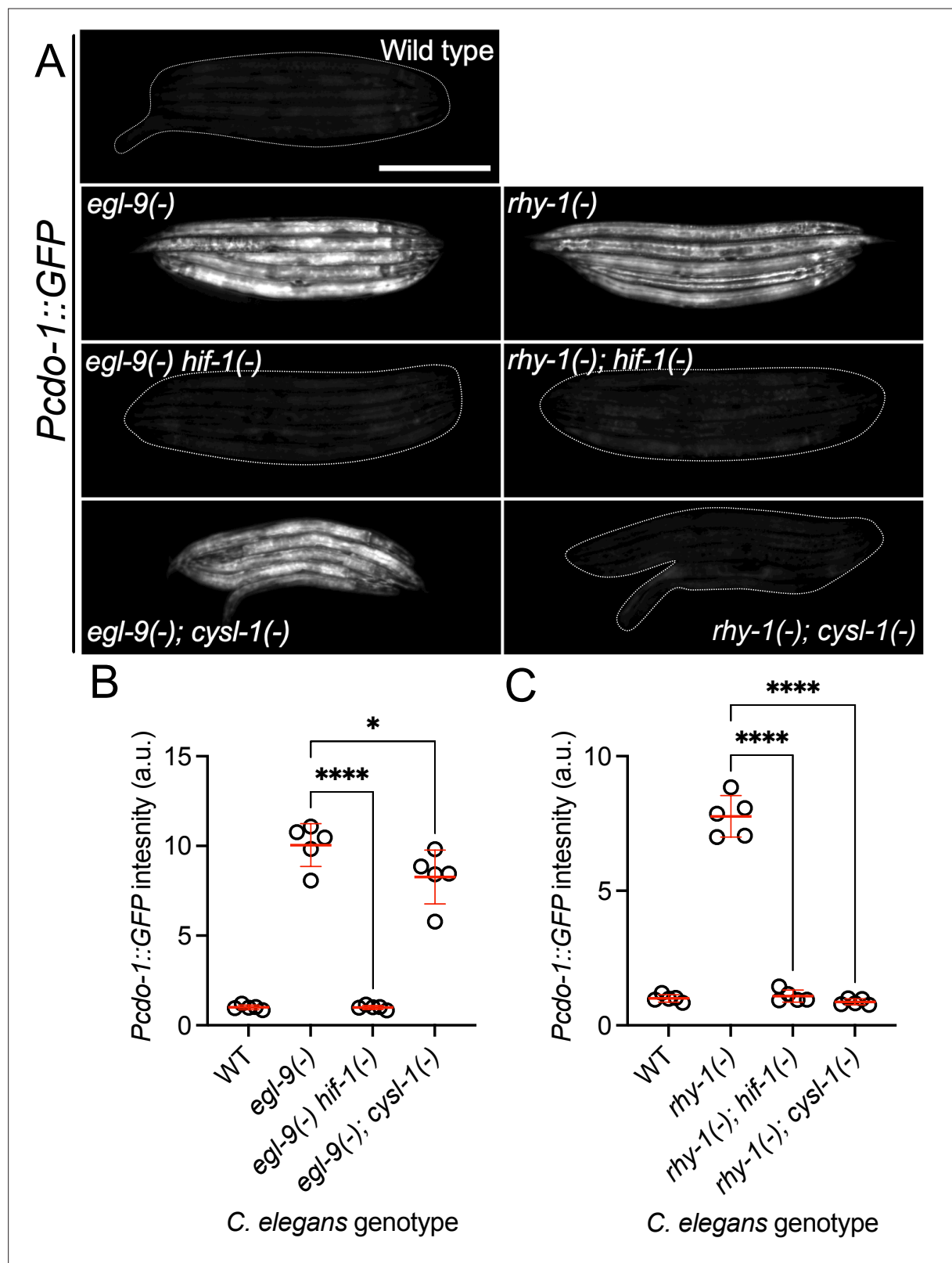


Figure 2. *cdo-1* transcription is activated by HIF-1 downstream of RHY-1, CYSL-1, and EGL-9. (A) Expression of the *Pcdo-1::GFP* transgene is displayed for wild-type, *egl-9(sa307)*, *egl-9(sa307) hif-1(ia4)* double mutant, *egl-9(sa307); cysl-1(ok762)* double mutant, *rhy-1(ok1402)*, *rhy-1(ok1402); hif-1(ia4)* double mutant, and *rhy-1(ok1402); cysl-1(ok762)* double mutant *C. elegans* animals at the L4 stage of development. Scale bar is 250 μm. White dotted line outlines animals with basal GFP expression. For GFP imaging, exposure time was 100ms. (B, C) Quantification of the data displayed in (A). Individual

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datapoints are shown (circles) as are the mean and standard deviation (red lines). *n* is 5 individuals per genotype. Data are normalized so that wild-type expression of *Pcdo-1::GFP* is 1 arbitrary unit (a.u.). *, $p < 0.05$, ****, $p < 0.0001$, ordinary one-way ANOVA with Dunnett's post hoc analysis. Note, wild-type, *egl-9(-)*, and *rhy-1(-)* images in panel A and quantification of *Pcdo-1::GFP* in panels B and C are identical to the data presented in (**Figure 1D and E**). They are re-displayed here to allow for clear comparisons to the double mutant strains of interest.

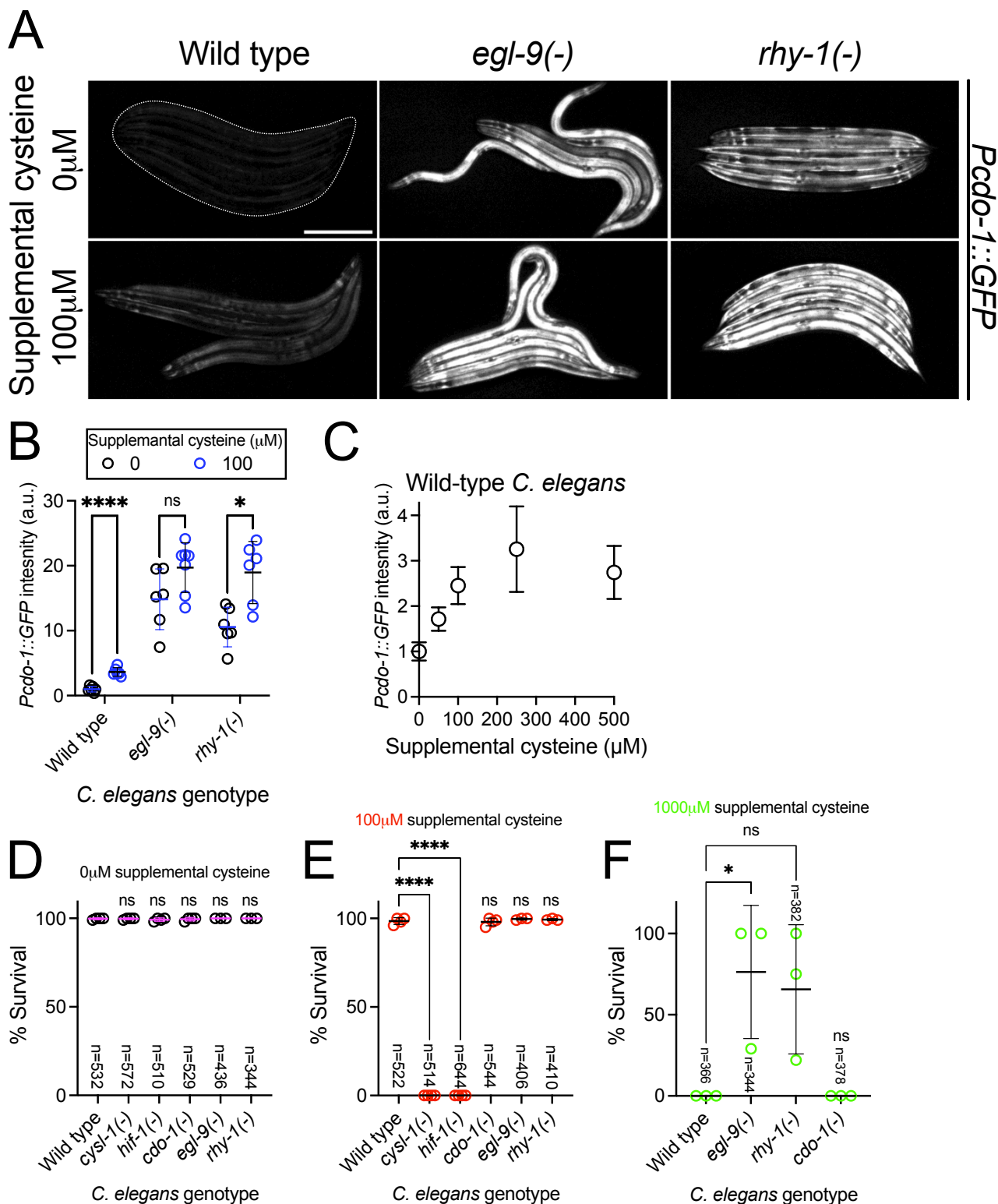


Figure 3. High levels of cysteine activate *cdo-1* transcription and are lethal to *hif-1* and *cysl-1* mutant animals. **(A)** Expression of the *Pcdo-1::GFP* transgene is displayed for wild-type, *egl-9(sa307)*, and *rhy-1(ok1402)* young-adult *C. elegans* exposed to 0 or 100 μ M supplemental cysteine. Scale bar is 250 μ m. White dotted line outlines animals with basal GFP expression. For GFP imaging, exposure time was 100ms. Supplemental cysteine did not impact the mortality of the animals being imaged. **(B)** Quantification of the data displayed in **(A)**. Individual datapoints are shown (circles) as are the

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mean and standard deviation (black lines). *n* is 6 or 7 individuals per genotype. Data are normalized so that expression of *Pcdo-1::GFP* in wild-type *C. elegans* exposed to 0 μ M supplemental cysteine is equal to 1 arbitrary unit (a.u.). *, $p < 0.05$, ****, $p < 0.0001$, multiple unpaired t test with Welch's correction. **(C)** Quantification of the *Pcdo-1::GFP* expression is displayed for wild-type young-adult *C. elegans* exposed to 0, 50, 100, 250, or 500 μ M supplemental cysteine. Mean and standard deviation are displayed. *n* is 6 or 7 individuals per concentration of supplemental cysteine. **(D–F)** The percentage of animals that survive overnight exposure to **(D)** 0, **(E)** 100, or **(F)** 1000 μ M supplemental cysteine. Individual datapoints (circles) represent biological replicates. Three or four biological replicates were performed for each experiment and the total individuals scored amongst all replicates is displayed (*n*). *, $p < 0.05$, ****, $p < 0.0001$, ordinary one-way ANOVA with Dunnett's post hoc analysis. ns indicates no significant difference was identified.

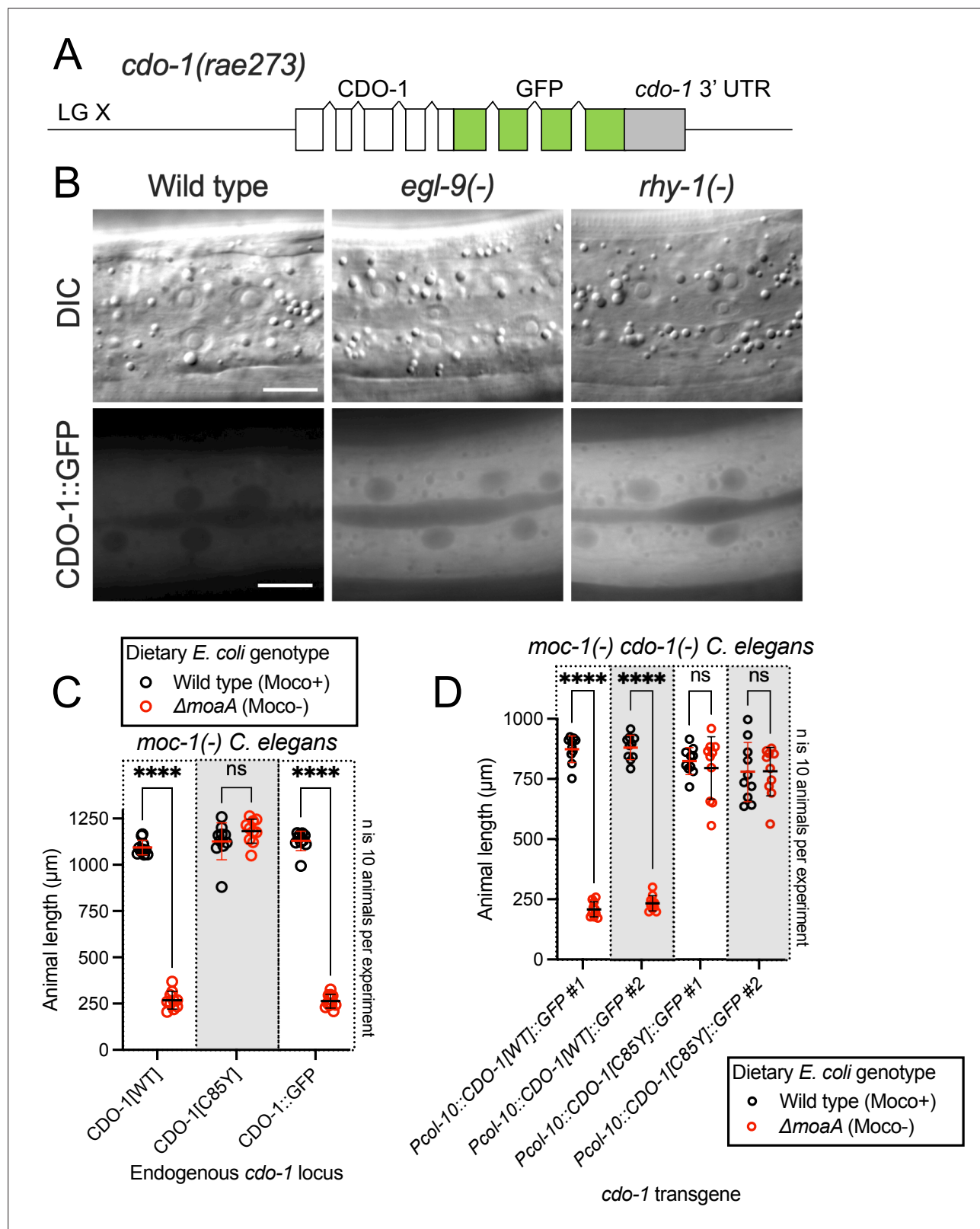


Figure 4. Hypodermal CDO-1 accumulates in the cytoplasm when *egl-9* or *rhy-1* are inactive and is sufficient to promote sulfur amino acid metabolism. (A) Diagram of *cdo-1(rae273)*, a CRISPR/Cas9-generated allele with GFP inserted into the *cdo-1* gene, creating a functional C-terminal CDO-1::GFP fusion protein expressed from the native *cdo-1* locus. (B) Differential interference contrast (DIC) and fluorescence imaging are shown for wild-type, *egl-9(sa307)*, and *rhy-1(ok1402)* *C. elegans* expressing CDO-1::GFP encoded by *cdo-1(rae273)*. Scale bar is 10 μm . For GFP imaging, exposure time was

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200 ms. An anterior segment of the Hyp7 hypodermal cell is displayed. CDO-1::GFP accumulates in the cytoplasm and is excluded from the nuclei.

(C) *moc-1(ok366)*, *moc-1(ok366) cdo-1(mg622)*, and *moc-1(ok366) cdo-1(rae273)* animals were cultured from synchronized L1 larvae for 72 hr on wild-type (black, Moco+) or $\Delta moaA$ mutant (red, Moco-) *E. coli*. (D) *moc-1(ok366) cdo-1(mg622)* double mutant animals expressing *Pcol-10::CDO-1::GFP* or *Pcol-10::CDO-1[C85Y)::GFP* transgenes were cultured for 48 hr on wild-type (black, Moco+) or $\Delta moaA$ mutant (red, Moco-) *E. coli*. Two independently derived strains were tested for each transgene. For panels C and D, animal lengths were determined for each condition. Individual datapoints are shown (circles) as are the mean and standard deviation. Sample size (**n**) is 10 individuals for each experiment. ****, $p < 0.0001$, multiple unpaired t test with Welch's correction. ns indicates no significant difference was identified.

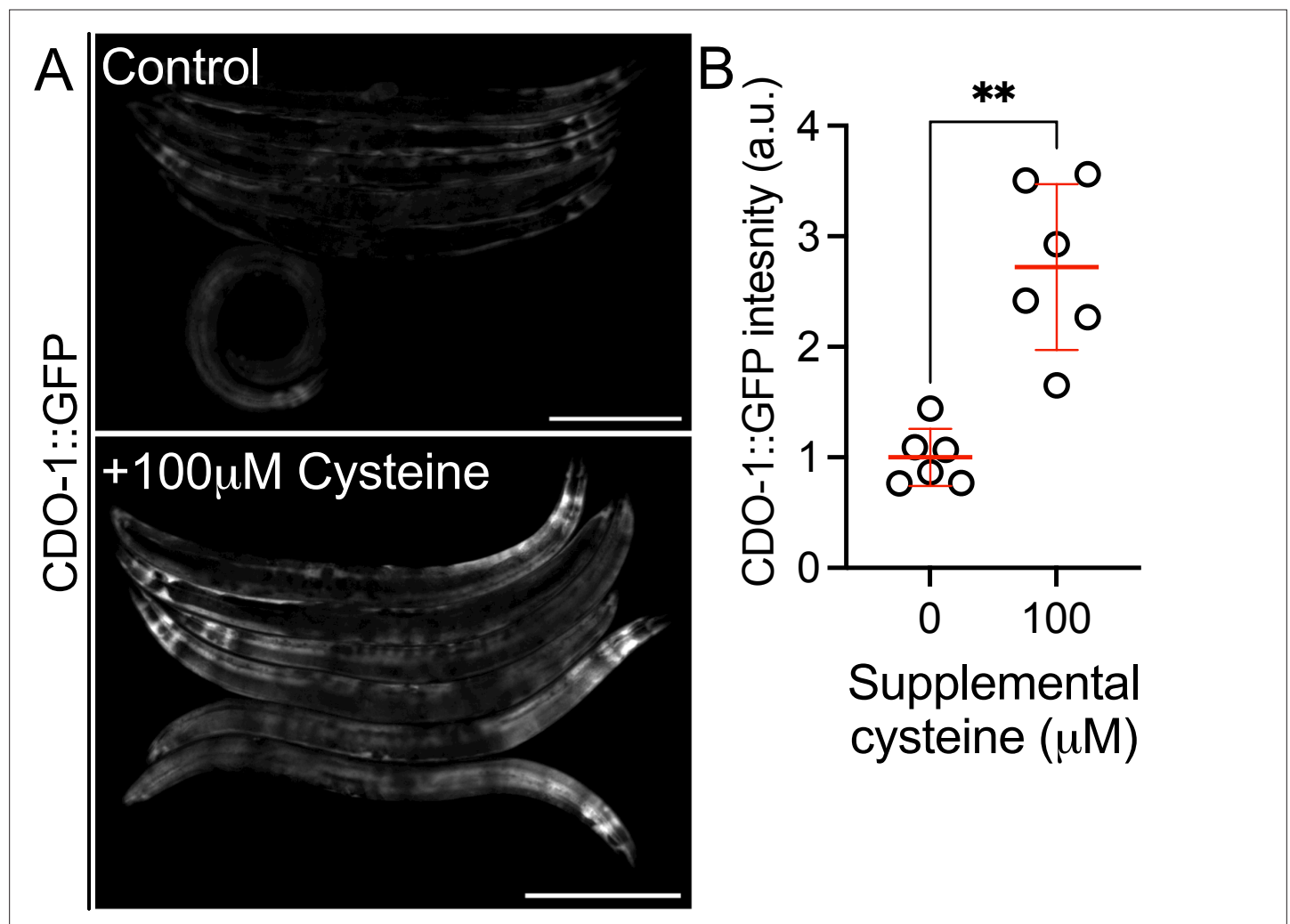
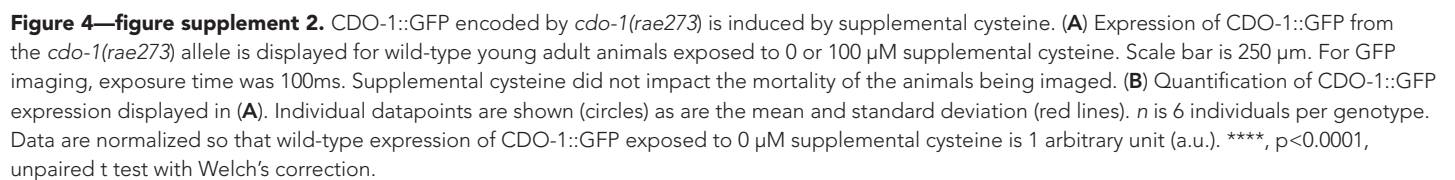


Figure 4—figure supplement 1. A functional CDO-1::GFP fusion protein is induced by loss of *egl-9* or *rhy-1*. **(A)** Expression of CDO-1::GFP from the *cdo-1(rae273)* allele is displayed for wild-type, *egl-9(sa307)*, and *rhy-1(ok1402)* animals at the L4 stage of development. Scale bar is 250 μ m. For GFP imaging, exposure time was 100ms. **(B)** Quantification of CDO-1::GFP expression displayed in **(A)**. Individual datapoints are shown (circles) as are the mean and standard deviation (red lines). *n* is 7 individuals per genotype. Data are normalized so that wild-type expression of CDO-1::GFP is 1 arbitrary unit (a.u.). **, $p < 0.01$, ordinary one-way ANOVA with Dunnett's post hoc analysis.



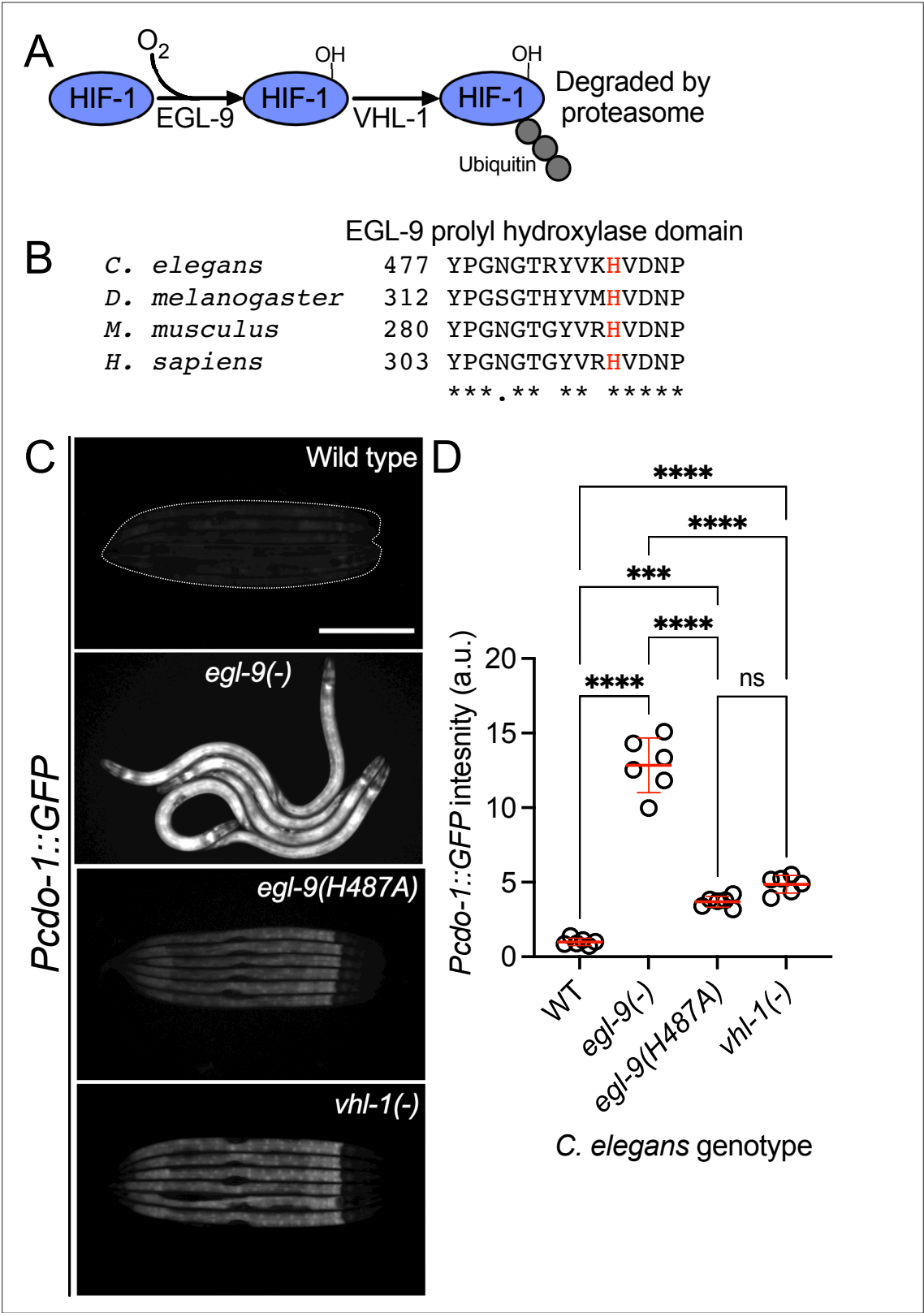


Figure 5. *egl-9* inhibits *cdo-1* transcription in a largely prolyl-hydroxylase and VHL-1-independent manner. **(A)** The pathway of HIF-1 processing during normoxia is displayed. EGL-9 uses O₂ as a substrate to hydroxylate (-OH) HIF-1 on specific proline residues. Prolyl hydroxylated HIF-1 is bound by VHL-1 which facilitates HIF-1 polyubiquitination and targets HIF-1 for degradation by the proteasome. **(B)** Amino acid alignment of the EGL-9 prolyl hydroxylase domain from *C. elegans*, *D. melanogaster*, *M. musculus*, and *H. sapiens*. '*' indicate perfect amino acid conservation while '.' indicates weak conservation. **(C)** Fluorescence microscopy images of *C. elegans* expressing *Pcd-1::GFP* in the wild type, *egl-9(-)*, *egl-9(H487A)*, and *vhl-1(-)* backgrounds. Scale bar represents 100 μm. **(D)** Quantification of *Pcd-1::GFP* intensity (a.u.) in the different genotypes. **** indicates p < 0.0001, ns indicates not significant.

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similarity amongst species compared. Highlighted (red) is the catalytically essential histidine 487 residue in *C. elegans*. Alignment was performed using Clustal Omega (EMBL-EBI). **(C)** Expression of *Pcdo-1::GFP* promoter fusion transgene is displayed for wild-type, *egl-9(sa307, -)*, *egl-9(rae276, H487A)*, and *vhl-1(ok161)* *C. elegans* animals at the L4 stage of development. Scale bar is 250 μ m. White dotted line outlines animals with basal GFP expression. For GFP imaging, exposure time was 500ms. **(D)** Quantification of the data displayed in **(C)**. Individual datapoints are shown (circles) as are the mean and standard deviation (red lines). *n* is 6 individuals per genotype. Data are normalized so that wild-type expression of *Pcdo-1::GFP* is 1 arbitrary unit (a.u.). ***, $p < 0.001$, ****, $p < 0.0001$, ordinary one-way ANOVA with Tukey's multiple comparisons test. ns indicates no significant difference was identified.

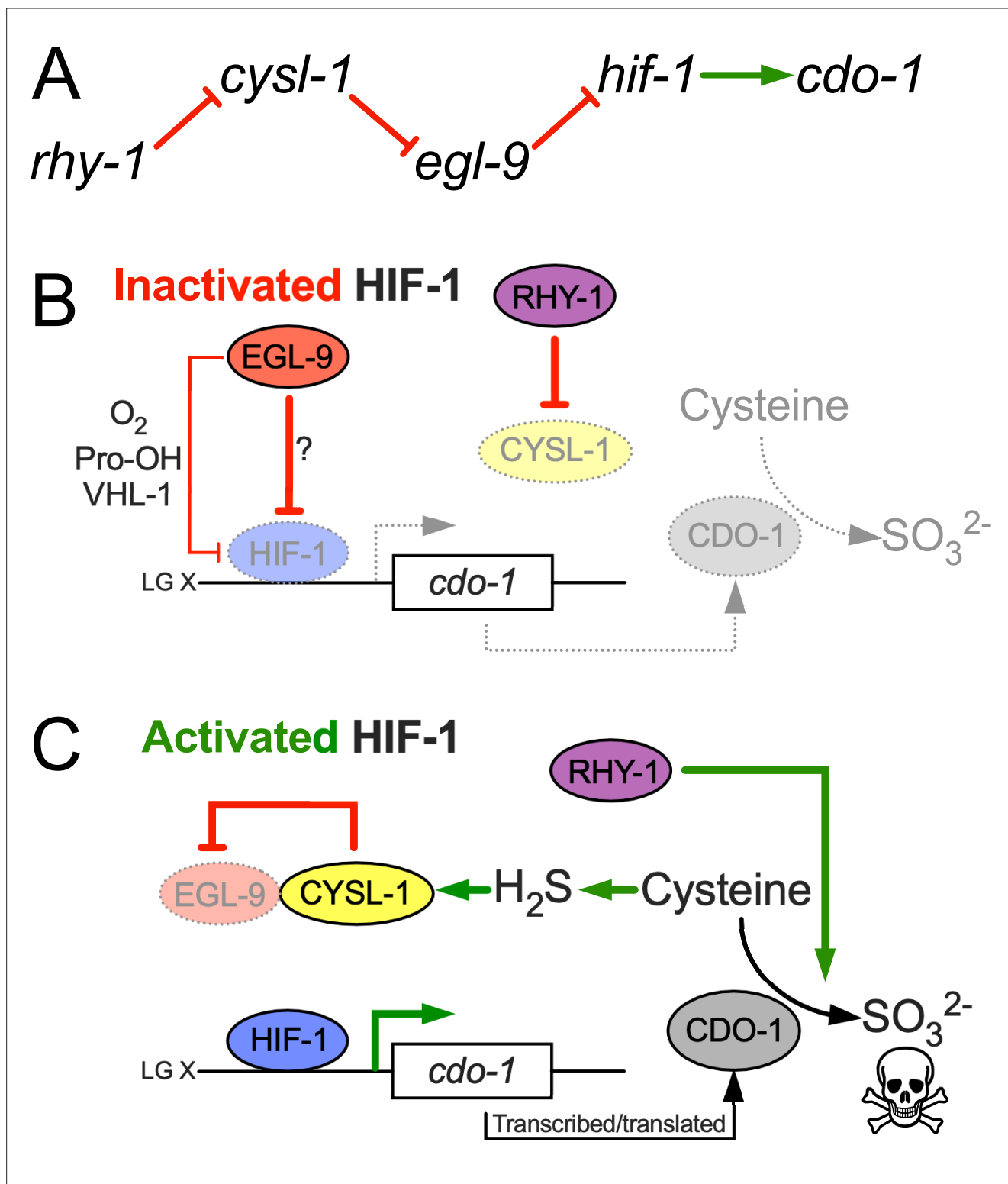


Figure 6. Model for the regulation of cysteine metabolism by HIF-1. (A) Proposed genetic pathway for the regulation of *cdo-1*. *rhy-1*, *cysl-1*, and *egl-9* act in a negative-regulatory cascade to control activity of the HIF-1 transcription factor, which activates transcription of *cdo-1*. (B) Under basal conditions (inactivated HIF-1), EGL-9 negatively regulates HIF-1 through 2 distinct pathways; one pathway is dependent upon O_2 , prolyl hydroxylation (Pro-OH), and VHL-1, while the second acts independently of these canonical factors. Under these conditions *cdo-1* transcription is kept at basal levels and cysteine catabolism is not induced. (C) During conditions where HIF-1 is activated (high H_2S), CYSL-1 directly binds and inhibits EGL-9, preventing HIF-1 inactivation. Active HIF-1 binds the *cdo-1* promoter, driving transcription and promoting CDO-1 protein accumulation. High CDO-1 levels promote

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the catabolism of cysteine leading to production of sulfites (SO_3^{2-}) that are toxic during Moco or SUOX-1 deficiency. HIF-1-induced cysteine catabolism requires the activity of *rhy-1*.