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<tr>
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Cytochrome P450 Drives a HIF-regulated Behavioral Response to Reoxygenation by C. elegans

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Abstract:

Oxygen deprivation followed by reoxygenation causes pathological responses in many disorders, including ischemic stroke, heart attacks and reperfusion injury. Key aspects of ischemia-reperfusion can be modeled by a C. elegans behavior, the O2-ON response, which is suppressed by hypoxic preconditioning or inactivation of the O2-sensing HIF (hypoxia-inducible-factor) hydroxylase EGL-9. From a genetic screen, we found that the cytochrome P450 oxygenase CYP-13A12 acts in response to the EGL-9/HIF-1 pathway to facilitate the O2-ON response. CYP-13A12 promotes oxidation of polyunsaturated fatty acids into eicosanoids, signaling molecules that can strongly affect inflammatory pain and ischemia-reperfusion injury responses in mammals. We propose that roles of the EGL-9/HIF-1 pathway and cytochrome P450 in controlling responses to anoxia-reoxygenation are evolutionarily conserved.

One Sentence Summary:

A genetic screen using a C. elegans behavioral model of ischemia-reperfusion injury identifies a gene that drives response to anoxia-reoxygenation and encodes a cytochrome P450.

Main Text:

Ischemia-reperfusion-related disorders, such as strokes and heart attacks, are the most common causes of adult deaths worldwide (1). Blood delivers O2 and nutrients to target tissues, and ischemia results when the blood supply is interrupted. The restoration of O2 from blood flow after ischemia, known as reperfusion, can exacerbate tissue damage (2). How organisms prevent ischemia-reperfusion injury is poorly understood. Studies of the nematode C. elegans led to
discovery of an evolutionarily conserved family of O$_2$-dependent enzymes (EGL-9 in *C. elegans* and EGLN2 in mammals) that hydroxylate the HIF transcription factor and link hypoxia to HIF-mediated physiological responses (3-7). Exposure to chronic low concentrations of O$_2$ (hypoxic preconditioning) or direct inhibition of EGLN2 strongly protects mammals from stroke and ischemia-reperfusion injury (2, 8, 9). Similarly, EGL-9 inactivation in *C. elegans* blocks a behavioral response to reoxygenation, the O2-ON response (characterized by a rapidly increased locomotion speed triggered by reoxygenation after anoxia) (10, 11), which is similar to mammalian tissue responses to ischemia-reperfusion: (i) reoxygenation drives the O2-ON response and is the major pathological driver of reperfusion injury, (ii) hypoxic preconditioning can suppress both processes, and (iii) the central regulators (EGL-9/HIF) of both processes are evolutionarily conserved. How the EGL-9/HIF-1 and EGLN2/HIF pathways control the O2-ON response and ischemia-reperfusion injury, respectively, is largely unknown.

To seek EGL-9/HIF-1 effectors important in the O2-ON response, we performed an egl-9 suppressor screen for mutations that can restore the defective O2-ON response in egl-9 mutants (fig. S1A). We identified new alleles of hif-1 in this screen; because EGL-9 inhibits HIF-1, hif-1 mutations suppress the effects of egl-9 mutations (10). We also identified mutations that are not alleles of hif-1 (Figs. 1A-1C and fig. S1B). hif-1 mutations recessively suppressed three defects of egl-9 mutants: the defective O2-ON response, defects in egg-laying and the ectopic expression of the HIF-1 target gene *cysl*-2 (previously called *K10H10.2*) (fig. S1C) (10, 12). By contrast, one mutation, *n5590*, dominantly suppressed the O2-ON defect but did not suppress the egg-laying defect or the ectopic expression of *cysl*-2::GFP (Figs. 1D, 1E and fig. S2). *n5590* restored the sustained phase (starting at 30s post-reoxygenation) better than it did the initial phase (within 30s post-reoxygenation) (Figs. 1A-1C). egl-9; hif-1; *n5590* triple mutants displayed a normal O2-
ON response, just like the wild type and *egl-9; hif-1* double mutants (fig. S1D). Thus, *n5590* specifically suppresses the *egl-9* defect in the sustained phase of the O2-ON response.

We genetically mapped *n5590* and identified an M46I missense mutation in the gene *cyp-13A12* by whole-genome sequencing (Fig. 2A, fig. S3A and Table S1A). Decreased wild-type *cyp-13A12* gene dosage in animals heterozygous for a wild-type allele and the splice acceptor null mutation *gk733685*, which truncates the majority of the protein, did not recapitulate the dominant effect of *n5590* (Fig. 2B). *gk733685* homozygous mutants similarly did not recapitulate the effect of *n5590* (Fig. 2C). Thus, *n5590* does not cause a loss of gene function. By contrast, increasing wild-type *cyp-13A12* gene dosage by overexpression restored the sustained phase of the O2-ON response (Fig. 2D), and RNAi against *cyp-13A12* abolished the effect of *n5590* (Fig. 2E). We conclude that *n5590* is a gain-of-function allele of *cyp-13A12*.

*cyp-13A12* encodes a cytochrome P450 oxygenase (CYP). CYPs can oxidize diverse substrates (13-15). The *C. elegans* genome contains 82 CYP genes, at least two of which are polyunsaturated fatty acid (PUFA) oxygenases that generate eicosanoid signaling molecules (fig. S3B) (16, 17). The closest human homolog of CYP-13A12 based on BLASTP scores is CYP3A4 (fig. S4). We aligned the protein sequences of CYP-13A12 and CYP3A4 and found that *n5590* converts methionine 46 to an isoleucine, the residue in the corresponding position of normal human CYP3A4 (fig. S4). Methionines can be oxidized by free radicals, which are produced in the CYP enzymatic cycle, rendering CYPs prone to degradation (18, 19). Using transcriptional and translational GFP-based reporters, we identified the pharyngeal marginal cells as the major site of expression of *cyp-13A12* (fig. S5) and observed that the abundance of CYP-13A12::GFP protein was decreased by prolonged hypoxic preconditioning and also decreased in *egl-9* but not in *egl-9; hif-1* mutants (Fig. 2F and fig. S5). The *n5590* mutation prevented the decrease in CYP-
13A12::GFP abundance by hypoxia or egl-9. Thus, n5590 acts, at least in part, by restoring the normal abundance of CYP-13A12, which then promotes the O2-ON response in egl-9 mutants.

We tested whether CYP-13A12 was normally required for the O2-ON response in wild-type animals. The cyp-13A12 null allele gk733685 abolished the sustained phase of the O2-ON response; the initial phase of the O2-ON response was unaffected (Fig. 3A). A wild-type cyp-13A12 transgene fully rescued this defect (Fig. 3B). A primary role of CYP-13A12 in the sustained phase of the O2-ON response explains the incomplete rescue of the defective O2-ON response of egl-9 mutants by n5590 during the initial phase (Fig. 1C). The activity of most and possibly all C. elegans CYPs requires EMB-8, a CYP reductase that transfers electrons to CYPs (20). No non-CYP EMB-8 targets are known. emb-8(hc69) causes a temperature-sensitive embryonic lethal phenotype. We grew emb-8(hc69) mutants at the permissive temperature to the young-adult stage. A shift to the non-permissive temperature simultaneously with E. coli-feeding RNAi against emb-8 nearly abolished the O2-ON response (Figs. 3C and 3D) (Both the hc69 mutation and RNAi against emb-8 were required to substantially reduce the level of EMB-8 (17).) CYP-13A12 is thus required for the sustained phase of the O2-ON response, and one or more other CYPs likely act with CYP-13A12 to control both phases of the O2-ON response.

CYP oxygenases define one of three enzyme families that can convert PUFAs to eicosanoids, signaling molecules that affect inflammatory pain and ischemia-reperfusion responses of mammals (15, 21-23); the other two families, cyclooxygenases and lipoxygenases, do not appear to be present in C. elegans (17, 24). To test whether eicosanoids are regulated by EGL-9 and CYP-13A12, we used high-performance liquid chromatography (HPLC) coupled with mass spectrometry (MS) to profile steady-state amounts of 21 endogenous eicosanoid species from cell extracts of wild-type, egl-9(n586) and egl-9(n586); cyp-13A12(n5590) strains.
Only free eicosanoids have potential signaling roles (21, 22, 24), so we focused on free eicosanoids. The egl-9 mutation caused a markedly decreased overall amount of free eicosanoids, while the total amount of eicosanoid, including both free and membrane-bound fractions, was unaltered (Fig. 4A and fig. S6). Among the eicosanoids profiled, 17,18-DiHEQ (17,18-diolhydroxyeicosatetraenoic acid) was the most abundant species (fig. S6B). 17,18-DiHEQ is the catabolic hydrolase product of 17,18-EEQ (17,18-epoxyeicosatetraenoic acid), an epoxide active in eicosanoid signaling (25). Free cytosolic 17,18-EEQ and 19-hydroxyeicosatetraenoic acid (19-HETE) were present in the wild type but undetectable in egl-9 mutants (Figs. 4C-4F). egl-9(n586); cyp-13A12(n5590) mutants exhibited partially restored free overall eicosanoid levels as well as restored levels of 17,18-EEQ and 19-HETE (Figs. 4A-4F and fig. S6B). Thus, both EGL-9 and CYP-13A12 regulate amounts of free cytosolic eicosanoids.

We tested whether the O2-ON response requires PUFAs, which are CYP substrates and eicosanoid precursors. PUFA-deficient fat-2 and fat-3 mutants (26) exhibited a complete lack of the O2-ON response, although the acceleration in response to anoxia preceding the O2-ON response was normal (Fig. 4G and figs. S7A-S7C). The defective O2-ON response of fat-2 mutants was restored by feeding animals the C20 PUFA arachidonic acid (Fig. 4H) but not oleate, a C18 monounsaturated fatty acid that is processed by FAT-2 to generate C20 PUFAs (fig. S7D). These results demonstrate an essential role of PUFAs for the O2-ON response.

We suggest a model in which CYPs, which are strictly O2-dependent (27, 28), generate eicosanoids to drive the O2-ON response (Figs. 4I and fig. S8). In this model, EGL-9 acts as a chronic O2-sensor, so that during hypoxic preconditioning, the O2-dependent activity of EGL-9 is inhibited, HIF-1 is activated and unknown HIF-1 up-regulated targets decrease CYP protein abundance. The low abundance of CYPs defines the hypoxic preconditioned state. Without
hypoxic preconditioning, CYPs generate eicosanoids, which drive the O2-ON response. By contrast, with hypoxic preconditioning or in egl-9 mutants, the CYP amounts are insufficient to generate eicosanoids and the O2-ON response is not triggered. Neither C20 PUFAs nor overexpression of CYP-29A3 restored the defective O2-ON response of egl-9 mutants (figs. S9 and S10), indicating that this defect is unlikely caused by a general deficiency in C20 PUFAs or CYPs. Since the O2-ON response requires EMB-8, a general CYP reductase, but only the sustained phase requires CYP-13A12, we propose that CYP-13A12 and other CYPs act as acute O2 sensors and produce eicosanoids, which are short-lived and act locally (22) during reoxygenation to signal nearby sensory circuits that drive the O2-ON response.

In humans, a low uptake of PUFAs or an imbalanced ratio of ω3/ω6 PUFAs is associated with elevated risk of stroke, cardiovascular disease and cancer (21, 23, 29, 30). Cytochrome P450s and eicosanoid production also have been implicated in mammalian ischemia-reperfusion (15, 21). Nonetheless, the causal relationships among and mechanisms relating O2 and PUFA homeostasis, CYP and PUFA-mediated cell signaling and organismal susceptibility to oxidative disorders are poorly understood. We identify a novel pathway in which EGL-9/HIF-1 regulates CYP-eicosanoid signaling, demonstrate that PUFAs confer a rapid response to reoxygenation via CYP-generated eicosanoids and provide direct causal links among CYPs, PUFA-derived eicosanoids, and an animal behavioral response to reoxygenation. As molecular mechanisms of O2 and PUFA homeostasis are fundamentally similar and evolutionarily conserved between nematodes and mammals (7, 11, 26), we suggest that the C. elegans O2-ON response is analogous to the mammalian tissue/cellular response to ischemia-reperfusion injury and that the principle of CYP-mediated regulation and the molecular pathway including EGL-9/HIF-1 and CYPs in controlling responses to anoxia-reoxygenation are evolutionarily conserved.
References and Notes:


**Acknowledgments:** We thank C. Bargmann, A. Fire, A. Hart, Y. Iino, J. Powell-Coffman and C. Rongo for reagents; the *Caenorhabditis* Genetics Center and the Million Mutation Project
for strains. H.R.H. is an Investigator of the Howard Hughes Medical Institute and the David H. Koch Professor of Biology at MIT. Supported by NIH grant GM24663 (H.R.H), German Research Foundation grant ME2056/3-1 (R.M.), NSF Graduate Research Fellowship (N.B.), MIT Undergraduate Research Opportunities Program (S.Z.) and a Helen Hay Whitney Foundation postdoctoral fellowship (D.K.M.).

**Fig. 1.** *n5590** suppresses the defect of egl-9 mutants in the O2-ON response. (A) Speed graph of wild-type animals, showing a normal O2-ON response. Average speed values ± 2 SEMs (blue) of animals (n > 50) are shown with step changes of O2 between 20% and 0% at the indicated times. The mean speed within 0-120 s after O2 restoration is increased compared with that before O2 restoration (p < 0.01, one-sided unpaired t-test). The dashed green line indicates the approximate boundary (30s post-reoxygenation) between the initial and sustained phases of the O2-ON response. (B) Speed graph of egl-9(n586) mutants, showing a defective O2-ON response. (C) Speed graph of egl-9(n586); cyp-13A12(n5590) mutants, showing a restored O2-ON response mainly in the sustained phase (right of the dashed green line). The mean speed within 30-120 s after O2 restoration was significantly higher than that of egl-9(n586) mutants (p <0.01). (D) Speed graph of egl-9(n586); cyp-13A12(n5590)/+ mutants, showing a restored O2-ON response in the sustained phase. (E) *hif-1* but not *cyp-13A12(n5590)* suppressed the expression of *cysl-2::GFP* by egl-9(n586) mutants. GFP fluorescence micrographs of 5-7 worms aligned side by side carrying the transgene *nIs470 [Pcysl-2::GFP]* are shown. Scale bar, 50 µm.

**Fig. 2.** *n5590* is a gain-of-function allele of *cyp-13A12*. (A) Genetic mapping positioned *n5590* between the SNPs *pkP3075* and *uCE3-1426*. Solid grey lines indicate genomic regions for which recombinants exhibited a defective O2-ON response, thus excluding *n5590* from those regions. The locations of *n5590* and *gk733685* are indicated in the gene diagram of *cyp-13A12*. (B) Speed
graph of egl-9(n586); cyp-13A12(gk733685)/+ animals, showing a defective O2-ON response.

(C) Speed graph of egl-9(n586); cyp-13A12(gk733685) mutants, showing a defective O2-ON response. (D) Speed graph of egl-9(n586); nEx [cyp-13A12(+)] animals, showing a restored O2-ON response in the sustained phase (right of the dashed green line). (E) Speed graph of egl-9(n586); cyp-13A12(n5590); cyp-13A12(RNAi) animals, showing a suppressed O2-ON response. (F) Fractions of animals expressing CYP-13A12::GFP or CYP-13A12(n5590)::GFP (* p<0.01, two-way ANOVA with Bonferroni's test, n=4).

Fig. 3. Requirement of CYP-13A12 for a normal O2-ON response. (A) Speed graph of cyp-13A12(gk733685) loss-of-function mutants, showing an O2-ON response with a normal initial phase but a diminished sustained phase (left and right, respectively, of the dashed green line). (B) Speed graph of cyp-13A12(gk733685) mutants with a rescuing wild-type cyp-13A12 transgene, showing the O2-ON response with a normal initial phase and sustained phase. The mean speed within 30-120 s after O2 restoration was higher than that of cyp-13A12(gk733685) mutants (p <0.01, one-sided unpaired t-test, n >50). (C) Speed graph of emb-8(hc69) mutants growing at the permissive temperature of 15°C with simultaneous E. coli feeding RNAi against emb-8, showing a normal O2-ON response. (D) Speed graph of emb-8(hc69) mutants growing post-embryonically at the restrictive temperature of 25°C with simultaneous E. coli feeding-RNAi against emb-8, showing a reduced O2-ON response.

Fig. 4. Modulation of eicosanoid concentrations by EGL-9 and CYP-13A12. (A) Overall levels of free eicosanoids, calculated by adding the values of the profiled 21 eicosanoids in the wild type and egl-9(n586) and egl-9(n586); cyp-13A12(n5590) strains. (B) Schematic illustrating the conversion of arachidonic acid (AA, 20:4n-6) to 19-HETE and of EPA (20:5n-3) to 17,18-EEQ by CYPs. (C) Quantification of 19-HETE and 17,18-EEQ concentrations in the wild type
and egl-9(n586); cyp-13A12(n5590) and egl-9(n586) mutant strains. Amounts of free (membrane unbound) forms of 17,18-EEQ and 19-HETE from extracts of age-synchronized young adult hermaphrodites are shown. p < 0.01, one-way ANOVA post hoc test, n = 3. Error bars are SEMs.

(D-F) Representative HPLC-MS traces indicating free 17,18-EEQ levels based on the spectrograms of three MS samples: (D) wild type, (E) egl-9(n586), and (F) egl-9(n586) ; cyp-13A12(n5590). Peaks of 17,18-EEQ at its transition m/z (mass-to-charge ratio) were measured and extracted (MassHunter). The x-axis shows the retention time (minutes); the y-axis shows the abundance (counts), with specific integral values over individual peaks indicated above each peak. (G) Speed graph of fat-2 mutants, showing a defective O2-ON response. Animals were supplemented with the solvents used in (H) as a control. (H) Speed graph of fat-2 mutants, showing the O2-ON response rescued by C20 PUFA (AA) supplementation. (I-J) Model of how EGL-9 and CYPs control the O2-ON response under (I) normoxic conditions and (J) conditions of hypoxic preconditioning or in egl-9 mutants (see text for details). The light blue indicates low protein activity, low amounts of eicosanoids or a defective O2-ON response.

Supplementary Materials

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Supplementary Materials for

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**Materials and Methods**

**EMS Mutagenesis, Genetic Screens, Mapping and Whole-genome Sequencing**

To screen for *egl-9* suppressors, we mutagenized *egl-9(n586)* mutants carrying the *cysl-2::GFP* transgene *nls470* with ethyl methanesulfonate (EMS) and observed the F2 progeny in one of three ways using: (1) a dissecting microscope and GFP fluorescence to isolate suppressors of *cysl-2::GFP* overexpression, (2) a dissecting microscope to isolate suppressors of the defective egg-laying behavior, and (3) a compound microscope and a nitrogen gas-flow chamber to isolate suppressors of the defective O2-ON response.

We refer to mutations that cause diminished *cysl-2::GFP* fluorescence or restored egg-laying behavior under conditions of normoxia (21% O₂) as GFP or Egl suppressors, respectively. To isolate suppressors of the defective O2-ON response of *egl-9* mutants, we used a nitrogen gas-flow chamber system with a 100% nitrogen gas source, flow-meters, and a lid with gas tubing inlets and outlets mounted on a Petri plate freshly seeded with *E. coli* OP50 (10). Animals with restored rapid acceleration immediately (0 - 60s) after reoxygenation (by removal of the plate lid) were identified as suppressors of the defective O2-ON response. False positives were eliminated by retesting animals in the next generation using a population assay (>50 animals) (10).

To map the *egl-9* suppressor mutation *n5590*, we first generated a polymorphic Hawaiian *egl-9(n586)* strain by repeatedly crossing *egl-9(n586)* with the Hawaiian wild-type strain CB4856. *nls470; egl-9(n586); n5590* mutants were then crossed with the Hawaiian *egl-9(n586)* strain for genetic mapping. F2 animals were isolated, and clonal F3 populations were assayed for
the O2-ON response. Mapping using SNP analysis (50) positioned \textit{n5590} between the SNPs \textit{pkP3075} and \textit{uCE3-1426} on chromosome III (Fig. 2A).

Whole-genome sequencing and data analyses were performed as described (51). Two protein-coding mutations were identified in the \textit{n5590} interval defined above: a T-to-G mutation in the gene \textit{Y39E4A.2} and a G-to-A mutation in the gene \textit{cyp-13A12}. As described in the text, we showed that the \textit{egl-9} suppressor \textit{n5590} is a gain-of-function mutation of \textit{cyp-13A12}.

**Behavioral Analyses**

The O2-ON response was measured using a multi-worm tracker with a gas-flow chamber system and quantified by customized MatLab algorithms as previously described (10). For experiments using the C20 PUFA arachidonic acid as a food supplement, arachidonic acid salts (Cayman Chemical) were dissolved in ethanol at 1 mg/ml, and 50 µL was spread evenly onto NGM plates before drying briefly and cultivating OP50 \textit{E. coli} on the plates. \textit{C. elegans} was then transferred to the PUFA-supplemented plates.

To quantify egg-laying behavior, we scored the developmental stages of newly laid eggs of young adult hermaphrodites that had been transferred to fresh NGM plates with OP50 (52).

**Determination of eicosanoid levels**

Endogenous CYP-derived eicosanoids were assayed for the N2 wild-type strain as well as for \textit{egl-9(n586)} and \textit{egl-9(n586); cyp-13A12(n5590)} mutant strains. For each experiment (three independent cultures per strain), 12,000 stage-synchronized larvae were allocated to four fresh Petri plates (diameter = 94 mm) and further cultivated at 22.5°C until the young adult stage (24 hrs post-L4). To generate a synchronous culture of first-stage (L1) larvae, a population of well-
fed animals was collected from Petri plates by rinsing and then filtered through a 10 \( \mu \)m membrane (SM 16510/11, Sartorius, Goettingen, Germany), a pore size that retains all but L1 larvae. These larvae were allowed to grow to be young adults and then were filtered again to eliminate L1 larvae of the next generation and so retain exclusively young adults. Subsequently, animals were prepared for LC-MS/MS analysis essentially as described previously (17). Briefly, aliquots corresponding to 30 mg wet weight were mixed with internal standard compounds (10 ng each of 20-HETE-d6, 14,15-EET-d8 and 14,15-DHET-d11; from Cayman Chemicals, Ann Arbor, MI, USA) and either subjected to alkaline hydrolysis (total eicosanoids) or directly extracted with methanol/water (free eicosanoids) followed by solid-phase extraction of the metabolites. Sample preparation, HPLC and MS conditions as well as the monitoring of multiple reactions for the analysis of the CYP-eicosanoid profile were as described previously (25). The protein concentration of each sample was measured after hydrolysis (53).

**Mutations and Strains**

*C. elegans* strains were cultured as described previously (54). The N2 Bristol strain (54) was used as the reference wild-type strain, and the polymorphic Hawaiian strain CB4856 (55) was used for genetic mapping and SNP analysis. Mutations used were as follows: LG III, cyp-13A12(n5590 and gk733685) (gk733685 was obtained from the Million Mutation Project (56)), emb-8(hc69) (20), cdk-5(ok626) (41); LG IV, fat-2(wa17), fat-3(wa22, ok1126) (57); LG V, egl-9(sa307, n586) (5, 58), hif-1(ia4, n5513, n5527) (59, 60).

Transgenic strains were generated by germline transformation as described (61). Transgenic constructs were co-injected (at 20 - 50 ng/\( \mu l \)) with mCherry reporters, and lines of mCherry-positive animals were established. Gamma irradiation was used to generate integrated transgenes. Transgenic strains used were as follows: nEx2015 [P\_cyp-13A12::GFP; P\_unc-54::mCherry]; nEx2016 [P\_cyp-13A12::GFP; P\_unc-54::mCherry]; nEx2017 [cyp-13A12(+)]; nEx2018 [cyp-13A12(n5590)]; nIs587 [P\_cyp-13A12::GFP; P\_unc-54::mCherry]; nIs588 [P\_cyp-13A12::cyp-
Molecular biology

Constructs were generated using the PCR-fusion technique (62), the Gateway system (Invitrogen) and the Infusion cloning (Clontech) technique (63). Primer sequences are shown in Table S1.

Statistical analyses

One-sided unpaired t-tests were used to compare the mean speeds of all animals within 60 or 120 seconds before or after O2 restoration (10). p<0.01 indicates speed differences that are significant, as noted in each figure. Fisher's exact tests were used after egg-laying behavioral assays to compare the distributions of the six categories of embryos from the wild type and various mutants. Two-way ANOVA was used to calculate p values to test for significance of the effects of genotypes and different conditions in the O2-ON response.

Bioinformatics

The BLASTP program from NCBI was used to search for proteins homologous to CYP-13A12 (64). Multiple sequence alignments were generated and analyzed using ClusterW2 (65), and the results were displayed and annotated using JalView (66). Schematic gene structures and annotations were generated using the Exon-Intron Graphic Maker (http://wormweb.org/exonintron).

Supplementary Text

Transcriptional and translational GFP reporters with the cyp-13A12 promoter or with the promoter and coding sequence were both most strongly expressed in the pharynx (fig. S5). The GFP-stained pharyngeal cells extended processes along the anterior pharyngeal bulb and
exhibited finger-like protrusions, and we identified these cells as the pharyngeal marginal cells (MCs). MCs intercalate with pharyngeal muscles and might structurally reinforce these muscles (31). However, MCs contain abundant mitochondria, suggesting that these cells might perform active non-structural roles (32). The O2-ON response involves rapid reoxygenation and occurs independently of known aerotaxic neural O₂ sensors (10, 11, 33-35); we hypothesize that MCs actively signal reoxygenation by converting PUFAs to membrane-diffusible eicosanoids, which are sensed by nearby sensory neurons and trigger the O2-ON response via neural circuits that control forward/backward locomotion (36, 37).

In egl-9 mutants or animals with prolonged (24 hrs) hypoxic preconditioning (10), the O2-ON response is suppressed because CYP-13A12 is decreased through the EGL-9/HIF-1 pathway (Figs. 2F and 4J). Our GFP reporter experiments indicate that regulation of CYP-13A12 by EGL-9/HIF-1 occurs primarily by regulation of the abundance of CYP proteins (Fig. 2F and fig. S5). HIF-1 activation can facilitate protein ubiquitination and homeostasis and pro-survival effects of hypoxic preconditioning likely require suppression of protein translation in C. elegans (38-40). We suggest that one or more transcriptional targets of HIF-1 regulate the abundance of CYP-13A12 and likely that of other CYPs, since CYP-13A12 does not control the initial phase but EMB-8, a general CYP reductase, affects both the initial and sustained phases (Fig. 3). egl-9(n685); cyp-13A12(n5590) mutants might have been restored for the sustained phase of the O2-ON response because they have restored the abundance of CYP-13A12 and thus partially restored the ability to produce eicosanoids from PUFAs. Because cdk-5 mutations suppress the defective LIN-10 trafficking in egl-9 mutants (41), we also tested cdk-5 mutants and found that cdk-5 mutations did not suppress cysl-2::GFP expression, the defective egg-laying behavior, or
the defective O2-ON response or of *egl-9* mutants (figs. S11A-S11B), indicating that EGL-9 regulates the O2-ON response independently of CDK-5.

Our conclusions are consistent with findings that mammalian CYP proteins closely related to *C. elegans* CYP13A12 are expressed in tissues that display ischemia-reperfusion injury and are involved in eicosanoid signaling. For example, CYP3A4 is the most closely related human homolog of *C. elegans* CYP13A12 (162/501 or 32% amino acid identity with CYP13A12) and is mainly expressed in the liver but is also highly expressed in the brain (42-44), consistent with our hypothesis that CYP3A4 modulates ischemic processes in these organs. We note that members of particular CYP protein families share high similarity in general, and CYP13A12 is also homologous to other mammalian CYPs, including the CYP5A1 thromboxane synthase (148/481 or 31% amino acid identity with CYP13A12), which generates the eicosanoid thromboxane and is widely expressed in the vasculature (45), suggesting that CYP5A1-generated eicosanoids function in the vasculature. Increased production of HETE-type eicosanoids is also associated with ischemia-reperfusion processes (46, 47). The modulation of ischemia-reperfusion by CYPs in mammals is thus unlikely mediated by a single CYP, such as CYP3A4, just as the O2-ON response of *C. elegans* is likely mediated by one or more other CYPs in addition to CYP-13A12. Rather, we suggest that the principle of CYP-mediated regulation and the novel molecular pathway including EGL-9/HIF-1 and CYPs in regulating responses to anoxia-reoxygenation are conserved; different mammalian CYPs might act in different tissues and organs. The observation that potent inhibitors of CYPs, such as sulfaphenazole, cause strong protection against ischemia-reperfusion injury (15, 48, 49) is consistent with our model. Using *C. elegans* genetics and a behavioral model of ischemia/reperfusion, we demonstrate a direct causal role of CYPs in the response to anoxia-reoxygenation and therefore suggest a similarly causal role of CYPs in modulating mammalian ischemia-reperfusion processes.
Supplementary Figures

A

\[ nls470 \text{ IV: } egl-9(n586) \text{ V} \]
\[ (cysl-2::GFP+, Egl and defective O2-ON response) \]
\[ \downarrow \text{EMS} \]
\[ \downarrow \text{F1} \]
\[ \downarrow \text{F2 single animals examined} \]

Three classes of suppressor mutants:
i. CYSL-2::GFP: GFP-
ii. Egg laying: non-Egl
iii. Locomotion: O2-ON defect restored

B

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C

\[ egl-9(n586); hif-1(ia4)/ + \]
\[ O2-ON response suppressed \]

D

\[ egl-9(n586); hif-1(ia4); cyp-13A12(n5590) \]
\[ O2-ON response restored \]
fig. S1. Genetic screens for *egl-9* suppressors

(A) Schematic of the screens for *egl-9* suppressors. Approximately 15,000 haploid genomes were screened. (B) Summary of the non-*hif-1* suppressor mutant isolates, showing that each of the three aspects of the *egl-9* mutant phenotype was separately suppressed. (C) Speed graph of *egl-9; hif-1/+* mutants with a defective O2-ON response, showing the recessive suppression of *egl-9* by *hif-1*. Cross progeny of *egl-9; hif-1* and *egl-9* mutants were assayed for the O2-ON response. (D) Speed graph of *egl-9(n586); hif-1(ia4); cyp-13A12(n5590)* triple mutants, showing a normal O2-ON response. Canonical alleles of *egl-9* and *hif-1* were used.
fig. S2. n5590 did not suppress the defective egg-laying of egl-9 mutants

(A-D) hif-1 but not cyp-13A12(n5590) suppressed the egg-laying defect of egl-9(n586) mutants (p < 0.001, Fisher's exact test). Fractions of the developmental stages of eggs laid by animals carrying various mutations are shown.
fig. S3. The predicted catalytic CYP domain of CYP-13A12 and its protein family homology

(A) Primary structure of CYP-13A12, which is predicted by SOSUI (67) to be membrane-spanning with the C-terminal end in the cytoplasm. The membrane-spanning segment is indicated with red, the predicted CYP domain is indicated with blue and the methionine residue mutated in \( cyp-13A12(n5590) \) is indicated with green. (B) Four categories of predicted \( C. \) elegans CYP genes based on their amino acid sequence similarities to human counterparts. Known biochemical functions for CYP-22A1 (a.k.a. DAF-9), CYP-33E2 and CYP-29A3 as lipid hydroxylases are noted (16, 17, 68, 69).

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<th>Known C. elegans functions</th>
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<td>PCB hydroxylase?</td>
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fig. S4. Similarity between *C. elegans* CYP-13A12 and human CYP3A4 proteins

(A) Protein sequence alignment showing homology between CYP-13A12 and human CYP3A4. The arrowhead indicates the *n5590* Met461le mutation.
fig. S5. Expression pattern of cyp-13A12 and effects of hypoxia, cyp-13A12(n5590) and egl-9 mutations on CYP-13A12

(A) A diagram of the C. elegans pharynx, showing the locations of the nuclei of the nine marginal cells (green). The process of one anterior marginal cell is shown with tiny protrusions that intercalate with pharyngeal muscles. (B) Merged image of Nomarski and GFP fluorescence micrographs showing expression of cyp-13A12 in the pharynx. Scale bar, 50 µm. The transcriptional reporter used was an extrachromosomal array of genotype nEx [Pcyp-13A12::GFP]. (C) Merged image of high-magnification Nomarski and GFP fluorescence micrographs showing expression of cyp-13A12 in a marginal cell with finger-like protrusions, one of which is indicated by the arrow. Unlike translational reporters (see below), patterns or levels of GFP expression in such cyp-13A12 transcriptional
reporters are not significantly altered by hypoxic preconditioning or egl-9 mutations. (D) Representative fluorescence micrographs indicating expression of a P_\text{cyp-13A12}::cyp-13A12::GFP transgene of translational fusion protein in various strains or after 24 hrs hypoxic preconditioning (10). The reporter contains the promoter and genomic coding regions of cyp-13A12 fused with GFP. Note that CYP-13A12::GFP proteins, which were present in marginal cells (green arrows), were down-regulated by hypoxia and in egl-9 mutants, compared with in wild-type animals. (E) Representative fluorescence micrographs indicating expression of a P_\text{cyp-13A12}::cyp-13A12(n5590)::GFP transgene of translational fusion protein in various strains or after 24 hrs hypoxic preconditioning. CYP-13A12(M46I)::GFP proteins were not down-regulated by hypoxia or in egl-9 mutants. Equal exposure times were used. Also note that the background fluorescence from body wall muscles (grey arrow) corresponds to fluorescence emission bleed-through from the co-injection marker P_\text{unc-54}::mCherry. The background fluorescence is not present in the transcriptional reporter because the transcriptional GFP reporter is at least one magnitude brighter than the translational GFP reporter.
fig. S6. EGL-9 and CYP-13A12-regulated eicosanoids
(A) Levels of free (membrane-unbound) 17,18-DiHEQ and 14,15-DiHEQ profiled by HPLC/MS. (B) Levels of free (membrane-unbound) 14,15-DHET, 11,12-DHET, 8,9-DHET, 5,6-DHET, 19-HETE, 20-HETE, 14,15-EET, 11,12-EET, 8,9-EET, 5,6-EET, 11,12-DiHEQ, 8,9-DiHEQ, 5,6-DiHEQ, 19-HEPE, 20-HEPE, 17,18-EEQ, 14,15-EEQ, 11,12-EEQ, and 8,9-EEQ profiled by HPLC/MS. (C) Levels of total (both free and membrane-bound) 17,18-DiHEQ and 14,15-DiHEQ profiled by HPLC/MS. (D) Levels of total (both free and membrane-bound) 14,15-DHET, 11,12-DHET, 8,9-DHET, 5,6-DHET, 19-HETE, 20-HETE, 14,15-EET, 11,12-EET, 8,9-EET, 5,6-EET, 11,12-DiHEQ, 8,9-DiHEQ, 5,6-DiHEQ, 19-HEPE, 20-HEPE, 17,18-EEQ, 14,15-EEQ, 11,12-EEQ, and 8,9-EEQ profiled by HPLC/MS. (E) Levels of total 17,18-EEQ and 19-HETE profiled by HPLC/MS. Error bars, SEMs, n = 3.
fig. S7. The O2-ON response requires C20 PUFA biosynthesis

(A) A schematic of a PUFA biosynthetic pathway showing biochemical functions of FAT-2 and FAT-3. Note that fat-2 and fat-3 mutants completely lack C20 PUFAs. (B) Speed graph of fat-3 null mutants, showing a defective O2-ON response. Animals were supplemented with PUFA solvents used in (C) as a control. (C) Speed graph of fat-3 mutants, showing the O2-ON response was rescued by C20 PUFA (arachidonic acid) supplementation. (D) Speed graph fat-2 mutants, showing the O2-ON response was not rescued by supplementation with the C18 MUFA oleate.
A  Normal behavioral state in wild type

B  Behavioral state in egl-9 mutants or wild-type animals after hypoxic preconditioning

C  Behavioral state in egl-9; cyp-13A12(n5590) mutants

D  Behavioral state in cyp-13A12 loss-of-function mutants
fig. S8. Model for the control of the O2-ON response by EGL-9, CYPs and PUFAs.

(A) Model for the molecular pathway by which EGL-9, CYP, PUFA-eicosanoids coordinately control the O2-ON response under normal conditions. CYP enzymes, including the PUFA oxygenase CYP-13A12, act as acute molecular O2-sensors during reoxygenation to promote the O2-ON response. The HIF hydroxylase EGL-9, by contrast, acts as a chronic O2 sensor to suppress the O2-ON response by hypoxic preconditioning (10) via regulation of CYPs. The regulation of CYP-13A12 by HIF-1 occurs primarily at the protein level, likely via an unidentified HIF-1 transcriptional target that decreases CYP-13A12 protein levels. The biosynthesis of PUFAs, known physiological substrates of CYP enzymes, is mediated by the FAT-2 and FAT-3 lipid desaturases in a parallel pathway. The defective O2-ON response of egl-9 mutants is not caused by a reduced activity of the FAT-2/FAT-3/PUFA pathway (fig. S9A); furthermore, HIF-1 activation might enhance but not reduce PUFA biosynthesis (70).

In panels (A) - (D), the light blue indicates low levels of protein activity, eicosanoids, or a failure of the O2-ON response. (B) egl-9 mutation causes HIF-1 activation and down-regulation of CYP-13A12 and other CYPs, resulting in a down-regulation of eicosanoids and suppression of the O2-ON response. (C) The gain-of-function mutation cyp-13A12(n5590) restores eicosanoid levels, leading to restoration of the sustained phase of the defective O2-ON response of egl-9 mutants. In contrast to cyp-13A12, over-expression of another CYP gene cyp-29A3 did not restore the defective O2-ON response in egl-9 mutants (figs. S10A-S10B), indicating that restoration of the O2-ON response in egl-9 mutants by cyp-13A12(n5590) is not caused by a general increased function of PUFA oxygenases. (D) The loss-of-function mutation cyp-13A12(gk733685) causes a specific defect in the sustained phase of the O2-ON response.
fig. S9. The defective O2-ON response of egl-9 mutants is not caused by a constitutive deficiency in PUFAs.

(A) Speed graph of egl-9 mutants, showing the defective O2-ON response was not rescued by exogenous C20 PUFA (arachidonic acid) as a dietary supplement.
fig. S10. Specificity of CYP-13A12 in rescuing the O2-ON response by egl-9 mutants.

(A) Speed graph of egl-9 mutants, showing the defective O2-ON response was not rescued by overexpression of cyp-29A3(+). (B) Speed graph of egl-9 mutants, showing the defective O2-ON response was rescued by overexpression of cyp-13A12(+) in the sustained but not the initial phase. The mean speed within 30-120 s after O2 restoration is significantly higher than that of egl-9(n586) mutants (p <0.01).
fig. S11. EGL-9 and CYPs regulate the O2-ON response independently of CDK-5.

(A) Speed graph of egl-9 mutants, showing a defective O2-ON response. (B) Speed graph of egl-9; cdk-5 mutants, showing the defective O2-ON response was not rescued by loss of cdk-5.
Supplementary Table S1

(A) Protein-coding mutations within the genetically mapped interval identified by whole-genome sequencing of the n5590 mutant. These mutations seen after EMS mutagenesis were not present in the parental mutagenized strain.

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(B) Primers used for molecular cloning and the construction of transgenes

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References and Notes:


69. D. L. Motola et al., *Cell* 124, 1209 (Mar 24, 2006).

**Fig. 1**

**A**  
Wild type  
O2-ON response

**B**  
time (min)  
Animal speed (µm/sec)

**C**  
egl-9(n586); cyp-13A12(n5590)  
Sustained phase of O2-ON response restored

**D**  
time (min)  
Animal speed (µm/sec)

**E**

- **nls470 [Pcysl-2::GFP]**  
- **nls470; egl-9**  
- **nls470; egl-9; hif-1**  
- **nls470; egl-9; cyp-13A12(n5590)**

Images show different conditions and responses in the context of oxygen regulation and gene expression.
Fig. 2

A

LG III
CB4856
N2

SNP: pkP3075
SNP: uCE3-1426

n5590 (GOF)
gk733685 (LOF)
100 bp

B

0% O2
20% O2

egl-9(n586); cyp-13A12(gk733685)/+

O2-ON response suppressed

Animal speed (µm/sec)

Time (min)

C

0% O2
20% O2

egl-9(n586); cyp-13A12(gk733685)

O2-ON response suppressed

Animal speed (µm/sec)

Time (min)

D

0% O2
20% O2

egl-9(n586); nEx [cyp-13A12(+)]

Sustained phase of O2-ON response restored

Animal speed (µm/sec)

Time (min)

E

0% O2
20% O2

egl-9(n586); cyp-13A12(n5590); cyp-13A12(RNAi)

O2-ON response suppressed

Animal speed (µm/sec)

Time (min)

F

P_cyp-13A12::cyp-13A12::GFP
P_cyp-13A12::cyp-13A12(n5590)::GFP

Fraction of GFP+ animals

Wild type
Wild type + hypoxia
egl-9(n586)
egl-9(n586) hif-1(ia4)
**Fig. 3**

A. **cyp-13A12(gk733685)**
   - 0% O2
   - 20% O2
   - No sustained phase

B. **cyp-13A12(gk733685)**
   - nEx [cyp-13A12(+)]
   - Sustained phase

C. **emb-8(hc69)**
   - + emb-8 RNAi at 15°C
   - O2-ON response

D. **emb-8(hc69)**
   - + emb-8 RNAi at 25°C
   - O2-ON response reduced
A

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<td>1.8±0.6</td>
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B

\[ \text{AA (20:4 n-6)} \]

\[ \text{19-HETE} \rightarrow \]

\[ \text{EPA (20:5 n-3)} \]

\[ \text{17,18-EEQ} \]

C

- **19-HETE**
- **17,18-EEQ**

Free eicosanoids (ng/mg proteins)

![Graph showing free eicosanoids](image)

D

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E

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F

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G

- **fat-2(wa17)**
- Control
- O2-ON response suppressed

H

- **fat-2(wa17)** + C20 PUFA (AA)
- O2-ON response restored

I

- Normal behavioral state

J

- Behavioral state in egl-9 mutants or wild-type animals after hypoxic preconditioning