

Cytochrome P450 Drives a HIF-Regulated Behavioral Response to Reoxygenation by *C. elegans*

Dengke K. Ma,¹ Michael Rothe,² Shu Zheng,¹ Nikhil Bhatla,¹ Corinne L. Pender,¹ Ralph Menzel,³ H. Robert Horvitz^{1*}

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 *Caenorhabditis 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 reoxygenation after anoxia are evolutionarily conserved.

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 the discovery of an evolutionarily conserved family of O₂-dependent enzymes (EGL-9 in *C. elegans* and EGLN2 in mammals) that hydroxylate the HIF transcription factor and link hypoxia to hypoxia-inducible factor (HIF)-mediated physiological responses (3–7). Exposure to chronic low concentrations of O₂ (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. It remains unknown how the EGL-9–HIF-1 and EGLN2–HIF pathways control the O₂-ON response and ischemia-reperfusion injury, respectively.

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* (Fig. 1, A to C, 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* (Fig. 1, D and E, and fig. S2). *n5590* restored the sustained phase (starting 30 s after reoxygenation) better than it did the initial phase (within 30 s after reoxygenation) (Fig. 1, A to C). *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 a Met⁴⁶ → Ile 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). Similarly, *gk733685* homozygous mutants 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

RNA interference (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 about 82 CYP genes, at least two of which are polyunsaturated fatty acid (PUFA) oxygenases that generate eicosanoid signaling molecules (fig. S3B) (16, 17). On the basis of BLASTP scores, the closest human homolog of CYP-13A12 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 green fluorescent protein (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. The mutation *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 nonpermissive temperature simultaneously with *Escherichia coli*-feeding RNAi against *emb-8* nearly abolished the O₂-ON response (Fig. 3, C and D). [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, which are signaling molecules that affect inflam-

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matory 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 marked decrease in the overall amount of free eicosanoids, whereas the total amount of eicosanoids, 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

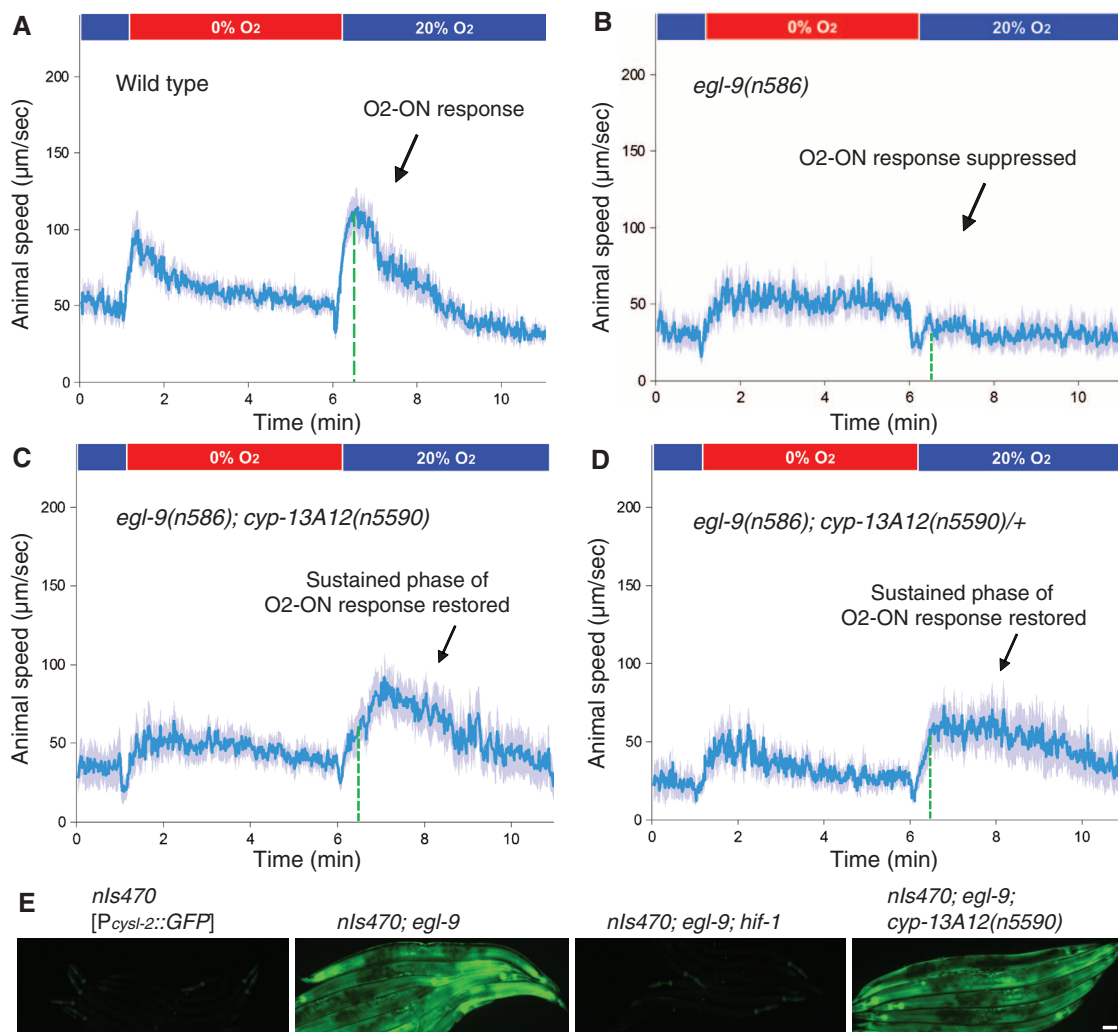
17,18-EEQ and 19-hydroxyeicosatetraenoic acid (19-HETE) were present in the wild type but undetectable in *egl-9* mutants (Fig. 4, C to F). *egl-9(n586); cyp-13A12(n5590)* mutants exhibited partially restored free overall eicosanoid levels as well as restored levels of 17,18-EEQ and 19-HETE (Fig. 4, A to F, 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 fig. S7, A to C). The defective O₂-ON response of *fat-2* mutants was restored by feeding animals arachidonic acid, a C20 PUFA (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 (Fig. 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 to be caused by a general deficiency in C20 PUFAs or CYPs. Because the O₂-ON response requires EMB-8, a general CYP reductase, but only the sustained phase requires CYP-13A12, we propose

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 \pm 2 SEM (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 to 120 s after O₂ restoration is increased relative to that before O₂ restoration ($P < 0.01$, one-sided unpaired t test). The dashed green line indicates the approximate boundary (30 s after 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 to 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 five to seven worms aligned side by side carrying the transgene *nls470* [*P_{cysl-2}::GFP*] are shown. Scale bar, 50 μ m.



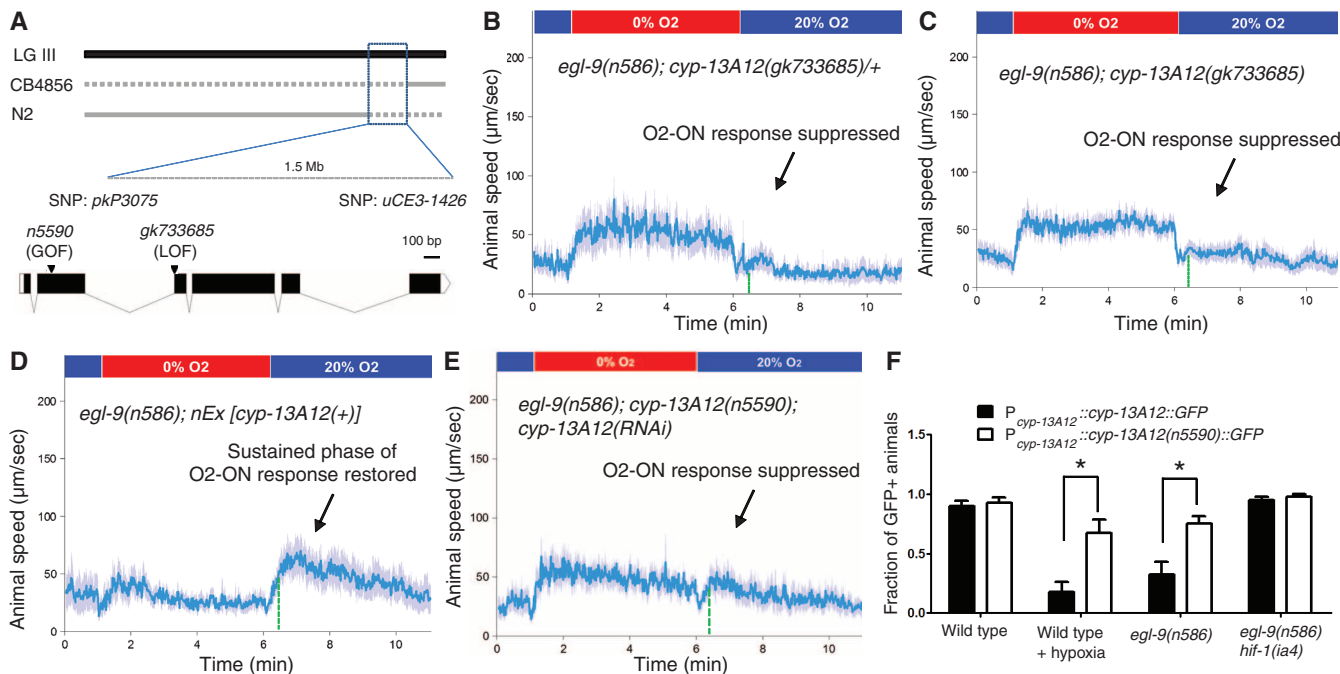
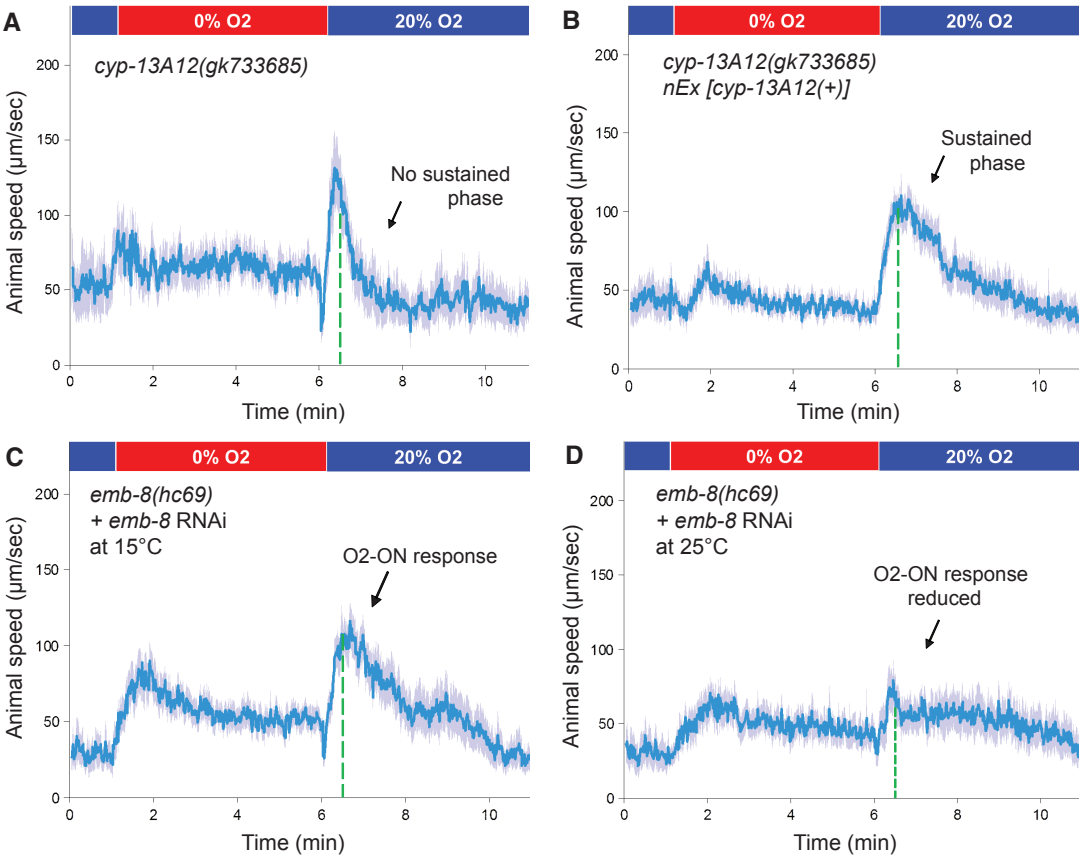


Fig. 2. *n5590* is a gain-of-function allele of *cyp-13A12*. (A) Genetic mapping positioned *n5590* between the SNPs *pkP3075* and *uCE3-1426*. Solid gray lines indicate genomic regions for which recombinants exhibited a defective O₂-ON response, thus excluding *n5590* from those regions. The locations 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 analysis of variance (ANOVA) with Bonferroni 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 to 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 grown 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 grown post-embryonically at the restrictive temperature of 25°C with simultaneous *E. coli*-feeding RNAi against *emb-8*, showing a reduced O₂-ON response.



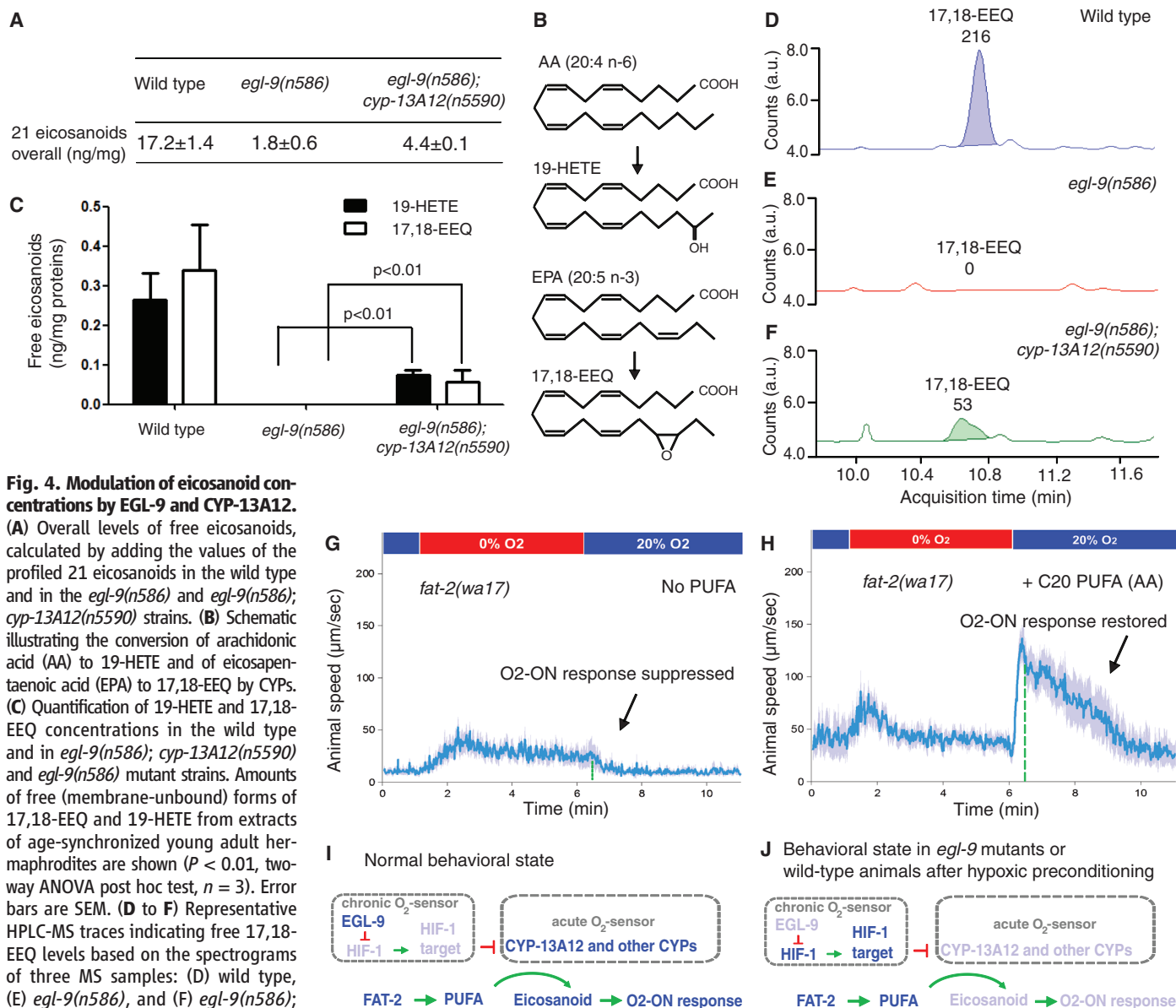


Fig. 4. Modulation of eicosanoid concentrations by EGL-9 and CYP-13A12.

(A) Overall levels of free eicosanoids, calculated by adding the values of the profiled 21 eicosanoids in the wild type and in the *egl-9(n586)* and *egl-9(n586); cyp-13A12(n5590)* strains. (B) Schematic illustrating the conversion of arachidonic acid (AA) to 19-HETE and of eicosapentaenoic acid (EPA) to 17,18-EEQ by CYPs. (C) Quantification of 19-HETE and 17,18-EEQ concentrations in the wild type and in *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 ($P < 0.01$, two-way ANOVA post hoc test, $n = 3$). Error bars are SEM. (D to 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* mu-

tants, showing the O₂-ON response rescued by C20 PUFA (AA) supplementation. (I and 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). Light blue indicates low protein activity, low amounts of eicosanoids, or a defective O₂-ON response.

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 $\omega 3$ -to- $\omega 6$ 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, little is known concerning the causal relationships among and mechanisms relating O₂ and PUFA homeostasis, CYP, and

PUFA-mediated cell signaling and organismal susceptibility to oxidative disorders. Our results identify a 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. Because the 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 and cellular

response to ischemia-reperfusion injury and that the molecular pathway including EGL-9-HIF-1 and CYPs in controlling responses to reoxygenation after anoxia is evolutionarily conserved.

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Supplementary Materials

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Materials and Methods

Supplementary Text

Figs. S1 to S11

Table S1

References (31–70)

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Robustness and Compensation of Information Transmission of Signaling Pathways

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Robust transmission of information despite the presence of variation is a fundamental problem in cellular functions. However, the capability and characteristics of information transmission in signaling pathways remain poorly understood. We describe robustness and compensation of information transmission of signaling pathways at the cell population level. We calculated the mutual information transmitted through signaling pathways for the growth factor–mediated gene expression. Growth factors appeared to carry only information sufficient for a binary decision. Information transmission was generally more robust than average signal intensity despite pharmacological perturbations, and compensation of information transmission occurred. Information transmission to the biological output of neurite extension appeared robust. Cells may use information entropy as information so that messages can be robustly transmitted despite variation in molecular activities among individual cells.

Signaling pathways transmit signals from growth factors to downstream gene expression, influencing various cell fate decisions such as cell differentiation (1). To control cellular responses by stimulation intensity, signaling pathways must reliably transmit stimulation intensity through their signaling activities. The reliability of signal transmission depends on the balance between signal intensity and variation. The smaller the signal variation, the more information can be transmitted through a pathway with the same dynamic range of signal in-

tensity. Even high-intensity signals cannot be reliably transmitted if the variation in signal intensity is large. In contrast, even signals with low intensity can be reliably transmitted if the variation in signal intensity is small (Fig. 1A). Thus, the reliability of signal transmission depends on both average (mean) intensity and variation. As a consequence, the number of controllable states of cellular responses is determined by the number of reliably transmitted signals. Intuitively, the larger the number of reliably transmitted signals, the more information the signal pathway can transmit. If cellular signaling pathways are treated as communication channels in the framework of Shannon's information theory (2–12), the amount of information that can be reliably transmitted through a cellular signaling pathway can be measured by mutual information, which corresponds to the logarithm of the average number of controllable states of a cellular response that can be defined by varied upstream signals (13–15).

We evaluated the information transmission from growth factors to the immediate early genes (IEGs) through various signaling pathways in PC12 cells. Nerve growth factor (NGF), pituitary

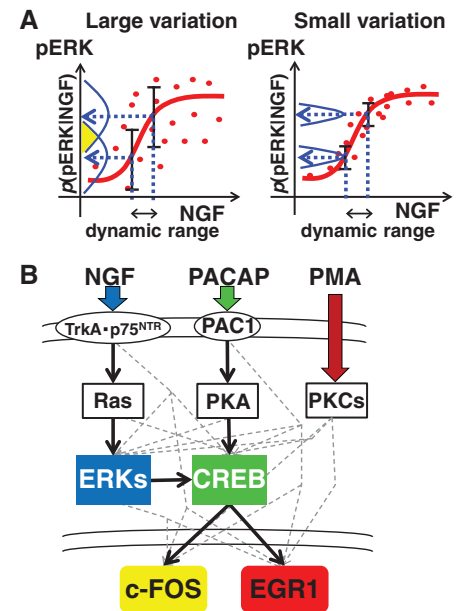


Fig. 1. Information transmission of signaling pathways. (A) Reliability of information transmission depends on both signal intensity and variation. More information can be transmitted with the same dynamic range of signal intensity if signal variation is smaller. Dots denote intensities of pERKs in individual cells, and lines denote the average intensity of pERKs. $p(\text{pERKs}|\text{NGF})$ denotes the distribution (a normalized histogram) of pERKs for a given dose of NGF. (B) Signaling pathways from growth factors, such as NGF, PACAP, and PMA to the IEGs, such as c-FOS and EGR1. Solid lines indicate the reported pathways for each growth factor, and gray dashed lines indicate other possible pathways. The colored boxes are the measured molecules, and white ovals are unmeasured molecules in this study.

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Editor's Summary

As the Worm Squirms

Restoration of oxygen supply to cells that have been deprived of oxygen actually causes further damage to cells and tissues. Such responses, known as reperfusion injury, contribute to the deadly effects of heart attacks and strokes in humans. Lack of oxygen is sensed directly by a prolyl hydroxylase known as EGL-9 in worms and EGLN2 in mammals. Inhibition of EGL-9 can reduce damage caused by reperfusion of tissues with oxygen, but how such beneficial effects are mediated is not clear. **Ma *et al.*** (p. 554, published online 27 June) used a genetic screen in the nematode *Caenorhabditis elegans*, which has a behavioral response to reoxygenation in which the animals increase their movement, to find factors that interact with EGL-9. They identified the cytochrome p450 oxygenase CYP-13A12 as such a factor. Some cytochrome p450 enzymes act on polyunsaturated fatty acids to make cellular signaling molecules known as eicosanoids. The effects of CYP-13A23 were mediated by eicosanoids. Because the regulatory pathways involved appear to be evolutionarily conserved, the results may aid understanding and management of reperfusion injury in humans.

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