Divergent mechanisms controlling hypoxic sensitivity and lifespan by the DAF-2/insulin/IGF-receptor pathway

Meghann E. Mabon
Washington University School of Medicine in St. Louis

Barbara A. Scott
Washington University School of Medicine in St. Louis

C. Michael Crowder
Washington University School of Medicine in St. Louis

Follow this and additional works at: https://digitalcommons.wustl.edu/open_access_pubs

Part of the Medicine and Health Sciences Commons

Recommended Citation
Mabon, Meghann E.; Scott, Barbara A.; and Crowder, C. Michael, "Divergent mechanisms controlling hypoxic sensitivity and lifespan by the DAF-2/insulin/IGF-receptor pathway," PLoS One. 4,11. e7937. (2009).
https://digitalcommons.wustl.edu/open_access_pubs/769

This Open Access Publication is brought to you for free and open access by Digital Commons@Becker. It has been accepted for inclusion in Open Access Publications by an authorized administrator of Digital Commons@Becker. For more information, please contact vanam@wustl.edu.
Divergent Mechanisms Controlling Hypoxic Sensitivity and Lifespan by the DAF-2/Insulin/IGF-Receptor Pathway

Meghann E. Mabon1,3, Barbara A. Scott1, C. Michael Crowder1,2,3*

1 Department of Anesthesiology, Washington University School of Medicine, St. Louis, Missouri, United States of America, 2 Department of Developmental Biology, Washington University School of Medicine, St. Louis, Missouri, United States of America, 3 The Division of Biology & Biomedical Sciences, Program in Developmental Biology, Washington University School of Medicine, St. Louis, Missouri, United States of America

Abstract
Organisms and their cells vary greatly in their tolerance of low oxygen environments (hypoxia). A delineation of the determinants of hypoxia tolerance is incomplete, despite intense interest for its implications in diseases such as stroke and myocardial infarction. The insulin/IGF-1 receptor (IGFR) signaling pathway controls survival of Caenorhabditis elegans from a variety of stressors including aging, hyperthermia, and hypoxia. daf-2 encodes a C. elegans IGFR homolog whose primary signaling pathway modulates the activity of the FOXO transcription factor DAF-16. DAF-16 regulates the transcription of a large number of genes, some of which have been shown to control aging. To identify genes that selectively regulate hypoxic sensitivity, we compared the whole-organismal transcriptomes of three daf-2 reduction-of-function alleles, all of which are hypoxia resistant, thermotolerant, and long lived, but differ in their range of severities for these phenotypes. The transcript levels of 172 genes were increased in the most hypoxia resistant daf-2 allele, e1370, relative to the other alleles whereas transcripts from only 10 genes were decreased in abundance. RNAi knockdown of 6 of the 10 genes produced a significant increase in organismal survival after hypoxic exposure as would be expected if down regulation of these genes by the e1370 mutation was responsible for hypoxia resistance. However, RNAi knockdown of these genes did not prolong lifespan. These genes definitively separate the mechanisms of hypoxic sensitivity and lifespan and identify biological strategies to survive hypoxic injury.

Introduction
Low ambient oxygen concentrations can induce cell death. However, cells vary greatly in the level and duration of hypoxia that is required to produce their death. Even within the same organism hypoxic sensitivity can range widely. In humans, neurons and cardiac myocytes are exquisitely hypoxia sensitive, which can lead to stroke and myocardial infarction. Thus, identification of the determinants of hypoxic sensitivity has both biological and medical significance.

We have previously shown that reduction-of-function mutations in the daf-2 gene confer powerful protection from hypoxic injury in the nematode Caenorhabditis elegans [1]. daf-2 encodes an insulin/IGF-receptor homolog that signals through a conserved PI-3 kinase signaling cascade to regulate negatively the activity of a FOXO transcription factor, DAF-16 [2,3]. daf-2 reduction-of-function mutants are not only hypoxia resistant but are also thermotolerant, long lived, and have a propensity to form dauer larvae, which are capable of long periods of hibernation in response to environmental stress [4]. Because of the very interesting biological processes controlled by the daf-2 – daf-16 pathway, considerable effort has been made to identify the downstream targets of the pathway [5,6,7,8,9,10,11,12]. The various methods used in these studies along with the different phenotypes screened for produced only modestly overlapping gene sets. Thus, DAF-16 appears directly or indirectly to control the expression of a large number of diverse genes that often influence only a specific daf-2 phenotype.

Multiple daf-2 alleles have been isolated, primarily in screens for mutants that constitutively form dauers. Gems et al. measured phenotypes for sixteen daf-2 alleles and found lack of concordance for the severity of the different phenotypes in several cases [13]. For example, daf-2(e1370) is moderately long lived and dauer constitutive but was the most thermotolerant of all alleles tested. On the other hand, daf-2(e1368) was considerably less thermotolerant and dauer constitutive than e1370 but was found to have a median lifespan about 50% longer than e1370. We measured the hypoxic sensitivity of thirteen daf-2 alleles and found a wide range of severities [1]. For example, e1370 was the most hypoxia resistant daf-2 allele tested while e1368 was very weakly resistant. The mechanism underlying this divergence of phenotypic severities among alleles is obscure.

In the current study utilizing cDNA microarrays and double stranded RNA-mediated interference (RNAi) of the expression of candidate genes, we take advantage of the daf-2 allelic variations to identify genes regulated by daf-2 that selectively alter hypoxic
sensitivity without affecting lifespan. The identity of these genes implicates novel pathways for the selective regulation of hypoxic sensitivity.

**Results**

**daf-2 Alleles Reveal Divergent Outputs for Hypoxic Survival and Lifespan**

We chose three *daf-2* alleles for our study *e1370, m596*, and *e1368*. We previously reported that after recovery from 20 hours of hypoxic incubation, whole organism survival for these alleles was 96%, 53%, and 23%, respectively, compared to 4% for the wild type strain N2 [1]. Gems et al. scored the dauer constitutive, thermotolerance, and long lifespan phenotypes of these alleles [13]. However, the severities of *daf-2* phenotypes have varied considerably among labs, particularly for lifespan. Thus, we scored the lifespan, thermotolerance, dauer constitutive, and hypoxia resistant phenotypes of all three *daf-2* alleles contemporaneously to confirm their phenotypic divergence. We again found that *e1370* was strongly hypoxia resistant, *m596* moderately resistant, and *e1368* weakly so (Figure 1A). The order of severities for the alleles was quite similar for thermotolerance (Figure 1B). However, for lifespan *m596* was the strongest allele having a mean lifespan of 31.3 days compared to 22.5 days for *e1370* and 18.8 for *e1368* (Figure 1C and Table 1). For the dauer constitutive phenotype, *m596* was the most severe allele; *e1370* was a close second while *e1368* was a much weaker allele (Figure 1D,E,F). The results are generally consistent with those previously reported by Gems et al. [13]. These authors reported that *e1370* was most thermotolerant, followed closely by *m596*, and more distantly by *e1368*.

**Transcriptional Profiling of daf-2 Alleles**

The transcriptional output of the *daf-2 - daf-16* pathway and the distinct *daf-2* allelic series for hypoxia versus lifespan and dauer

**Table 1. Lifespan analysis of daf-2 strains.**

| Strain           | Mean (d) | Maximum (d) | n  |
|------------------|----------|-------------|----|
| N2               | 14.9     | 29          | 87 |
| *daf-2(e1368)*   | 18.8*    | 37*         | 86 |
| *daf-2(m596)*    | 31.3*    | 51*         | 77 |
| *daf-2(e1370)*   | 22.5*    | 51*         | 86 |

Day 1 begins at first day of adulthood. ^p<0.01 vs N2; ^p<0.01 vs *e1368* and *m596.*

doi:10.1371/journal.pone.0007937.g001
formation offer a means to identify genes in the *daf-2 - daf-16* pathway that specifically regulate hypoxia. We hybridized whole genome oligonucleotide microarrays with cDNA derived from *e1370, m596,* or *e1368* L4 stage larval animals grown under normal conditions. Comparisons of the transcriptome across alleles revealed 182 genes that were statistically different (*p*<0.01) between *e1370* and *m596* and/or *e1368* (Figure 2A); Raw data have been deposited in the NCBI Gene Expression Omnibus database ([http://www.ncbi.nlm.nih.gov/geo/](http://www.ncbi.nlm.nih.gov/geo/), accession number GSE18601. 172 genes were up-regulated in *e1370* compared to *m596* and/or *e1368* (Table S1), and 10 were relatively down-regulated in *e1370* (Table 2, Figure 2B). The vast majority of the *e1370*-upregulated genes had a greater increase in expression relative to *m596* than relative to *e1368*. These results suggest that the majority of these genes may regulate lifespan, a trait where *e1370* diverges more from *m596* than *e1368*. The expression of only six of the 182 genes followed the hypoxic phenotypic allelic series, that is either up- or down-regulated in the order of *e1370, m596,* and *e1368* (Table 3). Of these, only one, C17C3.12, encodes a strong mammalian homolog, a mitochondrial acyl-CoA dehydrogenase, which functions catabolically in mammals in fatty acid oxidation. In general, genes involved in regulation of metabolic processes were highly represented in the 182 gene list (Figure 2C). Transcriptional/DNA/RNA processing genes were also abundant.

A number of previous studies have identified genes whose expression is regulated by *daf-2* [7,8,9,10,11,12,14,15]. These studies have implicated a large number of genes as being regulated by the *daf-2 - daf-16* pathway. However, the overlap among the datasets is relatively modest presumably due to the distinct methodologies employed by each study. Perhaps because of the experimental design of the microarrays that sought gene expression differences among *daf-2* alleles, the overlap between the 182 genes identified here and those from the other studies was also modest with only seven genes in common (Table 4). Four of the seven genes had expression changes that were qualitatively similar to those found previously. For example, the Y54G11A.13/*ctl-3* gene encodes one of three *C. elegans* catalases that are predicted to reduce peroxide free radicals. The *ctl-3* transcript is increased in *e1370* compared to both *e1368* and *m596*. Likewise using quantitative mass spectrometry methods, Dong et al. found that CTL-3 protein was increased in *e1370* compared to wild type worms [14]. Similarly, both our data and that from Murphy et al. [9] show increased expression in *e1370* mutants of unc-38, which encodes a subunit of a nicotinic acetylcholine receptor.

Ten genes were down-regulated in *e1370* (die) compared to the other alleles (Table 2, Figure 2B). These genes include *akt-2*, a homolog of the serine/threonine protein kinase Akt/PKB. *akt-2* along with its paralog, *akt-1*, functions downstream of *daf-2* in the *daf-2 - daf-16* pathway. Knockdown of *akt-1* and *akt-2* produce a partially redundant dauer constitutive phenotype [16], and an *akt-1* gain-of-function mutant partially suppresses the hypoxia resistance of *daf-2(e1370)* [1]. The role of AKT-1/2 downstream of DAF-2 is thought to be through phosphorylation of DAF-16, thereby inhibiting its nuclear localization and transcription factor activity [17,18]. These new data suggest that a positive feedback loop may exist that enhances the reduction-of-function phenotype of *e1370* compared to *m596* and *e1368* by reducing the levels of *akt-2* mRNA. Besides *akt-2*, two other *die* genes are predicted to have kinase or phosphatase activity, but their substrates are unknown. Other particularly interesting *die* genes include *acdh-2*, which as noted above has expression differences in the alleles that follow the hypoxic sensitivity allelic series, F54G2.1 - an isoform of the presynaptic vesicle fusion protein UNG-13, F36F2.3 - an E3 ubiquitin ligase, and T04A11.6 - an ATP-dependent DNA helicase. We previously found in a whole-genome RNAi screen for genes that promote hypoxic death that knockdown of several genes, predicted to control chromatin structure, also produce significant hypoxia resistance [19]. The current data show that the *daf-2* pathway acts in part through regulation of DNA/chromatin modifying enzymes to control hypoxic sensitivity. However, it is unclear how this protects the organism from hypoxic injury.

**Increased Hypoxic Survival by *die* Gene Knockdown**

Genes whose expression was increased in *e1370* relative to the other two alleles are candidates to encode hypoxia protective factors. We chose 10 genes with the highest normalized relative

---

**Figure 2. Expression of 182 genes are differentially regulated across *daf-2* alleles.** (A) Heat map of expression of 182 genes, either up- or down-regulated between *e1370* and *m596* and/or *e1368*, *p*<0.01, One-Way ANOVA. (B) Genes whose expression is downregulated in *e1370* (die) relative to *m596* and/or *e1368*, *p*<0.01. (C) Relative number of *daf-2* regulated genes that fall into the various functional categories. doi:10.1371/journal.pone.0007937.g002
Knockdown of all six genes produced strong hypoxia resistance (Figure 3A). We then tested the effect of RNAi to suppress genes that were tested could mediate responses either maladaptive or beneficial, or neither. Therefore, the suppression of the hypoxia resistance was incomplete for five of the six RNAi knockdowns. Thus, these genes do not regulate hypoxic sensitivity entirely via a DAF-16 dependent pathway.

The *die* genes are candidates to promote *daf-2*-regulated hypoxic sensitivity; thus, *die* gene reduction-of-function might produce hypoxic resistance. To test this hypothesis, we used RNAi to knockdown six of the ten *die* genes; of the four not tested, two, C17C3.12 and F28H6.1, were not in our library, one other, T22C1.8, did not grow when streaked, and the fourth, F36F2.3, was not in our library; one other, F58D2.1 did not grow when streaked, and the fourth, F36F2.3, were not in our library. One other, F28H6.1, was not in our library. These results show that reduction of function of any one of these genes is capable of converting the weakly hypoxia resistant *e1368* animals to strongly resistant ones. Also, the sensitized background of *e1368* revealed that F58D2.1 does in fact encode a hypoxic sensitivity promoting activity that was not apparent in a wild type background.

Given that expression of *sensitivity* entirely via a DAF-16 dependent pathway.

Table 2. Genes transcriptionally down-regulated in *e1370* vs *m596* or *e1368* – *die* genes.

| Sequence Name | Gene Name | Protein/Homology | Biological Process | Expression | p-value |
|---------------|-----------|------------------|--------------------|------------|---------|
| F36F2.3       | Predicted E3 ubiquitin ligase | Protein Degradation/ Modification/Turnover | ND         | 0.0019 | −0.6 −2.6 −2.3 |
| ZK262.8       | Synthetic lethal with Argonaute mutant | Unknown | ND | 0.0021 | −3.6 −1.9 −1.9 |
| T22C1.8       | Protein tyrosine phosphatase | Signaling | ND | 0.0027 | −5.2 −2.5 −2.1 |
| F54D11.3      | C. elegans-specific | Unknown | ND | 0.0048 | −7.1 −2.6 −2.7 |
| F58D2.1       | ZYG-11-like serine/threonine protein kinases | Unknown | ND | 0.0059 | −2.2 −1.5 −1.5 |
| C17C3.12      | acdh-2 | Mitochondrial short-chain acyl-CoA dehydrogenase | Metabolism | ND | 0.0060 | −2.4 −3.6 −1.5 |
| F28H6.1       | akt-2 | Homolog of the serine/threonine kinase | Signaling | Intestine, Neuronal | 0.0061 | −2.7 −1.3 −2.1 |
| F54G2.1       | Isoform 1 of protein UNC-13 homolog D | Unknown | Intestine, Neuronal | 0.0072 | −2.5 −1.6 −1.6 |
| T20D4.18      | snab-2 | Sra family integral membrane protein | Unknown | Hypodermal, Neuronal | 0.0077 | −2.1 −1.4 −1.5 |
| T04A11.6      | hmd-1 | ATP-dependent DNA helicase | Transcription/DNA/RNA Processing | Primarily Germine | 0.0086 | −2.9 −1.5 −1.9 |

Table 3. Genes regulated according to the allelic series.

| Sequence Name | Common Name | *e1370* vs *m596* | *e1368* vs *e1370*
|---------------|-------------|-----------------|-----------------|
| C14A4.8       |             | 2.19 0.90 0.85  |                  |
| Y37H2A.6      | *fbxa-211*  | 1.20 0.92 0.90  |                  |
| F4SD11.4      |             | 3.64 0.62 0.34  |                  |
| F4SD11.14     |             | 3.70 0.54 0.28  |                  |
| C17C3.12      | *acdh-2*    | 0.79 1.88 2.86  |                  |
| T26H5.1       |             | 0.83 1.17 1.20  |                  |

Table 4. Genes that overlap with other datasets.

| Sequence Name | Overlapping Study | Expression in *e1370* relative to *m596*, *e1368* | Expression in *e1370* relative to wild type* |
|---------------|-------------------|-----------------------------------------------|---------------------------------------------|
| C09G4.5       | Murphy            | Increased                                     | Decreased                                   |
| C47E8.5       | Dong              | Increased                                     | Decreased                                   |
| F15E11.1      | Dong              | Increased                                     | Increased                                   |
| F17C11.9      | Dong              | Increased                                     | Decreased                                   |
| F21F3.5       | Murphy            | Increased                                     | Increased                                   |
| F4SD11.14     | Dong              | Increased                                     | Increased                                   |
| Y54G11A.13    | Dong              | Increased                                     | Increased                                   |

Dong et al. *Nature* 424:277-83, 2003. Dong et al. Science 317:660-3, 2007.

'data from overlapping study.'
of the five die genes where knockdown produced hypoxia resistance in a wild type background. Knockdown of four of the five die genes produced significant thermotolerance (Figure 4). Only ZK262.8 RNAi did not protect from thermal stress (Figure 4B). These results are consistent with the strong correlation between thermotolerance and hypoxia resistance observed among various daf-2 alleles [1]. However, they also show that the mechanisms of thermal injury and hypoxic injury in C. elegans are not identical. Despite a considerable increase in both mean and maximum lifespan by daf-2 RNAi, none of the die gene RNAi’s resulted in a significant alteration in lifespan (Figure 5 and Table S2). Again, these results are consistent with the poor correlation between the lifespan and hypoxic sensitivity phenotypes of daf-2 alleles [1].

One possibility for the strong correlation between thermotolerance and hypoxia resistance is the 26.5°C temperature at which the hypoxic incubations are performed. As in all models of hypoxic injury, the sensitivity of C. elegans to hypoxia is temperature dependent [1]; the higher the temperature the more severe the hypoxic injury. However, a number of observations suggest that the hypoxic sensitivity differences among daf-2 alleles is not simply a manifestation of varied thermotolerance. First, two daf-2 alleles, e979 and e1369 with only moderate hypoxia resistance similar to that of m596, have thermotolerances at least as high as e1370 [15]. Second, when hypoxic incubations are performed at 20°C, e1370 and m579, which is highly hypoxia resistant under our assay conditions [1], are still highly hypoxia resistant, whereas m596 is not [20]. Finally, we asked whether high temperature incubation in buffer but in a room air environment produced significant wild type animal death that might account for the daf-2 allelic differences for hypoxic sensitivity at higher temperature. However, we observed no significant death of wild type animals after a 20 hour normoxic incubation at 28°C (Figure S1). Even a 71 hour incubation killed only 5% of animals.

**Discussion**

Utilizing daf-2 alleles with distinct orders of phenotypic severity for hypoxic sensitivity versus its other phenotypes as the basis for DNA microarray experiments, we identified 182 genes that were differentially expressed in animals with varying levels of hypoxia resistance. The expression of most of these genes was increased in the hypoxia resistant daf-2(e1370). These genes are candidates for antagonizing hypoxic death. However, we were unable to detect hypoxic hypersensitivity by RNAi knockdown of a subset of these genes. In general, we have found hypoxic hypersensitivity is difficult to produce in our model; this may reflect genetic redundancy and is reminiscent of the relatively uncommon phenotype of shortened lifespan as compared to prolonged lifespan. However, for the ten genes that were decreased in daf-2(e1370), we were able to test six of them and found that knockdown of each of them produced hypoxia resistance. These genes are of interest not only for the fact that they control hypoxic sensitivity but that they also do it in a relatively specific manner by having no detectable influence on lifespan. In this regard, ZK262.8 is the most intriguing because unlike the other five die genes with a hypoxia resistance knockdown phenotype, ZK262.8 knockdown does not confer thermotolerance. ZK262.8 is a poorly characterized gene with no clear homology to genes in other organisms. It was implicated in microRNA processing because of a synthetic lethal phenotype with a mutation in the Argonaute homolog gene, alg-2 [21]. ALG-2 is expressed in most, if not all, cells in C. elegans from embryonic to adult stages and is hypothesized to facilitate loading of pre-miRNAs into the Dicer complex [21]. However, it is unclear what role if any the ZK262.8 gene product has in miRNA processing.

Another hypoxic death promoting die gene identified is srab-21, which encodes a serpentine receptor that is likely to function in chemosensation [22]. In a whole genome RNAi screen for genes promoting hypoxic death, we identified eleven serpentine receptor genes where knockdown produced hypoxia resistance [19]. These genes represented about 5% of the genes identified in the screen. Given that these genes are thought to encode chemosensory receptors, normal chemosensory function appears to promote hypoxic sensitivity in C. elegans. Likewise for aging, mutations in
genes required for chemosensation prolong lifespan [23]. The prolonged lifespan of these mutants could be suppressed by mutations in *daf-16*, and a model was proposed whereby activation of chemosensory neurons increases insulin-like hormone signaling and thereby limits lifespan. However, the fact that *srab-21* knockdown does not prolong lifespan suggests that simply altering ligand activation of DAF-2 is an unlikely mechanism.

The identification of the ATP-dependent DNA helicase gene *him-6* as a *die* gene that promotes hypoxic death is consistent with previous results. In our whole genome RNAi screen, we identified a large number of DNA processing genes whose knockdown phenotype is hypoxia resistance [19]. Indeed, nucleic acid binding and processing enzyme genes represented the largest functional category with several helicases among them. *him-6* is expressed primarily in the germline as assessed by microarray experiments comparing animals with and without a germline [24]. Thus, some expression in somatic cells is possible. Germ cells from *him-6* mutant animals have increased sensitivity to ionizing radiation, but surprisingly have decreased germ cell apoptosis after genotoxic stress [25]. Thus, it is possible that the hypoxia resistance is secondary to defects in apoptosis but this remains to be determined.

We and others have identified a significant number of genes that regulate hypoxic sensitivity [1,19,26,27,28,29]. In no case, is there a complete understanding of the mechanism(s) whereby a gene protects the organism and its cells from hypoxic injury, and in most cases, as for the genes identified here, there is no mechanistic information at all. Future studies must necessarily focus on the most promising hypoxic sensitivity genes. Such genes would have clear mammalian orthologs, preferably with a considerable understanding of their cellular function already defined. Additionally, the most promising genes should be able to be modulated to control hypoxic injury without producing other side effects.
Thus, studies such as the one described here are important in order to identify specific pathways to target for hypoxic protection.

**Materials and Methods**

**Nematode Maintenance**

For strain maintenance worms were cultured at 20°C on nematode growth medium (NGM) agar plates seeded with OP50 bacteria [30]. The following strains were utilized in this study: N2 (wild-type), CB1370 *daf-2(e1370)* III, DR1565 *daf-2(m596)* III, DR1572 *daf-2(e1368)* III, and CF1038 *daf-16(mu86)* I. Strains were obtained from the *Caenorhabditis* Genetics Center funded by the NIH NCRR.

**Hypoxia Assay**

Worms were subjected to hypoxia as described previously [1]. Briefly, each plate was washed into one 1.5 mL tube with 1 mL of M9 buffer (22 mM KH$_2$PO$_4$, 2 mM Na$_2$HPO$_4$, 85 mM NaCl, 1 mM MgSO$_4$) [30]. Worms were allowed to settle by gravity, and 800 µL of M9 was removed. The tubes were then placed in the anaerobic chamber (Forma Scientific) at 26.5°C for 20, 22, or 24 hours, depending on the assay. Following the hypoxic insult, worms were placed on NGM plates spotted with OP50 bacteria using glass Pasteur pipets and recovered at 20°C for 16–20 hours prior to scoring. Animals were scored as dead if no pharyngeal pumping or spontaneous or evoked movement (touching with a platinum wire) was observed. A minimum of triplicate plates for each trial and three trials/RNAi were scored.

**Feeding RNAi**

One-generation feeding RNAi was performed as described previously [19,31]. Briefly, two gravid adult worms were left on RNAi plates with 2 day old bacterial lawns for 3 hours to obtain 30–50 eggs per plate. RNAi plates were composed of NGM agar supplemented with 50 µg/ml carbenicillin and 1 mM IPTG and seeded with the appropriate RNAi bacterial strain cultured in 2xYT with 50 µg/ml carbenicillin, 10 µg/ml tetracycline and 0.8 mM IPTG. Animals were grown at 20°C to young adulthood, one day past the L4 stage and then phenotyped.

**Lifespan Assays**

Lifespan assays were performed either on OP50 bacteria (Figure 1C) or on RNAi bacteria containing the L4440 empty vector control plasmid or the particular RNAi plasmid (Figure 5).

---

**Figure 5. No effects on lifespan by die gene RNAi knockdown.** Lifespan survival curves for animals grown on L4440 empty vector bacteria versus RNAi bacteria. (A) The effect of *daf-2* RNAi on lifespan is shown as a positive control and is significantly different from L4440 lifespan – p < 0.01 Mantel-Cox Log-rank test. (B-F) None of the other RNAis significantly lengthen lifespan at the p < 0.01 level. Lifespan assays were performed with 120 animals/RNAi grown at 20°C.

doi:10.1371/journal.pone.0007937.g005
Gravid adult worms were allowed to lay eggs for three hours onto OP50 or RNAi bacteria and picked off. The animals were cultured at 20°C until reaching the L4 stage and were then placed at a density of 30 worms/plate. For the daf-2 allele lifespan data, the L4 animals were transferred to fresh NGM plates seeded with OP50. For the daf-2 gene RNAi lifespan experiments, L4 animals were transferred to new RNAi plates. Worms were transferred daily while egg-laying and transferred every three days thereafter onto plates with freshly seeded OP50 or RNAi bacteria. Worms were scored daily for survival and censored for bagging, exploding, and crawling off the plate.

Thermotolerance Assay
Thermotolerance assays were performed in a 37°C water bath. Young adult worms, one day post L4 stage, were washed into tubes using M9 buffer and allowed to settle by gravity. Excess M9 was removed to 100 µL M9 buffer preheated to 37°C was added to each tube to a final volume of 500 µL. The tubes were incubated in the water bath according to the time course. Worms were removed from the tubes at the appropriate time using a glass Pasteur pipette and allowed to recover at 20°C for 16–20 hours on NGM plates spotted with OP50 bacteria and then scored for survival.

Dauer Arrest Assay
Ten gravid adult animals were allowed to lay eggs for one hour and picked off. Plates containing 30–50 eggs were put at 20°C, 23°C or 25°C. Animals were scored for dauer arrest when the non-dauer animals reached adulthood, 72 h or 96 h later. The fraction of non-adult animals that were partial dauers or arrested non-dauer larvae was not formally scored; however, the large majority of the non-adult animals were true dauers for all three strains as previously reported [13].

cDNA Microarrays
Animals from a synchronous egg lay were cultured at 20°C on NGM plates seeded with OP50, and total RNA was isolated from L4 staged daf-2(e1370), daf-2(m596) and daf-2(e1368) animals using Trizol reagent (Invitrogen). Dauer animals were separated from L4 larvae by differential pelleting in M9. RNA quality was measured using Nanodrop-1000 spectrophotometer (Thermo-Scientific). High quality RNA samples (OD 260/280>1.9) were used as inputs for microarrays. Eight micrograms of RNA was subjected to reverse transcription and subsequent hybridization using 3DNA Array 350 Kit (Genisphere, Inc, Hatfield, PA). Cy3- and Cy5-labeled samples were hybridized to C. elegans whole genome oligonucleotide microarrays (Genome Sequencing Center, Washington University in St. Louis). Slides were scanned on a ScanArray 3000 (Perkin Elmer, Waltham, MA) at 10 micrometer resolution. Three biological replicates (independent RNA isolations), each with a technical replicate (dye swap) were performed for each condition. Raw microarray fluorescent intensity data were processed and analyzed with GeneSpring GX 7.3.1 software (Agilent Technologies, Santa Clara, CA). Each time point was subjected to Lowess normalization (per spot and per array) separately. A flag filter was applied to include all genes that hybridized in three or more trials which narrowed the gene list to 14,317. One-way ANOVA (p<0.01) was performed to find genes that were statistically different between e1370 and the other two daf-2 alleles, m396 and e1368. This gene list included 102 genes. Raw data have been deposited in the NCBI Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/), accession number GSE18601.

Supporting Information
Table S1 List of genes upregulated in daf-2(e1370). Found at: doi:10.1371/journal.pone.0007937.s001 (0.06 MB XLS)
Table S2 Lifespan analysis of daf gene RNAi knockdown. Found at: doi:10.1371/journal.pone.0007937.s002 (0.03 MB XLS)

Figure S1 Comparison of normoxic and hypoxic lethality. Young adult N2 animals were incubated at 28 degrees in M9 buffer in either an incubator with room air atmosphere (normoxia) or in an incubator with <0.3% oxygen (hypoxia). The % of dead animals was scored after recovery from various incubation times. Each point represents the mean ±/− sd of a minimum of two trials with at least 16 animals/trial. Found at: doi:10.1371/journal.pone.0007937.s003 (2.48 MB TIF)

Acknowledgments
We thank Elaine Mardis and the Genome Center at Washington University in St. Louis for their help with the design and performance of the DNA microarray experiments.

Author Contributions
Conceived and designed the experiments: MEM CMC. Performed the experiments: MEM BAS CMC. Contributed reagents/materials/analysis tools: CMC. Wrote the paper: MEM BAS CMC.

References
1. Scott BA, Avidan MS, Crowder CM (2002) Regulation of hypoxic death in C. elegans by the insulin/IGF receptor homolog DAF-2. Science 296: 2388–2391.
2. Ogg S, Paradis S, Gottlieb S, Patterson GI, Gottlieb S, et al. (1997) The Fork head family member that can function to double the life-span of Caenorhabditis elegans. Nature 389: 994–999.
3. Lin K, Dorman JB, Rodan A, Kenyon C (1997) daf-16: An HNF-3/forkhead family member that can function to double the life-span of Caenorhabditis elegans. Nature 389: 994–999.
4. Riddle DL, Albert PS (1997) Genetic and environmental regulation of dauer larva development. In: Riddle DL, Blumenthal T, Meyer BJ, Priess JR, eds. Caenorhabditis elegans. Cold Spring Harbor: Cold Spring Harbor Laboratory Press. pp 739–768.
5. Lamitina ST, Strange K (2005) Transcriptional targets of DAF-16 insulin signaling pathway protect C. elegans from extreme hypertonic stress. Am J Physiol Cell Physiol 288: C467–474.
6. Lee SS, Kennedy S, Tolonen AC, Ruvkun G (2003) DAF-16 target genes that control C. elegans life-span and metabolism. Science 300: 644–647.
7. Murphy CT, McCarroll SA, Bargmann CI, Fraser A, Kamath RS, et al. (2003) Genes that act downstream of DAF-16 to influence the lifespan of Caenorhabditis elegans. Nature 424: 277–283.
8. Dong M-Q, Venable JD, Au N, Xu T, Park SK, et al. (2007) Quantitative Mass Spectrometry Identifies Insulin Signaling Targets in C. elegans. Science 317: 660–663.
9. Scott BA, Avidan MS, Crowder CM (2002) Regulation of hypoxic death in C. elegans by the insulin/IGF receptor homolog DAF-2. Science 296: 2388–2391.
10. McElwee J, Bubb K, Thomas JH (2003) Transcriptional outputs of the DAF-2 insulin receptor-like pathway in Caenorhabditis elegans. Nature 424: 277–283.
11. Lee SS, Kennedy S, Tolonen AC, Ruvkun G (2003) DAF-16 target genes that control C. elegans life-span and metabolism. Science 300: 644–647.
12. Yu H, Larsen PL (2001) DAF-16-dependent and independent expression targets of DAF-2 insulin-receptor-like pathway in Caenorhabditis elegans include FKBPs. J Mol Biol 314: 1017–1028.
13. Gems D, Sutton AJ, Sanderley ML, Albert PS, King KV, et al. (1998) Two pleiotropic classes of daf-2 mutation aﬀect larval arrest, adult behavior, reproduction and longevity in Caenorhabditis elegans. Genetics 150: 129–135.
14. Lamitina ST, Strange K (2005) Transcriptional targets of DAF-16 insulin signaling pathway protect C. elegans from extreme hypertonic stress. Am J Physiol Cell Physiol 288: C467–474.
15. Murphy CT, McCarroll SA, Bargmann CI, Fraser A, Kamath RS, et al. (2003) Genes that act downstream of DAF-16 to influence the lifespan of Caenorhabditis elegans. Nature 424: 277–283.
16. Dong M-Q, Venable JD, Au N, Xu T, Park SK, et al. (2007) Quantitative Mass Spectrometry Identifies Insulin Signaling Targets in C. elegans. Science 317: 660–663.

Supporting Information
Table S1 List of genes upregulated in daf-2(e1370). Found at: doi:10.1371/journal.pone.0007937.s001 (0.06 MB XLS)
Table S2 Lifespan analysis of daf gene RNAi knockdown. Found at: doi:10.1371/journal.pone.0007937.s002 (0.03 MB XLS)

Figure S1 Comparison of normoxic and hypoxic lethality. Young adult N2 animals were incubated at 28 degrees in M9 buffer in either an incubator with room air atmosphere (normoxia) or in an incubator with <0.3% oxygen (hypoxia). The % of dead animals was scored after recovery from various incubation times. Each point represents the mean ±/− sd of a minimum of two trials with at least 16 animals/trial. Found at: doi:10.1371/journal.pone.0007937.s003 (2.48 MB TIF)

Acknowledgments
We thank Elaine Mardis and the Genome Center at Washington University in St. Louis for their help with the design and performance of the DNA microarray experiments.

Author Contributions
Conceived and designed the experiments: MEM CMC. Performed the experiments: MEM BAS CMC. Analyzed the data: MEM BAS CMC. Contributed reagents/materials/analysis tools: CMC. Wrote the paper: MEM BAS CMC.
15. Halaschek-Wiener J, Khattria JS, McKay S, Pouzyrev A, Stott JM, et al. (2005) Analysis of long-lived C. elegans daf-2 mutants using serial analysis of gene expression. Genome Res 15: 603–615.

16. Paradis S, Ruvkun G (1998) Caenorhabditis elegans Akt/PKB transduces insulin receptor-like signals from AGE-1 PTK kinase to the DAF-16 transcription factor. Genes Dev 12: 2488–2498.

17. Lee KY, Hensch J, Ruvkun G (2001) Regulation of C. elegans DAF-16 and its human ortholog FKHRL1 by the daf-2 insulin-like signaling pathway. Curr Biol 11: 1950–1957.

18. Henderson ST, Johnson TE (2001) daf-16 integrates developmental and environmental inputs to mediate aging in the nematode Caenorhabditis elegans. Curr Biol 11: 1975–1980.

19. Mabon ME, Mao X, Jiao Y, Scott BA, Crowder CM (2009) Systematic identification of gene activities promoting hypoxic death. Genetics: in press.

20. Mendenhall AR, LaRue B, Padilla PA (2006) Glyceraldehyde-3-phosphate dehydrogenase mediates anaerobic response and survival in Caenorhabditis elegans. Genetics 174: 1173–1187.

21. Tops BBJ, Plasterk RHA, Ketting RF (2006) The Caenorhabditis elegans Argonautes ALG-1 and ALG-2: Almost Identical yet Different. Cold Spring Harbor Symposia on Quantitative Biology 71: 189–194.

22. Chen N, Pai S, Zhao Z, Mah A, Newbury R, et al. (2005) Identification of a nematode chemosensory gene family. Proceedings of the National Academy of Sciences of the United States of America 102: 146–151.

23. Apfeld J, Kenyon C (1999) Regulation of lifespan by sensory perception in Caenorhabditis elegans [see comments]. Nature 402: 804–809.

24. Reinke V, Smith HE, Nance J, Wang J, Van Doren C, et al. (2000) A global profile of germ-line gene expression in C. elegans. Mol Cell 6: 605–616.

25. Wicky C, Alpi A, Passamante M, Rose A, Garmer A, et al. (2004) Multiple Genetic Pathways Involving the Caenorhabditis elegans Bloom’s Syndrome Genes him-6, rad-51, and top-3 Are Needed To Maintain Genome Stability in the Germ Line. Mol Cell Biol 24: 5016–5027.

26. Anderson LL, Mao X, Scott BA, Crowder CM (2009) Survival from hypoxia in C. elegans by inactivation of aminoacyl-tRNA synthetases. Science 325: 630–633.

27. Samokhvalov V, Scott BA, Crowder CM (2008) Autophagy protects against hypoxic injury in C. elegans. Autophagy 4: 1034–1041.

28. Dasgupta N, Patel AM, Scott BA, Crowder CM (2007) Hypoxic Preconditioning Requires the Apoptosis Protein CED-4 in C. elegans. Curr Biol 17: 1954–1959.

29. Yuan A, Santi CM, Wei A, Wang ZW, Pollak K, et al. (2003) The sodium-activated potassium channel is encoded by a member of the Slo gene family. Neuron 37: 765–773.

30. Brenner S (1974) The genetics of Caenorhabditis elegans. Genetics 77: 71–94.

31. Timmons L (2006) Delivery methods for RNA interference in C. elegans. Methods Mol Biol 351: 119–125.