Introduction

Monounsaturated fatty acids (MUFAs) are key components of membrane phospholipids and triglycerides that play important roles in diverse cellular processes such as membrane function, energy storage, and signaling. MUFAs are synthesized from saturated fatty acids by delta-9 (Δ9) desaturases, also known as stearoyl-CoA desaturases (SCDs), which introduce a double bond between the 9th and 10th carbon of a saturated fatty acyl chain. Alterations in the ratio of MUFAs to saturated fatty acids are implicated in heart disease and cancer [1], the two leading causes of death in the United States [2]. The appropriate ratio between MUFAs and saturated fatty acids is maintained by the activity of the Δ9 desaturases, which are subject to complex regulation [3]. As a key control point in metabolic regulation, Δ9 desaturases could be therapeutic targets for treatment of obesity, diabetes, and cardiovascular disease.

The Δ9 desaturases are ubiquitous enzymes in eukaryotes, found in organisms from yeast to humans. Yeast have one Δ9 desaturase, Ole1p, and mutants that lack this activity are not able to survive without exogenous supplementation of unsaturated fatty acids [4]. Mice have four Δ9 desaturases, each having a unique expression pattern [5,6]. Mutant analysis has revealed distinct roles for SCD1 and SCD2. SCD1 is important for adult energy metabolism and lipid synthesis [7], while SCD2 is involved in lipid synthesis during embryonic development [8]. In humans, two SCD isoforms, hSCD1 and hSCD5, have been described [9,10]. A variety of environmental and physiological signals affect the expression of Δ9 desaturases. Diets rich in unsaturated fatty acids decrease Δ9 desaturase expression, while high carbohydrate consumption increases expression [3]. Decreased temperature leads to increases in Δ9 desaturase gene expression in poikilotherms [11]. In addition, endogenous hormones such as leptin and glucagon cause a decrease in Δ9 desaturase gene expression, while insulin has the opposite effect [3].

Sterol regulatory element binding proteins (SREBP)s and peroxisome proliferator-activator receptor protein-alpha (PPARα) have been identified as key transcriptional regulators of SCD1 gene expression in mammals [5]. The SREBP-1 gene encodes a transcription factor that stimulates expression of genes involved in fatty acid biosynthesis, including SCD1 [12], while the SREBP-2 gene product stimulates genes involved in cholesterol biosynthesis [13]. PPARα is one of a family of nuclear hormone receptors (NRH), that, upon ligand binding, acts as a heterodimer with the retinoid X receptor to induce transcription of target fat metabolism genes [14]. PPARδ and PPARγ are also involved in regulation of fatty acid metabolism [17]. These regulators have unique roles due to differences in their gene expression patterns and regulatory activities.

Caenorhabditis elegans is becoming recognized as an important model for the study of fat metabolism. These animals synthesize a wide variety of fatty acids using a Δ12 desaturase, an Δ3 desaturase, an Δ5 desaturase, a Δ6 desaturase, and three Δ9 desaturases [18,19]. C. elegans can also incorporate dietary

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Synopsis

The ratio of saturated to unsaturated fatty acids has a profound affect on the fluidity and function of cellular membranes. Animals, plants, and microorganisms regulate the synthesis of unsaturated fatty acids during changing environmental conditions, as well as in response to dietary nutrients. In this paper the authors use a combination of genetic and biochemical approaches to address the regulation of unsaturated fatty acid synthesis in the roundworm Caenorhabditis elegans. They identify a new transcription factor, NHR-80, that activates the expression of genes encoding delta-9 fatty acid desaturases, the enzymes responsible for catalyzing the insertion of double bonds into saturated fatty acid chains. These unsaturated fatty acids are critical components of membranes, as well as fat storage molecules. Experiments presented here demonstrate that the worms require adequate synthesis of unsaturated fatty acids for survival and that they maintain intricate regulation of the three delta-9 desaturase genes in response to different nutrients. Abnormalities in lipid metabolism lead to obesity and diabetes in humans; this study contributes to our understanding of the regulation of this metabolic pathway.

Results/Discussion

Identification of NHR-80 as a Regulator of Fatty Acid Metabolism

In our search to identify the desaturases and elongases involved in generation of unsaturated fatty acids in C. elegans, we performed a genetic screen to identify mutants with altered fatty acid profiles [18]. In the process of identifying the molecular nature of one mutation, we used RNAi against 156 genes at the end of Chromosome III to determine the fatty acid composition of live animals [20,21]. In an RNAi (RNA interference) screen, genes were identified that altered fat storage and many of these genes have mammalian counterparts known to function in fat metabolism [22]. In addition, mutant analysis offers insight into pathways known to regulate fat storage in both nematodes and mammals such as the insulin-signaling pathway [23]. A recent study established a role for NHR-49, as a regulator of lipid homeostasis [24]. The nhr-49 mutants have increased levels of the saturated fatty acid 18:0, higher fat accumulation, and a shorter lifespan than wild-type animals. NHR-49 is also required for inducing Δ9 desaturase expression in well-fed animals [25].

To gain a deeper understanding of fatty acid metabolism in C. elegans we have characterized the three Δ9 desaturase mutants using biochemistry, gene expression, and phenotypic analysis. While the three Δ9 desaturase single mutants, fat-5, fat-6, and fat-7 display few differences from wild type, we show that they compensate for loss of one isoform by regulated induction of the remaining Δ9 desaturase genes. This induction depends on NHR-80, a novel NHR that we have identified as a regulator of desaturase expression. Furthermore, the fat-5::fat-6::fat-7 triple mutant is unable to survive, revealing that endogenous production of monounsaturated fatty acids is essential for survival under standard growth conditions. The Δ9 desaturase genes and their transcriptional regulators are vital for maintaining optimal fatty acid unsaturation and proper membrane composition.

fatty acids into lipids, allowing researchers to modify the fatty acid composition of live animals [20,21]. In an RNAi (RNA interference) screen, genes were identified that altered fat storage and many of these genes have mammalian counterparts known to function in fat metabolism [22]. In addition, mutant analysis offers insight into pathways known to regulate fat storage in both nematodes and mammals such as the insulin-signaling pathway [23]. A recent study established a role for NHR-49, as a regulator of lipid homeostasis [24]. The nhr-49 mutants have increased levels of the saturated fatty acid 18:0, higher fat accumulation, and a shorter lifespan than wild-type animals. NHR-49 is also required for inducing Δ9 desaturase expression in well-fed animals [25].

Figure 1. Diagram of nhr-80, fat-5, fat-6, and fat-7 Genes and Mutations

(A) nhr-80 is composed of a zinc finger domain (green boxes) and a ligand-binding domain (light blue boxes). nhr-80(tm1011) contains a 446-bp deletion (light grey bar).

(B) fat-5, fat-6, and fat-7 all contain four trans-membrane domains (dark blue boxes) and three histidine boxes (red boxes). fat-5(tm420) consists of a 779-bp deletion (light grey bar). fat-6(tm331) contains a 1,232-bp deletion (light grey bar), and a 428-bp insertion (purple bar). The fat-7 alleles are point mutations with fat-7(wa36), creating a premature stop codon and fat-7(wa37) changing a conserved histidine into a tyrosine.

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Although two NHR mutant lines, nhr-49 and nhr-80, show increased 18:0 as compared with wild-type worms, not all NHR mutants cause these changes in fat metabolism [24]. Both of these transcription factors are proposed to be derived from the same ancestral gene that also is the progenitor of the mammalian gene encoding hepatocyte nuclear factor 4 [27], which in mammals, binds to fatty acids as ligands and is a key activator of lipid and cholesterol metabolism genes [28].

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In addition to the change in fatty acid composition, the *nhr-49* mutants display an increase in fat storage based on staining of whole worms with the lipophilic dye Nile red [24]. In the *nhr-80* mutants, we observed no increase in Nile red staining as compared to wild type (unpublished data) indicating no increase in fat storage. To confirm this, we tested fat storage in the *nhr-80* mutants by measuring the percent triglycerides in the total lipids. In the *nhr-80* mutants triglycerides comprised 44 ± 1% of the total lipids as compared to 45 ± 1% in wild type signifying no increase in fat storage. Thus the increased 18:0 and triglyceride synthesis. However, the altered fatty acid profile of *nhr-80* mutants indicates a role for *NHR-80* in the regulation of fatty acid metabolism in *C. elegans*.

NHR-80 Is Required for Normal Expression of Δ9 Desaturases

As NHR-80 is a transcription factor expressed in the intestine [26], the major site of fat metabolism in *C. elegans*, the increased 18:0 accumulation in the *nhr-80* mutants may be due to a reduced expression of the Δ9 desaturase genes. To test this we used quantitative RT-PCR (QPCR) to measure gene expression with primers designed to amplify *fat-5*, *fat-6*, and *fat-7*, along with the control genes *tbb-2* (β-tubulin) and *ubc-2* (ubiquitin-conjugating enzyme, E2). Relative expression of these genes was examined in wild-type and *nhr-80* mutant adult populations and we found that expression of all three Δ9 desaturases was decreased in the *nhr-80* mutants relative to wild type for eight experimental replicates (Figure 3). On average, *fat-5* and *fat-6* expression were reduced by 66% and 22% respectively, while *fat-7* expression was almost completely eliminated in the *nhr-80* mutants.

To determine if the expression pattern of *nhr-80* overlapped with the expression pattern of the Δ9 desaturases we created two green fluorescent protein (GFP)-fusion expressing lines for each of the Δ9 desaturase genes. Like *nhr-80*, all three Δ9 desaturase genes were expressed in the intestine in adult worms (Figure 3B), and in all four larval stages (unpublished data). The *fat-5* promoter::GFP expressing lines showed additional expression in the pharynx and tail cells after hatching and throughout the lifespan. The *fat-6* whole gene::GFP expressing lines displayed additional expression in the hypoderms in all life stages. The overlapping intestinal expression for all three Δ9 desaturase genes indicates possible functional redundancy. The potential role for *fat-5* in the pharynx and *fat-6* in the hypoderms remain to be determined; however, the constitutive expression of these genes in the intestine is consistent with a central role for Δ9 desaturation in normal *C. elegans* function.

To confirm the regulation of the Δ9 desaturases by NHR-80, lines expressing the GFP fusions were grown on *nhr-80(RNAi)* bacteria. Transformed adults were allowed to lay eggs on *nhr-80(RNAi)* and control bacteria. After 3 d, the progeny were examined for expression of *fat-5::GFP* and *fat-7::GFP* lines grown on *nhr-80(RNAi)*. Expression of *fat-5::GFP* was completely eliminated by the RNAi treatment. Expression of *fat-5* promoter::GFP was decreased but only in the intestine, not in the pharynx. Expression of *fat-6* whole gene::GFP was also slightly decreased. The reduction of *fat-5* and *fat-6* expression and the elimination of *fat-7* expression likely accounts for the changes in fatty acid composition observed in the *nhr-80* mutant. Similar to the *nhr-80* mutants, the *nhr-49* mutants exhibited an increased level of 18:0 accumulation and a decrease in expression of the Δ9 fatty acid desaturase genes by QPCR with *fat-5* and *fat-7* as the most reduced [24]. However, the *nhr-49* mutants have increased fat storage, which is not seen in the *nhr-80* mutants, and show decreased expression of two genes that encode proteins that participate in the mitochondrial β-oxidation pathway, an enoyl-CoA

**Figure 2.** Fatty Acid Composition of *nhr-80*

Relative abundance of selected fatty acid species expressed as percentage of total fatty acid as determined by gas chromatography analysis. The *nhr-80* mutants have significantly higher levels of 18:0 and lower levels of 16:0 and 18:1 Δ9 than wild type. Error bars represent the standard error. *significant differences between wild type and *nhr-80* mutant, p < 0.01.

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**Figure 3.** Expression of the Δ9 Desaturase Genes in *nhr-80*

(A) Gene expression by QPCR in the *nhr-80* mutant reveals a decrease in expression of the Δ9 desaturase genes relative to wild type. Error bars represent standard error.

(B) Transformed lines expressing Δ9 desaturase gene GFP fusions grown to adulthood on empty vector control bacteria or *nhr-80(RNAi)* bacteria. Exposure times for photographs were adjusted due to different GFP expression in the three genes, although the exposure time for the two treatments was kept the same for each genotype. The exposure time for the *fat-5::GFP* worms was 1/4 s, for the *fat-6::GFP* worms was 1/30 s, and for the *fat-7::GFP* worms was 1/8 s. After 4 d, there is a dramatic reduction in Δ9 desaturase gene expression in the intestine for *fat-5::GFP* and *fat-7::GFP* lines grown on *nhr-80(RNAi)*.

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hydratase gene (C29F3.1, ech-1) and an acyl-CoA synthetase gene (F28F8.2, acs-2). We tested the expression of ech-1 and acs-2 in the nhr-80 mutants by QPCR and found that there was no change in expression levels relative to wild-type expression. This is consistent with the normal level of fat storage seen in the nhr-80 mutants. Though both NHR-49 and NHR80 are required for Δ9 desaturase expression, their effects on fatty acid metabolism in C. elegans are not identical; NHR-49 appears to regulate a wider range of lipid homeostasis pathways.

**nhr-80 Mutants Do Not Die Early like nhr-49 Mutants**

It has been suggested that shifts in the ratio of saturated fatty acids to MUFAs in C. elegans may lead to a decreased lifespan. For example, the change in the ratio of 18:0 to 18:1 Δ9 from 1.9 in wild type to 4.3 in nhr-49 mutants has been proposed to cause a substantial reduction in lifespan from 15–18 d in wild type, to 6–8 d in nhr-49 mutants [24]. We examined the lifespan of the nhr-80 mutants (Figure 4) and found that they may have slightly shorter lifespans than wild type but live considerably longer than nhr-49 mutants despite having a similar fatty acid composition. In this experiment, the average lifespan of the nhr-80 mutant was 12.5 ± 0.5 d as compared to 13.9 ± 0.4 d in wild-type animals and 8.2 ± 0.2 d in nhr-49 mutants when grown at 25 °C. These data indicate a 10% decrease in mean lifespan between wild type and nhr-80 mutants, the difference between wild type and nhr-49 mutants is much greater with a 41% reduction in mean lifespan. The early death of the nhr-49 does not seem to be caused solely by an elimination of fat-7 expression or an increase in the ratio of 18:0 to 18:1 Δ9 since nhr-80 mutants also show these characteristics but do not have a dramatically shortened lifespan. It is possible that the shorter lifespan of the nhr-49 mutants is caused by metabolic changes due to other targets of NHR-49 regulation.

**C. elegans Δ9 Desaturases Are Redundant under Standard Growth Conditions**

Previous studies revealed that the three C. elegans Δ9 desaturase isoforms display different substrate specificities. While FAT-6 and FAT-7 preferentially desaturate stearic acid (18:0), similar to most of the characterized SCDs, FAT-5 prefers palmitic acid (16:0) and has little or no activity on stearic acid [19]. We obtained Δ9 desaturase single mutants to further characterize the roles of these three desaturases. We obtained fat-5(tm420) and fat-6(tm331) deletion alleles from the National BioResource Program for the Experimental Animal C. elegans, Japan (Figure 1B). The fat-5 allele has a 779-bp deletion early in the coding sequence that eliminates two of the conserved histidine boxes and two of the trans-membrane domains. The fat-6 allele has a 1,292-bp deletion and a 428-bp insertion. The deletion is early in the coding sequence and also eliminates two of the conserved histidine-rich regions and two trans-membrane domains. Both of these mutations are likely null. The fat-7(tm326) deletion allele is available but molecular analysis of this allele led us to believe that a more extensive genetic disruption had occurred that affects other genes in addition to fat-7. Alternative fat-7 alleles were isolated by TILLING (Targeting Induced Local Lesions IN Genomes) [29] and are single base pair changes (Figure 1B). The fat-7(tm326) allele is a ΔC to T mutation that leads to a premature stop codon that eliminates two trans-membrane domains and one of the conserved histidine boxes required for activity of the rat SCD enzyme [30], indicating that this allele is, at a minimum, a strong reduction-of-function allele. The fat-7(wa37) allele is a ΔC to T mutation that replaces a conserved histidine with tyrosine [19]. Because these histidines are expected to be required for Δ9 desaturase activity we expressed this allele in mutant yeast that lack Δ9 desaturase activity (ole1 mutants). The mutant fat-7(wa37) did not support growth of the ole1 mutant yeast, whereas expression of wild-type fat-7 did allow growth [19]. Phenotypic characterization including fatty acid composition and lifespan with fat-7(wa37) showed no difference from fat-7(wa36) therefore only data from fat-7(wa36) are reported here.

The C. elegans Δ9 desaturase mutants show subtle differences from wild type in their fatty acid profile when grown on an Escherichia coli lawn on NGM plates at 20 °C (Figure 5). Compared to wild type (4.1 ± 0.2%), the fat-5 mutants display decreased 16:1 Δ9 (3.4 ± 0.1%), which is the product of FAT5 desaturation based on the substrate specificity exhibited in yeast [19]. The fat-6 mutants exhibit a significant increase in their accumulation of the predicted substrate of FAT-6, 18:0 (9.6 ± 0.2%), over wild type (7.0 ± 0.2%).
The Δ9 desaturase mutants are indistinguishable from wild type in other characteristics tested including growth rate, reproduction, and behavior. The lack of phenotype indicates that subtle changes in fatty acid composition have no apparent effect and that the desaturases are functionally redundant. To determine if gene expression changes are involved in compensating for the lack of one isozyme, we examined expression of the Δ9 desaturase genes in the fat-5, fat-6, and fat-7 mutants (Figure 6). In the fat-6 mutants, fat-7 expression is increased approximately 4-fold over wild type and fat-5 expression is increased 2–3-fold over wild type. In the fat-7 mutant, expression of fat-6 and fat-5 is also slightly increased over wild type. The fat-5 mutant shows little difference from wild type in fat-6 and fat-7 expression.

Axenic Growth Reveals Substrate Specificity of the Δ9 Desaturases

The standard strain of E. coli on which C. elegans are maintained in the laboratory contains palmitic (16:0), palmitoleic (16:1 Δ9), and vaccenic (18:1 Δ11), but not oleic acid (18:1 Δ9) or polyunsaturated fatty acids [31]. When worms eat these bacteria they incorporate the fatty acids in their lipids. To test the fatty acid composition of the Δ9 desaturase mutants grown on a different food source we grew the C. elegans strains in axenic media devoid of bacteria. This liquid media provides amino acids, vitamins, growth factors, and heme [32]. Our measurements reveal that the axenic
media contains palmitic, palmitoleic, oleic and linoleic acids, but no vaccenic acid (unpublished data). Wild-type worms grow considerably more slowly under the axenic growth conditions, and the fatty acid profile is also dramatically different. In axenic culture, wild-type worms accumulate higher levels of 16:0, 18:0, and 18:1 Δ9, while they produce lesser amounts of 20:5 (Figure 5A and 5D).

The Δ9 desaturase mutants show greater differences in fatty acid composition when grown axenically than when grown on *E. coli* plates (Figure 5D–5F). Comparing the fatty acid composition of the *fat-5* mutant with wild type we observe an increase in 16:0 (19 ± 1% versus 12 ± 1%) and a decrease in 16:1 Δ9 (11 ± 0.4% versus 3.0 ± 0.3%) and 18:1 Δ11 (3.8 ± 0.1% versus 17 ± 1%) in the *fat-5* mutants. The *fat-6* mutants also display dramatic differences from wild type, with an increase in 18:0 (16.7 ± 0.8% versus 10.9 ± 0.7%) and a decrease in 18:1 Δ9 (11.3 ± 0.6% versus 21.8 ± 0.5%) in the *fat-6* mutants. The fatty acid composition of *fat-7* mutants does not differ significantly from wild type, indicating that *fat-6* can completely compensate for *fat-7* in axenic culture and therefore that *FAT-7* does not play an important role in maintaining proper fatty acid composition under axenic conditions. The dramatic reduction of 16:1 Δ9 and 18:1 Δ11 fatty acids in *fat-5* mutants and 18:1 Δ9 in *fat-6* mutants grown in axenic culture is the first evidence that these enzymes have the same substrate specificity in *C. elegans* as they do when expressed in yeast [19].

To determine whether the levels of Δ9 desaturase gene expression are modulated in response to diet we examined the expression of *fat-5*, *fat-6*, and *fat-7* genes in axenic media and on *E. coli* seeded plates using QPCR. We found that compared to worms grown on *E. coli*, *fat-5* expression increases about 6-fold in axenic media. In contrast, *fat-6* expression is maintained at similar levels while *fat-7* expression is dramatically decreased in axenic media (Figure S1).

Single Δ9 Desaturase Mutants Have No Early-Death Phenotype

Previous studies investigating the *C. elegans* Δ9 desaturases have used RNAi to deplete *fat-7* expression and have suggested that *fat-7* expression is required to maintain a normal lifespan [23,24]. Based on these results, it was proposed that the reduced expression of *fat-7* was the cause of the short lifespan in the *nhr-49* mutants [24]. However, the *fat-7* mutants used in our experiment as well as the other Δ9 desaturase mutants, *fat-5* and *fat-6*, do not exhibit an early death phenotype (Figure 4B). The average lifespan of the *fat-5* mutants is 15.8 ± 0.6 d, the *fat-6* mutant is 14.2 ± 0.5 d, and the *fat-7* mutant is 15.0 ± 0.5 d, as compared with a lifespan of 13.9 ± 0.4 d in wild-type animals. In this experiment, the *fat-5* mutant displayed a slight but significant (p < 0.01) increase in lifespan over wild type, while the *fat-6* and *fat-7* mutants were not significantly different from wild type in average lifespan.

Our experiments with the *fat-7* mutant do not support the requirement for *fat-7* for normal lifespan as proposed from studies using *fat-7(RNAi)* [23,24]. Additionally, *fat-7(RNAi)* revealed major changes in fatty acid composition and a reduction of fat storage [24] that was not observed in the *fat-7* mutants. The RNAi phenotype observed could be due to transitive secondary RNAi effect [33] as *fat-7* has 84% nucleotide identity with *fat-6* including eight regions of 21-44 nucleotides with 100% identity. Van Gilst et al. report that *fat-7(RNAi)* did not reduce *fat-6* expression when measured by QPCR [24]; however, we observe an elimination of *fat-6* expression when *fat-6* whole gene:GFP lines were grown on *fat-7(RNAi)* (unpublished data). In addition, it is possible that compensation by the third Δ9 desaturase, *fat-5*, is inhibited in the *fat-7(RNAi)*. Because the *fat-7* loss-of-function mutant is wild type for fatty acid composition and lifespan it must be concluded that *fat-7(RNAi)* is having off-target effects on the worm.

Δ9 Desaturase Activity and Monounsaturated Fatty Acids Are Required for Survival

Because the Δ9 desaturase genes appear to compensate for each other, we constructed a *fat-5;fat-6;fat-7* triple mutant lacking all three Δ9 desaturases. We expected these mutants would be unable to survive under standard growth conditions, so we supplemented the worms with a combination of 18:1 Δ9, 18:2 ω6, and 20:5 ω3 dietary fatty acids. After identifying the *fat-5;fat-6;fat-7* triple mutant, we moved the worms to plates without fatty acid supplementation and found that indeed these worms could not survive. Larvae that hatch from eggs laid on unsupplemented plates arrest in the L1 stage, while L3 and L4 stage larvae that are moved from supplemented to unsupplemented plates develop into thin, sterile adults with reduced movement and early death. The MUFA’s provided by the standard *E. coli* diet are not sufficient for survival in the *fat-5;fat-6;fat-7* triple mutant. Thus *C. elegans* have a requirement for a certain level of Δ9 desaturation that cannot be met by the standard *E. coli* diet. The yeast Δ9 desaturase mutant, *odel*, is also unable to grow without supplementation [4]. The *fat-5;fat-6;fat-7* triple mutant is the first multicellular organism generated that lacks all endogenous Δ9 desaturase activity.

To examine genetic interaction between *nhr-80* and *fat-6*, the most highly expressed Δ9 desaturase, we constructed the *fat-6;* *nhr-80* double mutant using plates supplemented with dietary fatty acids. When we removed the *fat-6;* *nhr-80* double mutants to unsupplemented plates we found that these worms also did not survive. Since the *nhr-80(RNAi)* phenotype resembles the *nhr-80* mutants, we used RNAi in combination with the Δ9 desaturase mutants to study this interaction further. The *fat-6* mutants, when grown on *nhr-80(RNAi)* from eggs, become thin, slow growing, and reproductively inviable after 4 d of growth (Figure 7). They also accumulate very high levels of 18:0 (Figure 7B). The *fat-6* mutants grown on *nhr-80(RNAi)* accumulate 34 ± 1% of their fatty acids as 18:0 as compared to 9.1 ± 0.1% when *fat-6* is grown on control bacteria or 14 ± 2% when wild-type worms are grown on *nhr-80(RNAi)* bacteria. Although 18:0 also accumulates in the *fat-5* and *fat-7* mutants grown on *nhr-80(RNAi)*, the extent of 18:0 accumulation is not as dramatic as observed in *fat-6* (Figure 7B) and they do not show a synthetic lethality (Figure 7A).

One explanation for the synthetic lethality of *fat-6;* *nhr-80* double mutants is that *NHR-80* is required for the increased *fat-5* and *fat-7* expression in the *fat-6* mutant. To test this we examined the expression of the Δ9 desaturase genes in the *fat-5*, *fat-6*, and *fat-7* mutants grown on *nhr-80(RNAi)*. We found that expression of *fat-7* in the *fat-6;* *nhr-80* is less than 10% of the expression of *fat-7* in the *fat-6* mutants grown on control bacteria, consistent with the notion that *NHR-80* is required to induce the expression of *fat-7* (Figure 7C). We
The overall amount of Δ9 desaturase gene expression is approximately equal for all worms grown on nhr-80(RNAi), but only fat-6 displays synthetic lethality with nhr-80. This could be due to the composition of the Δ9 desaturase gene expression. When wild type, fat-5, or fat-7 are grown on nhr-80(RNAi) fat-6 is the major gene expressed suggesting its central importance for Δ9 desaturation activity. The fat-6 mutants lack fat-6 expression and compensate by substantially increasing fat-7 expression when grown on control bacteria. It is noteworthy that under these conditions fat-7 expression is increased 37-fold, perhaps indicating that fat-7 is not as effective at Δ9 desaturation as fat-6 due to differences in tissue specific expression, translation efficiency or protein stability. When the fat-6 mutants are grown on nhr-80(RNAi) they are unable to compensate with an increase in fat-7 expression to an appropriate level and this may cause their reduced survival. Thus NHR-80 is required for increasing fat-7 expression in situations where higher fat-7 levels are necessary and consequently defines a critical regulator of fatty acid metabolism.

Our characterization of the novel NHR-80 and the family of C. elegans Δ9 desaturase mutants enhances our understanding of the regulation of lipid homeostasis. Maintaining appropriate fatty acid composition is essential and without sufficient Δ9 desaturase activity both the fat-5/fat-6/fat-7 triple mutants and the fat-6/nhr-80 double mutants are unable to survive. The integration of endogenous and environmental signals by NHRs such as NHR-80 precisely regulates the expression of the Δ9 desaturase genes and the production of monounsaturated fatty acids leads to optimal membrane fluidity and fat storage.

Materials and Methods

Culture of nematodes. Unless otherwise noted, C. elegans were grown on nematode growth media (NGM) plates with OP50 strain of E. coli as a food source [34]. The wild-type strain used is strain N2. Mutant strains obtained from Shohei Mitani and Edwin Cuppen were outcrossed at least four times to the N2 strain. The nhr-80(RNAi) construct, as well as the others used in the screen of Chromosome III, are from the Ahringer RNAi library [35] and were used as described [36]. As a control for RNAi experiments, nematodes were grown on NGM plates with the HT115 strain of E. coli transformed with pPD129.35 (pL4400) empty vector plasmid. The axenic culture media consisted of 3% soy peptone, 3% yeast extract, 0.5 mg/ml hemoglobin in 1 M KOH, and 20% ultra-high temperature pasteurized skim milk [32]. Worms were grown in this liquid culture at room temperature (22–23 °C) with constant shaking. To make plates supplemented with dietary fatty acids a 0.1 M stock solution of fatty acid sodium salts (NuCheck Prep, Elysian, Minnesota, United States) in water was prepared fresh for each supplementation experiment. The fatty acid stock was added slowly to NGM containing 0.1% tergitol. Plates were prepared fresh for each supplementation experiment. The fatty acid stock was added slowly to NGM containing 0.1% tergitol. Plates were poured, covered and allowed to dry in the dark at room temperature overnight. The OP50 strain of E. coli was added to each plate and allowed to dry for at least one night [21].

Fatty acid and lipid analysis. For fatty acid analysis, adult nematodes were washed from plates and allowed to settle. The excess water was removed from the worm pellet and 1 ml of 2.5% methanolic H2SO4 was added and incubated at 80 °C for 1 h to generate fatty acid methyl esters, which were extracted by adding 1.5 ml water and 0.2 ml hexane. The hexane was sampled for determination of fatty acid composition by gas chromatography on an SP-2380 fused silica capillary column (Supelco, Bellefonte, Pennsylvania, United States) using an Agilent (Palo Alto, California, United States) 6890 series gas chromatograph [18].

For lipid analysis, about 0.5 ml of adult nematodes were collected in a glass tube and frozen. Lipids were extracted by incubation in (1:1) methanolic H2SO4 was added and incubated at 80 °C for 1 h to generate fatty acid methyl esters, which were extracted by adding 1.5 ml water and 0.2 ml hexane. The hexane was sampled for determination of fatty acid composition by gas chromatography on an SP-2380 fused silica capillary column (Supelco, Bellefonte, Pennsylvania, United States) using an Agilent (Palo Alto, California, United States) 6890 series gas chromatograph [18].

Figure 7. Effects of nhr-80(RNAi) in the Δ9 Desaturase Mutant Background

(A) Photographs showing adult worms after 4 d of growth on nhr-80(RNAi) and empty vector control bacteria. The fat-6 mutants grown on nhr-80(RNAi) are thin, pale, and produce no viable progeny.

(B) Relative abundance of 18:0 expressed as a percentage of total fatty acid as determined by gas chromatography analysis. The fat-6 mutants grown on nhr-80(RNAi) (n = 5) accumulate much higher levels than fat-6 mutants grown on control (n = 7) and wild type grown on nhr-80(RNAi) (n = 6). *significant differences from growth on control bacteria, p < 0.01.

(C) Effects of nhr-80 on Δ9 desaturase gene expression in fat-5, fat-6, and fat-7 mutants. QPCR in fat-5, fat-6, fat-7, and wild type for worms grown on empty vector control bacteria (ev) and nhr-80(RNAi) (80) (n = 6). Values are expressed relative to fat-6 expression in wild-type worms grown on control bacteria. For all graphs error bars represent standard error.

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graphed the relative expression values, setting fat-6 expression in wild-type worms grown on control bacteria as 100%.

In wild-type worms on control bacteria fat-6 is the most highly expressed Δ9 desaturase gene and fat-5 and fat-7 are expressed at 3.6 ± 0.2% and 6.5 ± 0.6% of the level of fat-6 respectively. When the wild-type worms are grown on nhr-80(RNAi) we observe a similar relative decrease in Δ9 desaturase gene expression seen in the nhr-80 mutants (Figure 3A). Comparing the fat-5 and fat-7 mutants grown on control with those grown on nhr-80(RNAi) reveals a decrease in Δ9 desaturase gene expression. However, the biggest difference is seen in the fat-6 mutants. When these animals are grown on control bacteria fat-7 is increased in expression 37-fold over

wild type. When fat-6 is grown on nhr-80(RNAi) the fat-7 relative expression is only a 3-fold increase over wild type.
chloroform/methanol overnight at −20 °C. The samples were washed with 2.2 ml HClajra’s solution (0.2M H3PO4, 1M KCl) and the chloroform phase containing the lipids was isolated. The silica gel HL plates (Analttech, Newark, Delaware, United States) were activated by incubation at 110 °C for 1 h and 15 min. The samples were loaded onto the thin layer chromatography plates along with lipid standards (Sigma). The silicagel plates were developed in 65:43:3:2.5 chloroform/methanol/water/acetic acid solvent mixture until the solvent front was three-fourths of the way up the plate. The plate was dried, a new solvent mixture of 80:20 heptane/diethyl ether/acetic acid was added, and the plate was run until the solvent front reached the top of the plate. The marker lanes were visualized using iodine vapor and the corresponding bands for triglycerides and individual phospholipids in the silica gel were scraped into individual tubes. To quantify, 50 μl of 15% free fatty acid was added to each tube. GC analysis was performed on an Agilent 6890N with an internal standard and fatty acid analysis was performed by gas chromatography as described above [22].

**QPCR analysis.** Adult nematodes were harvested and frozen in liquid nitrogen. RNA was prepared using TRIzol Reagent (Invitrogen, Carlsbad, California, United States). A DNA-FREE RNA kit (Zymo Research, Orange, California, United States) was used for DNAse treatment and purification. After quantification, 1 μg of RNA was used in a reverse-transcription reaction with SuperScriptIII (Invitrogen) to generate cDNA. Primer sequences for the Δ9 desaturase genes and the reference genes were designed using PrimerQuest software (IDT). Each qPCR reaction contained 1 μl of cDNA, 0.2 μl of forward primer, 0.2 μl of reverse primer, 10 μl of SYBR Green SYBR Green qPCR Supermix UDG (Applied Biosystems). The qPCR was run and monitored on a MX3000P (Stratagene, La Jolla, California, United States). Relative abundance was determined using the ACT method and an average of the expression of the reference genes tub-2 and unc-2 to control for template levels [37].

**Construction of GFP fusions and microinjection.** Fusion PCR was used to create translational fat-5, fat-6, and fat-7 GFP constructs. The promoters and coding sequences of fat-6 and fat-7 and the promoter and first exon of fat-5 were amplified from genomic DNA. The upstream regulatory region for fat-5 was 4 kb, for fat-6 was 2.6 kb, and for fat-7 was 3.0 kb. GFP was amplified from the Fire vector pPD95.75 including the entire coding sequence and a termination sequence. These PCR products were fused together in a final PCR using nested primers [38]. These fusions were microinjected into lin-15 mutant C. elegans along with a rescuing plasmid, pJM23, containing the wild-type lin-15 gene [39,40]. Multiple independent lines of nematodes without the lin-15 phenotype were selected and examined for GFP expression using fluorescence microscopy on an Olympus IX70 microscope.

**Lifespan analysis.** Aging experiments were performed on adult nematodes grown at 25 °C. Worms were moved to plates containing 5-fluoro-2'-deoxyuridine (Sigma) at the fourth larval stage of development (L4). Live animals were assayed for movement in response to touch every 1–2 d [41].

**Generation of fat-5atf-5atf-7 triple mutants and fat-6nhr-80 double mutants.** The fat-6(m331)fat-7(uw36) hermaphrodites were crossed with fat-5(tm240)fat-7(uw36) males on plates supplemented with 18:1 Δ9. The F1 generation was moved to new 18:1 Δ9-supplemented plates and their progeny were moved to plates supplemented with a combination of 18:1 Δ9, 18:2 Δ6, and 20:5 Δ3. After the F2 generation reproduced, the adults were harvested for single worm PCR to determine the genotype [42]. The fat-5 and fat-6 mutations were monitored using the difference in amplification size between wild-type and mutant alleles due to the large deletions. The wild-type products were 1,100 bp for fat-5 and 1,457 bp, for fat-6 compared with the mutant products of 321 bp and 652 bp, respectively. All cross-progeny were homozygous for the fat-7 single base pair mutation.

To generate nhr-80fat-6 double mutants we crossed fat-6 males with nhr-80 hermaphrodites on 18:1 Δ9 supplemented plates and isolated the F1 generation onto new supplemented plates. The F2s were moved to OP50 seeded plates and allowed to reproduce then single worm PCR was used to identify nhr-80fat-6 double mutants. The nhr-80 wild-type allele generated a PCR product of 745 bp, whereas the nhr-80(tm1011) mutant allele generated a product 298 bp in length.

**Supporting Information**

**Figure S1.** Expression of Δ9 Desaturase Genes in Wild-Type Worms Grown on E. coli (OP50) Seeded Plates or Axenic Liquid Media

The percent expression shown is relative to fat-6 expression on E. coli plates, which is set at 100%. In wild-type worms grown in axenic culture the expression of fat-5 is increased and the fat-7 expression is nearly eliminated relative to expression in wild-type worms grown on E. coli (OP50) plates. Relative to fat-6 expression, fat-5 and fat-7 expression is higher in wild-type worms grown on E. coli (OP50) compared to wild-type worms grown on E. coli (HT115) (Figure S7C). Error bars are SEM, n = 3 replicates for plate grown and n = 6 replicates for axenic cultured nematodes.

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**Table S1.** Sequence of DNA Primers Used in These Studies

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**Accession Numbers**

The GenBank (http://www.ncbi.nlm.nih.gov/Genbank) accession numbers for genes used in this study are nhr-80 (H10E21.3) (AY294179), fat-5 (W06D12.3) (AF260242), fat-6 (VZK8221.1) (AF260244), and fat-7 (F10D2.9) (AF260243).

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**Author contributions.** TJB, JB, and JLW conceived and designed the experiments. TB performed the experiments and analyzed the data. TJB, JB, and JLW wrote the paper.

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**Competing interests.** The authors have declared that no competing interests exist.

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