**daf-31 Encodes the Catalytic Subunit of N Alpha-Acetyltransferase that Regulates *Caenorhabditis elegans* Development, Metabolism and Adult Lifespan**

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**Abstract**

The *Caenorhabditis elegans* dauer larva is a facultative state of diapause. Mutations affecting dauer signal transduction and morphogenesis have been reported. Of these, most that result in constitutive formation of dauer larvae are temperature-sensitive (ts). The daf-31 mutant was isolated in genetic screens looking for novel and underrepresented classes of mutants that form dauer and dauer-like larvae non-conditionally. Dauer-like larvae are arrested in development and have some, but not all, of the normal dauer characteristics. We show here that daf-31 mutants form dauer-like larvae under starvation conditions but are sensitive to SDS treatment. Moreover, metabolism is shifted to fat accumulation in daf-31 mutants. We cloned the daf-31 gene and it encodes an ortholog of the arrest-defective-1 protein (ARD1) that is the catalytic subunit of the major N alpha-acetyltransferase (NatA). A daf-31 promoter::GFP reporter gene indicates daf-31 is expressed in multiple tissues including neurons, pharynx, intestine and hypodermal cells. Interestingly, overexpression of daf-31 enhances the longevity phenotype of daf-2 mutants, which is dependent on the forkhead transcription factor (FOXO) DAF-16. We demonstrate that overexpression of daf-31 stimulates the transcriptional activity of DAF-16 without influencing its subcellular localization. These data reveal an essential role of NatA in controlling *C. elegans* life history and also a novel interaction between ARD1 and FOXO transcription factors, which may contribute to understanding the function of ARD1 in mammals.

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# Introduction

Animal development is a complex process that involves hierarchical gene regulatory networks and is influenced by environmental conditions. When food is abundant, the post-embryonic development of *C. elegans* consists of four larval stages (L1–L4) and the adult. During the L1 stage, environmental factors determine whether *C. elegans* molts to an L2 larva or a pre-dauer L2d larva [1]. At least three environmental cues have been defined: food supply, temperature, and a constitutively secreted dauer-inducing pheromone that signals population density [2]. The L2 larva is developmentally committed to continued growth, whereas the L2d larva can molt to a dauer larva if food is scarce and the animals are overcrowded, or to an L3 larva should conditions improve.

Mutations affecting dauer larval development include dauer-defective (*daf-d*) mutations that prevent entry into the dauer stage, and dauer-constitutive (*daf-c*) mutations that mandate entry into the dauer stage [2]. Based on epistatic relationships between *daf-c* and *daf-d* mutations, more than twenty genes controlling dauer formation have been ordered in a genetic pathway [2] representing generation of the pheromone signal [3], response by chemosensory neurons [4,5] and transduction of the signal to other cells. Three functionally overlapping neural pathways control the developmental response to environmental cues. They involve DAF-7/TGF-β [6,7], DAF-11/cyclic GMP [8], and DAF-2/insulin-like [9,10] pathways, which relay the environmental signals to a nuclear hormone receptor, DAF-12 [11], to control dauer versus non-dauer morphogenesis.

Mutations in two genes, *daf-9* and *daf-15*, lead to non-conditional formation of detergent-sensitive dauer-like larvae [12]. These mutants form dauer larvae constitutively and display some characteristics of dauer larvae formed under starvation, such as a high density of intestinal and hypodermal storage granules. *daf-9* encodes a cytochrome P450 related to those involved in the biosynthesis of steroid hormones in mammals [13,14]; it was found to specify a step in the biosynthetic pathway for a DAF-12 steroid ligand called dafachronic acid [15–17]. *daf-15* encodes the C.
**Author Summary**

The development of a living organism is influenced by the environmental conditions such as nutrient availability. Under starvation conditions, the *C. elegans* larvae will enter a special developmental stage called dauer larva. An insulin-like signaling pathway controls dauer formation as well as adult lifespan by inhibiting the activity of FOXO transcription factor DAF-16 that regulates expression of stress-resistant genes. Here we isolate a new gene called daf-31; this gene encodes a protein that regulates *C. elegans* larval development, metabolism and adult lifespan. This protein has been found in other species to be part of an enzyme that functions to modify other proteins. We show that overexpression of our newly discovered protein stimulates the transcriptional activity of DAF-16. Interestingly, abnormal regulation of human proteins similar to DAF-31 results in tumor formation. It is known that human FOXO proteins prevent tumorigenesis. Therefore, it is possible that abnormal DAF-31 activity may lead to tumor growth by reducing DAF-16 activity. Thus, the present study may not only contribute to understanding the role of a universal enzyme in controlling development, metabolism and lifespan in other organisms besides worms but may also shed light on the mechanisms of tumorigenesis in humans.

*elegans* ortholog of Raptor [18] that is proposed to interact with *C. elegans* target-of-rapamycin kinase (LET-363/CeTOR) to control *C. elegans* larval development [18]. Both daf-9 and daf-15 also regulate fat metabolism and adult lifespan [13,14,18].

The dauer-like mutants represent a mutant class distinct from the previously defined daf-9 and daf-15 mutants. Unlike most daf-9 mutants, the dauer-like mutants are not ts, and they do not complete dauer morphogenesis. The daf-d genes such as daf-12 have non-conditional alleles and fail to respond to pheromone [1], but unlike the dauer-like mutants they can execute non-dauer development. The dauer-like mutants define a third class of mutants, one in which the animals are incapable of executing either complex dauer or non-dauer development.

The daf-31 mutant was isolated in genetic screens to identify genes similar to daf-9 and daf-15 [17]. The overall aim of the present study was to clone the daf-31 gene and characterize the DAF-31 function. Our genetic epistasis analysis suggests daf-31 functions downstream of or in parallel to daf-3, daf-12 and daf-16 dauer-defective genes, and acts upstream of or in parallel to daf-15/raptor. We cloned the daf-31 gene by positional cloning and showed that it encodes an ortholog of arrest-defective-1 protein (ARD1), the catalytic subunit of the major N alpha-acetyltransferase (NatA). Moreover, our data reveal that daf-31 has an essential role in controlling *C. elegans* larval development, metabolism and adult longevity.

**Results**

*da*--*f*--31 mutations cause developmental larval arrest

Entry into the dauer stage is determined by the pheromone/food ratio, with high pheromone and low food supply favoring dauer formation [2]. Dauer larva is considered an alternative L3 larval stage. Compared to L3 larva (Figure 1A), the dauer larva has a constricted pharynx (Figure 1B) and a special cuticle with dauer alae along the lateral surface of the cuticle (body beneath focal plane). (E) unc-24(e138)daf-31(m655) dauer larva with dauer alae (body beneath focal plane). Arrows indicate isthmus of pharynx in panels A, B and D. Arrowheads indicate dauer alae in panels C and E. N2 and unc-24;daf-31/T1 animals were grown on NG agar plates at 20°C. Unc dauer larvae (unc-24;daf-31) were identified after animals were starved. (F–H) Representative pictures showing fat accumulation detected by Sudan Black B in daf-2 mutants (F), N2 (G) and daf-31 mutants (H). N2, daf-2(e1370) and daf-31(m655)/N2(T1) animals were placed on NG agar plates, incubated at 20°C until they entered L3 or dauer-like stages, then collected for staining. Scale bars: 10 μm.

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**Figure 1. Characteristics of daf-31 mutant dauer larvae.** (A) N2 L3 larva pharynx. (B) N2 dauer larva with fully constricted pharynx. (C) N2 dauer larva with dauer alae along the lateral surface of the cuticle (body beneath focal plane). (D) unc-24(e138)daf-31(m655) dauer larva with fully constricted pharynx. (E) unc-24(e138)daf-31(m655) dauer larva with dauer alae (body beneath focal plane). Arrows indicate isthmus of pharynx in panels A, B and D. Arrowheads indicate dauer alae in panels C and E. N2 and unc-24;daf-31/T1 animals were grown on NG agar plates at 20°C. Unc dauer larvae (unc-24;daf-31) were identified after animals were starved. (F–H) Representative pictures showing fat accumulation detected by Sudan Black B in daf-2 mutants (F), N2 (G) and daf-31 mutants (H). N2, daf-2(e1370) and daf-31(m655)/N2(T1) animals were placed on NG agar plates, incubated at 20°C until they entered L3 or dauer-like stages, then collected for staining. Scale bars: 10 μm.

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daf-31 is epistatic to daf-d genes but acts upstream of or in parallel to daf-15/raptor

To position daf-31 in the dauer formation pathway, we examined the epistatic relationship between daf-31 and daf-d genes including daf-3, daf-12, and daf-16. The daf-31 mutation is epistatic to all three daf-d mutations as judged by the ratio of progeny (1:2:1) (Table 1). For the epistasis analysis with daf-16, the ratio of progeny is 1:2 because nT1 homozygous animals are lethal. We repeated the epistasis analysis of daf-31 and daf-12 by using the daf-12(rh61 rh411) null allele [11] and obtained a similar result (Table 1). These epistatic relationships suggest that daf-31 functions downstream of or in parallel to daf-3, daf-12, and daf-16 in dauer formation.

To examine the epistatic relationship of daf-31 and daf-15, we injected dsRNA of daf-15 into unc-24daf-31/nT1 young adult worms. Wild-type (N2) animals were treated equally and used as controls. We examined the phenotype of progeny reproduced at various time periods after injection. The progeny reproduced between seven and eighteen hours after injection arrested development at a dauer-like stage three days after egg lay (Table 2). These dauer-like animals have a similar phenotype to daf-15 mutants. Thus, regarding dauer entry, it appears that the daf-15 mutation is epistatic to the daf-31 mutation. We scored the recovery of both N2 and unc-24daf-31 dauer-like animals two days after dauer-like arrest. 48% of N2 dauer-like worms remained at dauer-like stage and the rest of the animals recovered and grew to L4 larval or adult size (Table 2). By contrast, 100% of unc-24daf-31 animals stayed at dauer-like stage (Table 2). For unc-24daf-31 dauer-like larvae without daf-15 RNAi treatment, the majority of these animals died within five days. However, surviving animals all grew to L4 larval or adult size (n = 52). Taken together, these data indicate that daf-15 is epistatic to daf-31 as unc-24daf-31 mutants treated by daf-15 RNAi form dauer-like larvae similar to daf-15 mutants. Moreover, these two mutants have a synergistic effect on C. elegans development because no unc-24daf-31 dauer-like larvae treated by daf-15 RNAi recovered. Thus, these two genes may function in the same pathway and daf-31 is upstream of daf-15. However, the possibility that these two genes act in parallel cannot be excluded.

daf-31 encodes an ortholog of ARD1

A positional cloning strategy was used to identify the daf-31 gene on chromosome IV between unc-24 and fem-3 (Figure S2A). daf-31 was found to lie between the physical SNP markers T09A12 and F17E9 (Figure S2A). A genomic fragment corresponding to the K07H8.3 open reading frame fully rescued the genetic daf-31 null mutant [daf-31(m655)] phenotype, i.e. the transgenic animals did not form dauer-like larvae, but grew to fertile adults. Sequence analysis of the daf-31 gene in the mutant revealed a 393 bp deletion which removed 151 bp of promoter upstream of the ATG start codon and 242 bp of daf-31 coding region downstream of the ATG start codon, which may completely block daf-31 transcription as both the essential promoter region and the N-terminus portion of the gene were deleted (Figure S2B). Primers were designed to flank the deletion region and PCR analysis of mutant worms’ genomic DNA detected a 1,449 bp band (393 bp smaller than the wild-type band) in both homozygous and heterozygous daf-31(m655) mutant worms (Figure S2C).

The daf-31 gene encodes the ortholog of ARD1 with a predicted molecular weight of 21.2 kDa. ARD1 is the catalytic subunit of NatA that catalyzes the acetylation of proteins beginning with Met-Ser, Met-Gly and Met-Ala [20]. Amino acid sequence alignment showed that DAF-31 shares 75% identity with the Drosophila melanogaster ARD1, 77% identity with mouse ARD1, 72% identity with Broussonetia papyrifera ARD1 and 46% identity with yeast ARD1 (Figure S2D).

Table 1. Epistatic tests between daf-31 and daf-d mutations for dauer formation.

| Strain | Progeny (%) | Ratio (N²) |
|--------|-------------|------------|
| daf-31/unc-24; daf-3(m1376) | dauer-like (22.2%) WT (52.4%) Unc (25.4%) | dauer: WT = 1: 2.4: 1.1 (2874) |
| daf-31/unc-24; daf-12(m20) | dauer-like (24.7%) WT (51.4%) Unc (23.9%) | dauer: WT = 1: 2: 0.9 (1294) |
| daf-31/unc-24; daf-12(rh61 rh411) | dauer-like (22.9%) WT (50.7%) Unc (26.4%) | dauer: WT = 1: 2: 1.1 (3083) |
| unc-24daf-31/nT1+control vector RNAI | dauer-like Unc (33.2%) WT (66.8%) | dauer: WT = 1: 2 (3516) |
| unc-24daf-31/nT1+daf-16 RNAI | dauer-like Unc (33.8%) WT (66.2%) | dauer: WT = 1: 2 (3847) |

*Percentage of total animals scored.

†Total number of animals scored.

1daf-16 RNAI fully suppressed dauer formation of daf-2(e1370) mutants at 25 C (all 3152 daf-2 mutants treated with control vector RNAI formed dauer larvae. By contrast, only two out of 3278 daf-2 mutants treated with daf-16 RNAI entered dauer stage.)

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Table 2. Epistatic test between daf-31 and daf-15 mutations for dauer recovery.

| Strain                        | dauer (%) | N^b |
|-------------------------------|-----------|-----|
|                               | Day 3     | Day 5 |     |
| N2+daf-15 RNAi                | 100%      | 48.3% | 578 |
| unc-24daf-31+daf-15 RNAI      | 100%      | 100%  | 55  |

*Percentage of total animals scored.

^bTotal number of animals scored (reproduced between seven and eighteen hours after daf-15 dsRNA injection).

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Given that there is only a single mutant allele of daf-31, we used RNAi to inhibit daf-31 in the N2 background to confirm the daf-31 mutant phenotype. Inhibition of daf-31 by feeding animals with E. coli that express daf-31 dsRNA did not induce a dauer-like phenotype. From our previous work, dsRNA injection can create a stronger mutant phenotype similar to that of a genetic null mutant [18]. In vitro synthesized daf-31 dsRNA was injected into gonads of N2 young adult worms and the progeny displayed a dauer-like phenotype similar to the daf-31(m655) mutants. The starvation-induced dauer morphology of daf-31(m655) mutants, such as dauer alae formation and contrasted pharynx (described in Figure 1) could not be examined using this RNAi method. Therefore, we examined fat accumulation in daf-31 RNAi-treated animals. Similar to the daf-31(m655) mutant, daf-31 RNAi-treated animals accumulated fat as detected by both Sudan Black and Nile red staining of fixed animals (Figure S3). Based on these results, we conclude that the daf-31 mutant phenotypes described in this study most likely resulted from daf-31 mutation instead of secondary mutations in the background.

daf-31 is expressed in multiple tissues

In order to characterize the daf-31 expression pattern, we constructed a daf-31 promoter::gfp reporter construct. In N2 animals, GFP expression was detected from L1 to the adult stage in multiple tissues including the hypodermis, pharynx, intestine, and neurons (Figure 2). To confirm the GFP expression pattern of daf-31 promoter fusion reporter, we constructed daf-31 transcriptional fusion reporter genes in which the GFP open reading frame was fused to the full-length daf-31 genomic DNA in frame either at the N-terminus or at the C-terminus. Both translation fusion reporter genes fully rescued the dauer-like phenotypes of daf-31 mutants. However, we did not observe GFP expression in the rescued daf-31 mutant worms, a phenomenon previously reported with other GFP fusion gene mutants (see Table S1). Compared to controls, the mean lifespan of rrf-3 mutants treated with daf-31 RNAi was shortened by four days (Figure 3B and Table S1).

To test if daf-31 mutations influence the longevity phenotype of daf-2 mutants, we treated the daf-2(pk1426);daf-31(m655) mutant with daf-31 RNAi. The mean lifespan of daf-31 RNAI-treated rrf-3;daf-2 mutants was five days shorter compared to that of control animals (p = 0.0005, log-rank test) (Figure 3C and Table S1). Thus, inhibition of daf-31 partially suppressed the longevity phenotype of daf-2 mutants. Based on this result, we postulate that overexpression of daf-31 may further increase the lifespan of daf-2 mutants.

To test this, daf-2(e1370) and daf-16(mgDf47);daf-2(e1370) mutants overexpressing daf-31 were constructed. The overexpression of daf-31 was confirmed by qRT-PCR (Figure S4). As shown in Figure 3D, daf-31 overexpression increased the lifespan of daf-2 mutants (p<0.0001, log-rank test) (Figure 3D and Table S1). The mean lifespan was increased by eight days and the maximum lifespan was extended by seven days (Figure 3D and Table S1). This increased lifespan was due to daf-31 overexpression as daf-31 RNAi treatment completely abrogated it (Figure 3E and Table S1). Interestingly, daf-31 overexpression failed to extend the lifespan of daf-16;daf-2 double mutants (Figure 3F and Table S1), indicating that DAF-16 is required for daf-31 overexpression to enhance the daf-2 longevity phenotype. We also measured the lifespan of N2 animals overexpressing the daf-31 gene. As shown in Figure S5 and Table S1, daf-31 overexpression did not extend the lifespan of N2 worms. In fact, it slightly decreased the lifespan of N2 worms. Finally, to confirm daf-31 functions through daf-16 in C. elegans lifespan regulation, we tested if daf-31 RNAi can further decrease the lifespan of RNAi-sensitive daf-16 mutants (daf-16;rrf-3). We found daf-31 RNAi had no obvious effect on the lifespan of daf-16;rrf-3 mutants (Figure S6 and Table S1).

daf-31 overexpression stimulates daf-16 transcriptional activity

The forkhead transcription factor DAF-16/FOXO controls the transcription of an array of genes essential for lifespan extension and oxidative stress resistance including the antioxidant enzyme superoxide dismutase (sod) gene and beta-carotene 15,15'-monoxygenase gene (bemo-2) [22]. We used qRT-PCR to measure the expression level of sod-3 and bemo-2 in daf-31 overexpression strains and control animals. As shown in Figure 4A, the expression level of sod-3 was significantly increased when daf-31 was overexpressed in the N2 background and in daf-2 mutants. Similar to sod-3, the expression of bemo-2 is also significantly increased when daf-31 is overexpressed in the daf-2 mutant background (Figure 4B). Taken together, these data indicate overexpression of daf-31 stimulates the transcriptional activity of DAF-16.

Reduction of daf-2 insulin-like signaling activity increases C. elegans lifespan by promoting nuclear localization of DAF-16.
[23,24]. We crossed the DAF-16::GFP reporter gene into daf-31 overexpressing animals to examine if daf-31 overexpression influences the subcellular localization of DAF-16. We found the percentage of animals showing DAF-16 nuclear localization was not significantly different between daf-31 overexpressing animals and control worms (Figure 4C and D).

daf-31 overexpression does not confer stress resistance
daf-2 mutants are resistant to environmental stresses such as high temperature [25,26]. We examined if daf-31 overexpression could enhance the thermotolerance of daf-2 mutants. As reported previously [25], the survival rate of daf-2 mutants at 35°C is doubled compared to N2 worms (Figure S7 and Table S2). However, the mean survival for N2 and N2 overexpressing daf-31 was similar (9.8 hours vs. 10 hours) (p = 0.2420, log-rank test) (Figure S7 and Table S2). Similarly, daf-31 overexpression did not increase the survival of daf-2 mutants at 35°C (p = 0.4623, log-rank test) (Figure S7 and Table S2).

daf-31 overexpression enhances reproduction
We tested the influence of daf-31 overexpression on the reproduction of N2 and daf-2 mutant animals. daf-31 overexpression increased the brood size of N2 animals and daf-2 mutants by about 23% and 30%, respectively (Figure S8). While daf-31 overexpression increased the daf-2 mutant lifespan in a daf-16 dependent manner, daf-31 overexpression increased the reproduction of daf-2 mutants significantly in a daf-16 independent way. Overexpression of daf-31 increased the brood size of daf-16;daf-2 mutants by 40% (P<0.01, t-test) (Figure S8).

Discussion
We demonstrated that daf-31 mutants form dauer-like larvae that share some characteristics of wild-type dauer larvae such as fat accumulation. Many daf genes, especially those from the insulin-like signaling pathway, are involved in the regulation of lifespan [27]. Mutations in daf-2, which encodes an insulin/IGF-1 receptor [9], convey a temperature-sensitive Daf-c phenotype, and the adults live twice as long as wild-type animals [9,28,29]. Mutations in some genes downstream of daf-2, such as age-1 and pdk-1, also extend lifespan [30,31]. Conversely, mutations in other downstream genes, including daf-18 and daf-16, shorten lifespan [32–34].

We examined whether daf-31 is also involved in aging and found that daf-31 partially mediates the effect of reduced daf-2/IGF signaling pathway on C. elegans lifespan. Moreover, overexpression of daf-31 enhances the longevity phenotype of daf-2 mutants depending on the activity of DAF-16. Supporting this lifespan data, the expression levels of sod-3 and bmo-2, the transcriptional targets of the DAF-16 FOXO3 transcription factor, are up-regulated in the daf-31 overexpression strains. Thus, it is reasonable to argue that DAF-31 regulates C. elegans lifespan by influencing DAF-16 transcriptional activity and daf-31 overexpression stimulates DAF-16 activity. Indeed both DAF-31 and DAF-16 are expressed in neurons and intestine, two major tissues essential for regulation of C. elegans lifespan by the DAF-2/IGF signaling pathway [35–37]. However, overexpression of daf-31 has no influence on the subcellular localization of DAF-16. It is consistent with the lifespan data that overexpression of daf-31 does not increase the lifespan of N2 animals. Thus, overexpression of DAF-31 only extends C. elegans lifespan in the daf-2 mutant background in which DAF-16 has entered the nucleus due to inhibition of the IGF signaling.

Previous studies show that the stress-resistance phenotype can be uncoupled from the longevity phenotype [38]. Indeed, although daf-31 overexpression further increases the long-lived lifespan of daf-2 mutants, it has no effect on the thermotolerance of daf-2 mutants. Interestingly, daf-31 overexpression increases the reproduction of both wild-type animals and daf-2 mutants, which is not
dependent on DAF-16, suggesting DAF-31 functions through DAF-16 for lifespan regulation but not for reproduction. Since DAF-16 is required for the stress resistance of daf-2 mutants, it is likely that daf-31 overexpression extends the daf-2 mutant lifespan through DAF-16-dependent mechanisms other than increasing stress-resistance. DAF-31 is found in multiple tissues including neurons. It is known that many C. elegans neurons are refractory to RNAi treatment in wild-type background [39]. It is possible that neuronal DAF-31 activity is more important for lifespan regulation because daf-31 RNAi treatment only shows influence on lifespan of RNAi-sensitive mutants. Supporting this assumption, it has been reported that daf-16/FOXO activity in neurons accounted for only 5–20% of the lifespan extension seen in daf-2 mutants [37]. Since DAF-31 may only influence DAF-16 activity in neurons, its overexpression only increases the daf-2 mutant lifespan modestly.

We cloned the daf-31 gene and sequence analysis indicates DAF-31 is a worm ortholog of ARD1 that was first identified in yeast [40]. ARD1 is the catalytic subunit of the major NatA that transfers an acetyl group from acetyl coenzyme A to the N-terminal of nascent polypeptides. Yeast ARD1 mutants fail to enter stationary phase and sporulate during nitrogen deprivation [40]. The yeast stationary phase is comparable to C. elegans dauer stage and is essential for survival when nutrients are limited. C. elegans enters dauer stage during starvation or under high concentration of pheromone. Our data show daf-31 mutants could not complete dauer morphogenesis in response to pheromone and starvation, which indicates daf-31 is required for dauer formation. Thus, both yeast ARD1 and worm DAF-31 play an important role in the developmental switch in response to the environmental nutrient limitation. Additionally, daf-31 mutants could not grow to fertile adults in an environment with abundant

Figure 3. Influence of daf-31 on C. elegans lifespan. (A) N2 animals treated with daf-31 RNAi have a similar lifespan to those treated with the vector RNAi control. daf-31 RNAi significantly decreases the lifespan of RNAi-sensitive rrf-3 mutants (B) and RNAi-sensitive daf-2 mutants (rrf-3;daf-2) (C). (D) daf-31 overexpression enhances the longevity phenotype of daf-2 mutants. (E) daf-31 RNAi abrogates the effect of daf-31 overexpression on the daf-2 mutant lifespan. (F) daf-16 mutations block the further lifespan extension of daf-2 mutants conferred by daf-31 overexpression. doi:10.1371/journal.pgen.1004699.g003
food suggesting its essential role in normal development. Similar to our observation, it has been reported that loss of Ard1 is lethal for D. melanogaster and affects cell survival or proliferation, indicating ARD1 is required for D. melanogaster development [41]. In addition to developmental arrest, the daf-31 mutants shift metabolism to fat accumulation. Interestingly, yeast ARD1 mutants not only fail to enter stationary phase but also do not accumulate as much carbohydrates as wild-type yeast strains [40]. Thus, the function of ARD1 in regulating development and metabolism appears conserved from yeast to C. elegans.

N-terminal acetylation is one of the most common posttranslational protein modifications. It is estimated to occur on 50% of yeast proteins [20], 71% of D. melanogaster cytosolic proteins [20] and 84% of human proteins [42]. NatA plays the most prominent role in N-terminal acetylation. It would be interesting to know whether the pleiotropic phenotypes of ard1 mutants result from global changes of protein N-acetylation or from acetylation status of specific protein substrates. It has been reported that human ARD1 directly acetylates β-catenin and enhances its transcriptional activity [43]. We show that overexpression of DAF-31 stimulates the transcriptional activity of DAF-16. It would be interesting to examine if DAF-31 overexpression acetylates DAF-16. Alternatively, a suppressor screening of daf-31 mutants may help to identify the essential substrates of the DAF-31 acetyltransferase and contribute to understanding the mechanisms by which ARD1 influences development, metabolism and aging. Moreover, emerging evidence has revealed that abnormal regulation of ARD1 is associated with tumorigenesis and ARD1 represents a novel cancer drug target [44,45]. Identification of DAF-31 substrate proteins may uncover new therapeutic targets of cancer diseases.

Materials and Methods

Culture conditions and C. elegans strains

All strains were grown on NG agar plates seeded with E. coli strain OP50 [46]. Mutations are listed by linkage groups as follows: LG I: daf-16(mgDf47); LG II: rrf-3(pk1426); LG III: daf-2(e1370); LG IV: unc-24(e138), daf-31(m655); LG X: daf-3(e1376), daf-12(m20), daf-12(rh61rh411). All mutants are derived from the wild-type Bristol N2 strain.

To make the daf-16(mgDf47); daf-2(e1370)III double mutant, daf-2(e1370) males were mated with daf-16(mgDf47) hermaphrodites. Ten F1 adults (daf-16/+; daf-2/) were incubated at 25°C. P2 dauer larvae (either +/+; daf-2 or daf-16/++; daf-2) were transferred to a fresh plate at 15°C for recovery. Then adults were shifted to 25°C. Since daf-16(mgDf47) can suppress the daf-2(e1370) Daf-c phenotype, non-dauer adults from the next generation were daf-16(mgDf47); daf-2(e1370) double mutants.

Double mutants were constructed for epistatic tests between daf-31 and daf-12 mutants at 20°C. However, daf-16 RNAi was used for epistasis analysis between daf-31 and daf-16. daf-16 RNAi fully suppressed dauer formation of daf-2(e1370) mutants. daf-12(m20) and daf-12(rh61rh411) mutations were used to construct the strain+daf-31(m655)/unc-24(e138)+; daf-12(m20) and +daf-31(m655)/unc-24(e138)+; daf-12(rh61rh411) using
standard genetic methods. The *daf-12*(rh61/rh411) mutation was confirmed by sequencing. +*daf-31*(m655)/unc-24(e138); *daf-3*(e1376) was constructed to determine the epistatic relationship between *daf-31* and *daf-3*. As the *daf-31*(m655) mutation was not marked by a genetic mutation in *daf-31;daf-3* and *daf-31;daf-12* mutant worms, the *daf-31*(m655) deletion mutations were confirmed by using single worm PCR. Representative gel pictures are shown in Figure S9. Injection of RNAi was used to inhibit the *daf-15* gene in *unc-24(e138);daf-31(m655);nT1* to examine the epistatic relationship between *daf-15* and *daf-31*.

To construct the *daf-31* overexpressing strains, the full-length *daf-31* genomic DNA, including its native 760-bp promoter and 3’-UTR was cloned into pGEM-T (Promega); this construct successfully rescued the *daf-31* dauer-like mutant phenotype. Multiple copies of the construct were integrated into chromosomal DNA by γ-irradiation to make an N2 transgenic line overexpressing *daf-31*. Then the *daf-31* overexpressing chromosome was introduced into both *daf-2(e1370)* and *daf-16(mgDf47);daf-2(e1370) mutants by genetic crosses.

To make a *daf-31* promoter-GFP transcriptional fusion, the 760-bp *daf-31* promoter was inserted into the GFP vector pPD95.70 (a gift from Dr. Andrew Fire at Stanford University) between the PstI and BamHI sites. The construct was injected into N2 adults at a concentration of 100 μg/ml. pRF4, which encodes a mutant collagen and induces a dominant roller (Rol) phenotype, was co-injected at the same concentration as a transformation marker. Rol animals were selected from the F2 generation and used to establish stable transgenic lines.

To evaluate the subcellular location of DAF-16, T356 (zIs356 [daf-16p::daf-16a/b::GFP+rol-6]) males were crossed to homozygotes. PCR products were cloned into the pGEM-T vector (Promega) following the manufacturer's instructions. T7 primer 5’-GTA ATA CGA CTC ACT ATA GG-3' and SP6 primer 5’-TAC GAT TTA GGT GAC ACT ATA G-3' were used in DNA sequencing reactions.

**RNAi**

Part of the *daf-31* coding region was amplified from *C. elegans* genomic DNA using primers 5’- CGG GAT CCA TTC GTT GTG CTC GCG TG -3' and 5’- CCC AAG CCT GCA GTG GTA TAG GCC TC -3’. The PCR products were then purified and cloned into the feeding RNAi vector L4440 (Addgene) between the BamHI and HinHIII sites. The RNAi construct was transformed into *E. coli* HT115 (DE3) and RNAi feeding was performed as previously described [48].

To inhibit *daf-15* and *daf-31* genes by injection of RNAi, a 1 kb *daf-15* cDNA fragment and the full-length *daf-31* cDNA were cloned into pGEM-T vector (Promega), respectively. The gene identity was confirmed by sequencing. The Riboprobe Combination System-SP6/T7 (Promega) was used to transcribe RNA in vitro according to the manufacturer's protocol. Double-stranded RNA was synthesized and injected as described by Fire et al. [49].

qRT-PCR

Synchronized N2 L1 larvae were treated with either control (empty) vector or *daf-31* RNAi by feeding as previously described [48]. Day 1 adult animals were collected for total RNA extraction using the Trizol kit (Zymo). Synchronized L1 larvae of *daf-31* overexpressing strains were allowed to grow on OP50 food plates. Day 1 adult animals were collected for total RNA extraction using the Trizol reagent (Zymo). The first strand cDNA was synthesized using the ImProm-II reverse transcription system (Promega). SYBR green dye (Quanta) was used for qRT-PCR to measure the expression level of *daf-31*, sod-3 and bemo-2 in corresponding worm samples. Reactions were performed in triplicate on an ABI Prism 7000 real-time PCR machine (Applied Biosystems). Relative-fold changes were calculated using the 2-ΔΔCT method. The primers used for qRT-PCR were: *daf-31*: 5’- GAA GAT CAC AAG GGA AAT GTT G-3’ and 5’- CTC TTG CGG TCT GAT CCA TC-3’; *act-1*: 5’- GAA TCA TGG AGA GGT ATC CTT ACC CTC-3’; *sod-3*: 5’- GCC GAT TTA GAG AAC GGA GAT CAC -3’ and 5’- TGA GAA TTC CGT CAT CTG TCC CCC GA-3’; *bemo-2*: 5’- GGA GAT GAC ACT CAT-3’ and 5’- TGG GTG CTC GCG TG-3’.

**Adult lifespan, thermotolerance and reproduction**

About 120–150 L4 larvae raised at 20°C were transferred to ten NG agar plates (twelve to fifteen animals per plate spread with either OP50 or RNAi food) and incubated at 25°C. The first day of adulthood is day 1 in the survival curves. During the reproductive period, adult animals were transferred daily to fresh plates. Thereafter, animals were transferred every ten days (OP50 food) or every six days (RNAi food). Animals were scored as alive, dead, or lost every other day. Animals that do not move in response to touching were scored as dead. Animals that died from causes other than aging, such as sticking to the plate walls, internal hatching or bursting in the vulval region, were scored as lost. GraphPad Prism was used for statistical analysis and generation of survival curves. For the thermotolerance experiment, day 1 adult animals were incubated at 35°C and survival was scored as described above. To measure reproduction of worms, L4 larvae...
growing at 20°C were transferred daily to fresh plates and the progeny were counted.

Supporting Information
Figure S1  Fat accumulation in daf-31 mutants. Nile red staining of fixed worms detects more fat droplets in daf-2(e1370) (A) and daf-31 mutants (C) than those in N2 animals (B). N2, daf-2(e1370) and daf-31(m655) V; nT1[unc-7(e754) let-71](IV;V) synchronized L1 larvae were placed on NG agar plates, incubated at 20°C until they entered L3 or dauer-like stages, then collected for staining. Scale bars: 10 μm.

(TIF)

Figure S2  daf-31 encodes an ortholog of ARD1. (A) Physical map of the daf-31 region of chromosome IV (corresponding to 0.54 map units). (B) Schematic structure of daf-31 genomic DNA. The closed black boxes represent exons and solid lines are introns. The open box represents the deletion in the daf-31(m655) mutant allele. (C) PCR detected a 393 bp deletion (the actual size of the lower deletion band is 1,449 bp) in daf-31 heterozygous and homozygous mutant worms. (D) Alignment of the DAF-31 protein with its orthologs. Identical amino acids are in black boxes.

(TIF)

Figure S3  Fat accumulation in daf-31 RNAi-treated wild-type animals. Sudan Black staining and Nile red staining of fixed worms detect more fat droplets in daf-2(e1370) (A and D, respectively) and daf-31 mutants (C and F, respectively) than those in N2 animals (B and E, respectively). Scale bars: 10 μm.

(TIF)

Figure S4  daf-31 RNAi knocks down the mRNA level of daf-31. qRT-PCR shows the reduced daf-31 mRNA level in daf-31 RNAi-treated wild-type worms and increased daf-31 mRNA level in daf-31 overexpressing worms. daf-31 RNAi treatment successfully knocks down daf-31 mRNA level in daf-31 overexpressing worms. ***, P<0.001 (t-test).

(TIF)

Figure S5  daf-31 overexpression does not extend wild-type C. elegans lifespan. N2 worms and daf-31 overexpressing animals were grown at 20°C in the presence of food. L4 hermaphrodites were picked up for lifespan experiments. The statistical analysis of lifespan data is presented in Table S1.

(TIF)

Figure S6  daf-31 RNAi does not influence the lifespan of RNAi-sensitive daf-16 mutants. daf-16; rrf-3 animals were fed E. coli that express daf-31 dsRNA or E. coli carrying the empty vector. The lifespan of RNAi-treated progeny were measured at 20°C.

(TIF)

Figure S7  Thermostolerance of animals overexpressing daf-31. daf-31 overexpression does not influence the resistance of N2 worms (p = 0.2420, log-rank test) and daf-2 mutant adults to heat stress at 35°C (P = 0.4623, log-rank-test).

(TIF)

Figure S8  daf-31 overexpression increases reproduction. daf-31 overexpression increases the total number of progeny of N2 and daf-2 mutants, and is not dependent on DAF-16. ** P<0.01, *** P<0.0001 (t-test).

(TIF)

Figure S9  The daf-31 deletion mutation in daf-31;daf-d mutants detected by single worm PCR. Representative gel pictures showing the daf-31 deletion mutation in all daf-31;daf-3 (A) and daf-31;daf-12 (B) homozygous mutants. Arrows indicate the 1,842 bp wild-type band and the 1,449 bp deletion band, respectively.

(TIF)

Table S1  Statistical analysis of daf-31 lifespan data.  ¹ Mean lifespan for each trial. ² Maximum lifespan for each trial. ³ Percentage of changes in mean lifespan relative to corresponding control for each trial. ¼ Numbers of animals counted for each trial. ⁵ p values (log-rank test) compared to corresponding control.

(DOCX)

Table S2  Statistical analysis of thermostolerance experimental data.  ¹ Mean survival for each trial. ² Maximum survival for each trial. ³ Percentage of changes in mean survival relative to corresponding control for each trial. ⁴ Numbers of animals counted for each trial. ⁵ p values (log-rank test) compared to corresponding control.

(DOCX)

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Author Contributions
Conceived and designed the experiments: DC JZ JM TK KJ. Performed the experiments: DC JZ JM TK KJ. Analyzed the data: DC JZ DLR KJ. Contributed reagents/materials/analysis tools: DC JZ JM DLR KJ. Wrote the paper: DC JZ JM TK DLR KJ.

References
1. Golden JW, Riddle DL (1984) A pheromone-induced developmental switch in Caenorhabditis elegans: Temperature-sensitive mutants reveal a wild-type temperature-dependent process. Proc Natl Acad Sci U S A 81: 819–823.
2. Riddle DL, Albert PS (1997) Genetic and environmental regulation of dauer larva development. In: C. elegans II (ed D L. Riddle, T. Blumenthal, B J. Meyer and J R . Priess), pp 739–768 NY: Cold Spring Harbor Laboratory Press.
3. Golden JW, Riddle DL (1983) A gene affecting production of the Caenorhabditis elegans dauer-inducing pheromone. Mol Gen Genet 198: 534–536.
4. Albert PS, Brown SJ, Riddle DL (1981) Sensory control of dauer larva formation in Caenorhabditis elegans. J Comp Neurol 198: 435–451.
5. Bargmann CI, Horvitz HR (1991) Control of larval development by chemosensory neurons in Caenorhabditis elegans. Science 251: 1243–1246.
6. Ren P, Lim CS, Johnsen R, Albert PS, Pilgrim D, et al. (1996) Control of C. elegans larval development by neuronal expression of a TGF-beta homolog. Science 274: 1389–1391.
7. Schackwitz WS, Inoue T, Thomas JH (1996) Chemosensory neurons function in parallel to mediate a pheromone response in C. elegans. Neuron 17: 719–728.
8. Birdy DA, Link EM, Vowell JJ, Tian H, Galaschio PL, et al. (2000) A transmembrane guanylyl cyclase (DAF-11) and Hsp90 (DAF-21) regulate a common set of chemosensory behaviors in Caenorhabditis elegans. Genetics 155: 85–104.
9. Kimura KD, Tissenbaum HA, Liu Y, Ruvkun G (1997) daf-2, an insulin receptor-like gene that regulates longevity and diapause in Caenorhabditis elegans. Science 277: 942–946.
10. Pierce SB, Costa M, Wisotzkey R, Devadhar S, Homburger SA, et al. (2001) MAP kinase signaling regulates the nuclear receptor DAF-16, an insulin receptor-like gene that regulates longevity and dauer development in C. elegans. Dev Cell 1: 672–686.
11. Anasetti A, Yeh WH, Tait D, Hedgecock EM, Riddle DL (2000) daf-12 encodes a nuclear receptor that regulates the dauer diapause and developmental age in C. elegans. Genetics 14: 1512–1527.
12. Albert PS, Riddle DL (1988) Mutants of Caenorhabditis elegans that form dauer-like larvae. Dev Biol 126: 270–293.
13. Gerisch B, Weitze C, Koher-Eisermann C, Rottiers V, Ansetti A (2001) A hormonal signaling pathway influencing C. elegans metabolism, reproductive development, and life span. Dev Cell 1: 841–851.
14. Jia K, Albert PS, Riddle DL (2002) DAF-9, a cytochrome P450 regulating C. elegans larval development and adult longevity. Development 129: 221–231.

15. Motola DL, Cummins CL, Rottiers V, Sharma KK, Li T, et al. (2006) Identification of ligands for DAF-12 that govern dauer formation and reproduction in C. elegans. Cell 124: 1209–1223.

16. Rottiers V, Motola DL, Gerisch B, Cummins CL, Nishiwaki K, et al. (2006) Hormonal control of C. elegans dauer formation and life span by a Rieske-like oxygenase. Dev Cell 10: 473–482.

17. Coldcutter IM (1995) Non-conditional dauer and dauer-like mutants of Caenorhabditis elegans [PhD dissertation]. Columbia (Missouri): Division of Biological Sciences, University of Missouri, Columbia.

18. Jia K, Chen D, Riddle DL (2004) The TORK pathway interacts with the insulin signaling pathway to regulate C. elegans larval development, metabolism, and life span. Development 131: 3897–3906.

19. Yen K, Le TT, Bansal A, Narasimhan SD, Cheng JX, et al. (2010) A comparative study of fat storage quantitation in nematode Caenorhabditis elegans using label and label-free methods. PLoS ONE 5:e12910.

20. Polevoda B, Sherman F (2003) N-terminal acetyltransferases and sequence requirements for N-terminal acetylation of eukaryotic proteins. J Mol Biol 325: 585–622.

21. Gunther CV, Georgi LL, Riddle DL (2000) A Caenorhabditis elegans type I TGF beta receptor can function in the absence of type II kinase to promote larval development. Development 127: 3337–3347.

22. Li J, Tewari M, Vidal M, Lee S (2007) The 14-3-3 protein FTT-2 regulates daf-16 signaling and environmental inputs to mediate aging in the nematode Caenorhabditis elegans. Curr Biol 11: 1975–1980.

23. 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.

24. Lin K, Hsin H, Libina N, Kenyon C (2001) Regulation of the Caenorhabditis elegans longevity protein DAF-16 by insulin/IGF-1 and germline signaling. Nat Genet 26: 139–145.

25. Libina N, Hsin H, Libina N, Kenyon C (2001) Regulation of the Caenorhabditis elegans longevity protein DAF-16 by insulin/IGF-1 and germline signaling. Nat Genet 26: 139–145.

26. Libhgos GJ, White TM, Melov S, Johnson TE (1995) Thermostolerance and extended life-span conferred by single-gene mutations and induced by thermal stress. Proc Natl Acad Sci U S A 92: 7340–7344.

27. Honda Y, Honda S (1999) The daf-2 gene network for longevity regulates oxidative stress resistance and Mn-superoxide dismutase gene expression in Caenorhabditis elegans. FESEB J 13: 1385–1393.

28. Kenyon C (2005) The plasticity of aging: insights from long-lived mutants. Cell 120: 449–460.

29. Kenyon C, Chang J, Gensch E, Rudner A, Tabtiang R (1993) A C. elegans mutant that lives twice as long as wild type. Nature 366: 461–464.

30. Gems D, Sundermeyer 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: 129–155.

31. Morris JZ, Tewari M, Vidal M, Lee SS (2007) The 14-3-3 protein FTT-2 regulates daf-16 signaling and environmental inputs to mediate aging in the nematode Caenorhabditis elegans. Curr Biol 11: 1975–1980.

32. Ogg S, Ruvkun G (1998) The C. elegans PTEN homolog, DAF-18, acts in the insulin receptor-like metabolic signaling pathway. Mol Cell 2: 887–893.

33. Ogg S, Paradis S, Gottlieb S, Patterson GI, Lee L, et al. (1997) The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in C. elegans. Nature 389: 994–999.

34. 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. Science 278: 1319–1322.

35. Apfeld J, Kenyon C (1990) Cell nonautonomy of C. elegans daf-2 function in the regulation of diapause and life span. Cell 65: 199–210.

36. Wolkow CA, Kimura KD, Lee MS, Ruvkun G (2000) Regulation of C. elegans life-span by insulin-like signaling in the nervous system. Science 290: 147–150.

37. Libina N, Berman JR, Kenyon C (2003) Tissue-specific activities of C. elegans DAF-16 in the regulation of lifespan. Cell 115: 409–502.

38. Fujii M, Tanaka N, Miki K, Hossain MN, Endoh M, et al. (2005) Coupling of longevity and pararquat resistance in mutants of the nematode Caenorhabditis elegans. Biosci Biotechnol Biochem 69: 2015–2018.

39. Timmons L, Court DL, Fire A (2001) Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in Caenorhabditis elegans. Gene 265: 103–112.

40. Whiteway M, Szostak JW (1985) The ARD1 gene of yeast functions in the switch between the mitotic cell cycle and alternative developmental pathways. Cell 43: 483–492.

41. Wang Y, Mijares M, Gall MD, Turan T, Javier A, et al. (2010) Drosophila variable nurse cells encode arrest defective 1 (ARD1), the catalytic subunit of the major N-terminal acetyltransferase complex. Dev Dyn 239: 2013–2027.

42. Arnesen T, Van Damme P, Polevoda B, Helsera K, Enjenth R, et al. (2009) Proteomics analyses reveal the evolutionary conservation and divergence of N-terminal acetyltransferases from yeast and humans. Proc Natl Acad Sci U S A 106: 8157–8162.

43. Linn JH, Park JW, Chun YS (2008) Human arrest defective 1 acetylases and activates beta-catenin, promoting lung cancer cell proliferation. Cancer Res 66: 10677–10682.

44. Arnesen T, Thompson PR, Varhaug JE, Lillehaug JR (2008) The protein acetyltransferase ARD1: a novel cancer drug target? Curr Cancer Drug Targets 8: 545–553.

45. Kuo HP, Hung MC (2010) Arrest-defective-1 protein (ARD1): tumor suppressor or oncogene? Am J Tradit Res 2: 56–64.

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

47. Pino EC, Webster CM, Carr CE, Soukas AA (2013) Biochemical and high throughput microscopic assessment of fat mass in Caenorhabditis elegans. J Vis Exp 73: 50180.

48. Kamath RS, Martinez-Campos M, Zippelren P, Fraser AG, Ahringer J (2001) Effectiveness of specific RNA-mediated interference through ingested double-stranded RNA in Caenorhabditis elegans. Genome Biol 2: RESEARCH0092.

49. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, et al. (1998) Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 391: 806–811.