

# Neurosecretory control of aging in *Caenorhabditis elegans*

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**ABSTRACT** In the nematode *Caenorhabditis elegans*, an insulin receptor signaling pathway regulates adult life span and developmental arrest at the dauer larval stage. Here we show that the *unc-64* and *unc-31* genes also function in this pathway. These two genes are involved in mediating  $\text{Ca}^{2+}$ -regulated secretion. Mutations in *unc-64* and *unc-31* increase adult life span and cause constitutive dauer formation. Both phenotypes are suppressed by mutations in *daf-16*, which also suppresses other mutations in this pathway. We present evidence that the site of action of *unc-64* is neuronal, suggesting that a neurosecretory signal regulates life span and dauer formation.

In *Caenorhabditis elegans*, genes that regulate life span have been identified by their effects on developmental arrest at the dauer larval stage (1). The dauer larva is an alternative third-stage larva that forms in response to unfavorable environmental conditions and is specialized for long-term survival (2). Mutations in many genes that cause constitutive dauer formation have been isolated. These genes define at least three parallel pathways that regulate dauer formation (3, 4). One of these, an insulin receptor signaling pathway, also regulates adult life span. Mutations in *daf-2*, which encodes a homolog of the insulin receptor (5), and *age-1*, which encodes a homolog of the phosphatidylinositol-3-OH kinase catalytic subunit (6), cause adults to live twice as long as wild types and cause constitutive dauer formation (1, 4). Both phenotypes are suppressed by mutations in *daf-16*, which encodes a Fork head transcription factor (7–10). The current model for this pathway is that activation of the DAF-2 insulin receptor by its ligand (not yet identified) leads to activation of AGE-1, which generates the 3-phosphoinositide second messengers  $\text{PIP}_2$  and  $\text{PIP}_3$ . These second messengers activate downstream signaling molecules such as the AKT-1 and AKT-2 kinases (11), which ultimately antagonize the activity of DAF-16. DAF-16 presumably acts in the nucleus to control transcription.

The *unc-64* and *unc-31* genes were originally identified in a screen for mutants affecting locomotion (12). *unc-64* and *unc-31* encode homologs of syntaxin and CAPS, proteins that mediate  $\text{Ca}^{2+}$ -regulated secretion (13–16). Here we show that *unc-64* and *unc-31* also regulate life span and dauer formation through the insulin receptor pathway.

## MATERIALS AND METHODS

**Strains.** All strains were derivatives of the wild-type strain N2 and were cultured as described (12).

**Dauer Formation Assays.** Parents were allowed to lay eggs for 4–6 hours at room temperature, and progeny were incubated at the assay temperature. Animals were counted  $\approx 43$  hours later at 27° and  $\approx 103$  hours later at 15°. Small differences in temperature around 27° can make significant differences in

the percentage of dauers formed (data not shown), so each set of assays included all of the relevant strains. The data compiled in Tables 1 and 3 combine the results from multiple assays in which the mean temperature probably varied slightly. Actual values varied from assay to assay, but the relative values of different strains were consistent in all experiments. For the dauer counts of *unc-64(md1259)/unc-64(e246)* and controls, *unc-64(e246 or md1259)/+* or wild-type males were mated to *dpy-18(e364) unc-64(e246)* hermaphrodites at 20° for 1 day. The mated hermaphrodites were allowed to lay eggs for 1 day at 20° and then were shifted to 26.8°. Non-Dpy dauers and non-dauers were counted the following day. Dauers and non-dauers were scored as Unc or non-Unc to determine the presence of the paternal *unc-64* mutation.

**Life Span Assays.** Animals were assayed as described (8). For the data presented in Table 2 and Fig. 2, animals were grown at 15° until the L4 or young adult stage to bypass the dauer stage and then were shifted to 20° for the remainder of the experiment. All other experiments were performed exclusively at 20°. Both *unc-64* and *unc-31* mutants grew to the adult stage at approximately the same rate as N2. *unc-31* mutant animals are defective in egg-laying (17) and frequently were killed by the internal hatching of larvae. Such animals were excluded from the assay results. All *P* values were determined by the Mann–Whitney *U* test by using INSTAT 2.01 software (<http://www.graphpad.com/instat3/instat.htm>).

***unc-64* Transgenic Constructs.** The *unc-64* B cDNA from pTX4 (14) was first modified to delete an upstream and out-of-frame ATG in the 5' untranslated region. An  $\approx 400$ -bp fragment of the 5' end of the gene was amplified by PCR, was digested with *Xba*I and *Hpa*I, and was ligated to pTX4 digested with *Xba*I and *Hpa*I. This resulted in a deletion of  $\approx 100$  bp of the *unc-64* 5' untranslated region, including the out-of-frame ATG. The construct was sequenced to confirm that no undesired mutations had been generated by PCR. The resulting cDNA was cloned into vectors containing the *hsp16–2*, *aex-3*, and *elt-2* promoters. *unc-64(e246); lin-15(n765)* animals were injected with *lin-15(+)* DNA (18) as a transgenic marker at 120 ng/ $\mu\text{l}$  and the appropriate *unc-64* construct at 20 ng/ $\mu\text{l}$ . For each construct, several transgenic lines were generated. Similar dauer phenotypes were seen for the different lines of a given construct, so only one line was analyzed in more detail to give the results seen in Table 3.

## RESULTS AND DISCUSSION

Developmental arrest of *C. elegans* at the dauer larval stage increases as the temperature increases (19, 20). Mutations in many genes cause constitutive dauer formation at 25°C. Of these, only *daf-2* and *age-1* mutants have an increased life span and are suppressed by mutations in *daf-16*. Though originally identified by their uncoordinated movement phenotypes (12), we found that the *unc-64(e246)* and *unc-31(e928)* mutations caused constitutive dauer formation at 27° but not at 25° (Table

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Table 1. Mutations in *unc-64* and *unc-31* cause constitutive dauer formation and increase life span

Genotype	Percent of dauers at 26.8 ± 0.2°C*	Days lived at 20°C†
N2 (wild-type)	12 ± 10	22 ± 5 (42)
<i>unc-64(e246)</i>	99 ± 2	39 ± 12 (46)
<i>unc-31(e928)</i>	93 ± 11	29 ± 12 (72)
<i>daf-16(m27)</i>	6 ± 5§	18 ± 3 (47)
<i>daf-16(m27); unc-64(e246)</i>	5 ± 4§	20 ± 5 (47)
<i>daf-16(m27); unc-31(e928)</i>	8 ± 6§	19 ± 5 (104)
<i>daf-5(e1385); unc-64(e246)</i>	99	
<i>daf-5(e1385); unc-31(e928)</i>	100	
+/ <i>unc-64(e246)</i> ‡	9	23 ± 5 (14)
<i>unc-64(e246)/unc-64(e246)</i> ‡	100	44 ± 14 (9)
<i>unc-64(md1259)/unc-64(e246)</i> ‡	94	38 ± 8 (17)
<i>unc-31(u280)</i>	100	29 ± 10 (40)

\*The top part of the table gives the mean ± SD of five independent experiments at temperatures ranging between 26.6 and 27.0°C. Each experiment had at least 100 animals of each strain. The middle two lines give the mean of two experiments at 26.6 and 27.0°C. The bottom part of the table gives the results from a single experiment at 26.8°C.

†The top part of the table combines the data from two independent experiments and gives the mean ± SD for the entire data set. No strain differed significantly from one experiment to the other (all *P* values > 0.15). The bottom part of the table gives the results from a single experiment. The number in parentheses is the number assayed.

‡This *unc-64(e246)* chromosome also carries *dpy-18(e364)*.

§Partial dauers (21).

1 and data not shown). An *unc-64(e246); unc-31(e928)* double mutant formed dauers at temperatures <25° (Table 2). These two results suggest that *unc-64* and *unc-31* regulate dauer formation but have weaker effects individually than the previously characterized dauer-constitutive genes. There are multiple genetic pathways regulating dauer formation (3, 4). To determine the pathway in which *unc-64* and *unc-31* function, we built double mutants between *unc-64* or *unc-31* and mutations that suppress each of the pathways. The dauer-constitutive phenotypes of *unc-64* and *unc-31* were completely suppressed by mutations in *daf-16* (Table 1), and the *unc-64; unc-31* double mutant also was suppressed completely by a mutation in *daf-16* (data not shown). In contrast, *unc-64* and *unc-31* were not suppressed by mutations in *daf-5* (Table 1), which suppresses mutations (e.g., *daf-7*) that disrupt the

Table 2. Mutations in *unc-64* and *unc-31* do not enhance the dauer and life span phenotypes of *daf-2* mutants

Genotype	Percent of dauers at 15°C*	Days lived at 20°C†
N2 (wild-type)	0	24 ± 7 (20)
<i>unc-64(e246)</i>	1	33 ± 14 (27)
<i>unc-31(e928)</i>	0	35 ± 8 (29)
<i>daf-2(e1370)</i>	0	57 ± 9 (31)
<i>daf-2(e1370) unc-64(e246)</i>	1	59 ± 13 (37)
<i>daf-2(e1370); unc-31(e928)</i>	1	47 ± 9‡ (62)
<i>unc-64(e246); unc-31(e928)</i>	67	43 ± 11 (36)

\*Results are the mean of two independent experiments at 15°C. More than 200 animals of each strain were assayed. We attempted dauer counts at 20°C, but *daf-2*, *daf-2 unc-64*, and *daf-2; unc-31* all formed a high percentage of dauers, making interpretation of synergy difficult.

†Worms were grown at 15°C until they were at the L4 or young adult stage and then were shifted to 20°C. Results are the mean ± SD from a single experiment. The number in parentheses is the number assayed.

‡Old *daf-2; unc-31* animals carry hatched but apparently dead L1 larvae in their body. This does not kill the worms immediately, but it could reduce life span and makes it difficult to interpret the life span data for this strain.

Table 3. *unc-64* acts in the nervous system

Relevant genotype	Expression*	Percent of dauers at 26.8 ± 0.2°C†
N2 (wild-type)	–	12 ± 12
<i>unc-64(e246)</i>	–	100 ± 0
<i>unc-64(e246); saEx357‡</i>	Neurons	70 ± 20
<i>unc-64(e246); saEx358‡</i>	Intestine	99 ± 1

\*Predominant tissue in which the transgene is expressed as determined by fusion of *gfp* to the identical promoter (data not shown). *saEx357* fuses *unc-64* to the *aex-3* promoter, which is expressed strongly in all neurons and weakly in intestine. *saEx358* fuses *unc-64* to the *elt-2* promoter, which is expressed in the intestine and parts of the pharynx.

†Results are the mean ± SD of five independent experiments at temperatures ranging between 26.6 and 27°C. Each experiment had at least 50 animals of each strain.

‡These strains carry a chromosomal *lin-15(n765)* mutation and *lin-15(+)* in the extrachromosomal array.

parallel type  $\beta$  transforming growth factor pathway. These data place *unc-64* and *unc-31* in the genetic pathway of the *daf-2* and *age-1* genes.

Because *unc-64* and *unc-31* affect the same dauer pathway as *daf-2* and *age-1*, we tested whether they also regulate adult life span. We found that both the *unc-64(e246)* and *unc-31(e928)* mutants had significantly longer life spans than the wild type (Table 1, Fig. 1), although the life span extension was not as great as for *daf-2* and *age-1*. These weaker life span phenotypes are consistent with the weaker dauer phenotypes of the *unc-64* and *unc-31* mutants. Furthermore, *daf-16(m27)* suppressed the life span extension of both *unc-64(e246)* and *unc-31(e928)* (Table 1 and Fig. 1). Thus, *unc-64* and *unc-31* appear to be new genes in the *daf-2/age-1* pathway regulating dauer formation and life span.

All of the results presented so far were with a single allele of each gene. To determine whether the dauer and life span phenotypes of these mutants were allele-specific, we examined an additional allele of each gene. An *unc-31(u280)* amber mutant (17) had dauer and life span phenotypes virtually identical to the *unc-31(e928)* mutant (Table 1). The *unc-31(e928)* mutation is a deletion that removes most of the *unc-31* gene, so it is presumably null (15). The *unc-64(e246)* mutation is a missense mutation (13, 14). Null alleles of *unc-64* result in larval lethality (14) and thus cannot be examined for

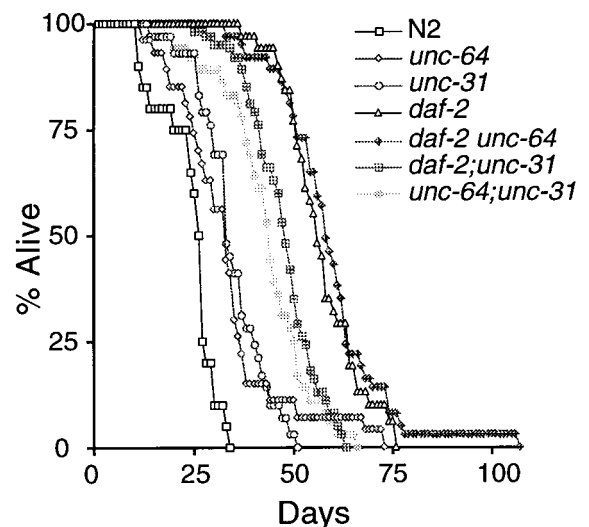


FIG. 1. *unc-64* and *unc-31* mutants have increased life spans and are suppressed by *daf-16*. Assays were performed at 20°. *unc-64* and *unc-31* have significantly longer life spans than N2 ( $P < 0.0001$  for *unc-64* and  $P = 0.0004$  for *unc-31*). This figure uses the same data set as Table 1.

dauer and life span phenotypes. *unc-64(md1259)*, a mutation in a splice site resulting in viable but unhealthy animals (14), caused constitutive dauer formation on its own at 27°, but broods were asynchronous and difficult to count, so we examined the phenotypes of *unc-64(md1259)* in trans to *unc-64(e246)*, which is fully recessive on its own. *unc-64(md1259)/unc-64(e246)* animals had dauer and life span phenotypes that were only slightly weaker than those of *unc-64(e246)* homozygotes (Table 1), indicating that these phenotypes are not allele-specific. We also built double mutants of *unc-64(e246)* and *unc-31(e928)* with *daf-16(mgDf50)*, a deletion of almost all of the *daf-16* coding sequence and presumed null allele (9). As with the non-sense mutation *daf-16(m27)* (10), the dauer-constitutive and long-lived phenotypes were completely suppressed by *daf-16(mgDf50)* (data not shown).

The *unc-64* and *unc-31* genes could act in a linear pathway with *daf-2* and *age-1* upstream of *daf-16* or in a parallel branch. To help distinguish these possibilities, we built double mutants of *unc-64(e246)* and *unc-31(e928)* with the *daf-2(e1370)* mutation. If *unc-64* and *unc-31* act in a linear pathway with *daf-2*, the double mutants should have phenotypes similar to the *daf-2* single mutant, but, if *unc-64* and *unc-31* act in parallel to *daf-2*, the double mutants might have stronger phenotypes, as seen for double mutants between *daf-2* and mutations in the parallel type  $\beta$  transforming growth factor pathway (9). Because the *daf-2* single mutant forms 100% dauers at 25° (4, 21), we measured dauer formation at 15°. As shown in Table 2, the double mutants do not have stronger dauer-constitutive phenotypes. In contrast, double mutants of *unc-64* or *unc-31* with *daf-7(e1372)* form 100% dauers at 15°, demonstrating that *unc-64* and *unc-31* are capable of enhancing the dauer-constitutive phenotype of mutants in a parallel pathway. Similar to the lack of synergy seen for the dauer-constitutive phenotype, the life spans of *daf-2 unc-64* and *daf-2;unc-31* were similar to or shorter than that of the *daf-2* single mutant (Table 2 and Fig. 2). Thus, we could find no evidence that *unc-64* and *unc-31* act in parallel to *daf-2*. In contrast, both the dauer and life span phenotypes of an *unc-64; unc-31* double mutant were

stronger than those of either single mutant (Table 2 and Fig. 2), suggesting that *unc-64* and *unc-31* are partially redundant.

A clue to *unc-64* and *unc-31* function in the *daf-2/age-1* pathway comes from their molecular identities. *unc-64* encodes a homolog of syntaxin, a protein involved in synaptic transmission and other types of  $Ca^{2+}$ -regulated secretion (13, 14). *unc-31* encodes a homolog of CAPS ( $Ca^{2+}$ -dependent activator protein for secretion) (15, 16), a protein required for  $Ca^{2+}$ -stimulated peptide secretion from a mammalian neuroendocrine cell line (22). Also, both mutants in *C. elegans* are resistant to the acetylcholinesterase inhibitor aldicarb, a phenotype characteristic of mutants defective in synaptic transmission (23). Thus, it is likely that the dauer and life span phenotypes of these mutants result from a defect in regulated secretion. An *unc-31::lacZ* reporter is expressed exclusively in neurons (15) whereas the UNC-64 protein is expressed in many types of secretory tissues, most prominently in the entire nervous system and intestine (14). Because the expression of these two genes coincides only in neurons, we predicted that their site of action in regulating dauer formation and life span would be neuronal. To further test this idea, we made transgenic animals carrying an *unc-64* cDNA fused to tissue-specific promoters and tested for rescue of *unc-64(e246)* mutant phenotypes. Because there are three distinct *unc-64* cDNAs (14), we made a fusion to the *hsp16-2* heat shock promoter as a measure of the maximal rescue expected from this particular cDNA. *unc-64(e246)* animals carrying the *hsp16-2::unc-64* fusion without heat shock showed weak rescue of the dauer-constitutive phenotype at 27° and no detectable rescue of the uncoordinated phenotype. With heat shock (1 hour at 33° during the L1 larval stage), there was stronger rescue of the dauer-constitutive (a mean of 50% dauers in four lines ranging from 25 to 73% vs. 100% for *unc-64* control) and uncoordinated phenotypes. However, this rescue was still not complete. We fused the same *unc-64* cDNA to the *aex-3* promoter, which expresses predominantly in the nervous system (ref. 24 and data not shown), and the *elt-2* promoter, which expresses predominantly in the intestine (ref. 25 and data not shown). Animals carrying the *aex-3::unc-64* fusion were partially rescued for the dauer phenotype whereas those carrying the *elt-2::unc-64* fusion were not (Table 3). Thus, *unc-64* acts at least partly in the nervous system to regulate dauer formation. Failure to achieve complete rescue by the *aex-3::unc-64* transgene could signify function of *unc-64* in additional tissues or could simply reflect somatic loss of the extrachromosomal array, insufficient transgene expression or a need for an alternative isoform. Consistent with these last two possibilities, these animals exhibited virtually no rescue of the uncoordinated phenotype, which is expected to have a neuronal focus.

Given the broad neuronal expression patterns of *unc-64* and *unc-31*, one could argue that the life span and dauer phenotypes of these mutants are caused by indirect effects on nervous system function. In addition to the suppression by *daf-16*, the following observations support a specific role for *unc-64* and *unc-31* in the *daf-2/age-1* pathway. One possible mechanism for increasing life span is caloric restriction, as has been shown for *C. elegans* mutants defective in eating (26). However, the *unc-64(e246)* and *unc-31* mutants do not significantly decrease pumping rate (14, 17), and their larval growth rates are approximately the same as that of the wild type. The increased life span is also unlikely to be caused by indirect effects on movement because many *unc* mutants do not affect life span (26). Finally, the phenotypes are probably not caused by general defects in synaptic transmission because other mutants disrupting general synaptic function do not have dauer-constitutive phenotypes at 27°, including *unc-11(e47)*, *unc-13(e51)*, *snt-1(md125)*, and *snb-1(md247)* (data not shown). *snt-1* and *snb-1* encode homologs of the synaptic vesicle proteins synaptotagmin and synaptobrevin (27, 28). Thus, the life span and dauer phenotypes of *unc-64* and *unc-31*

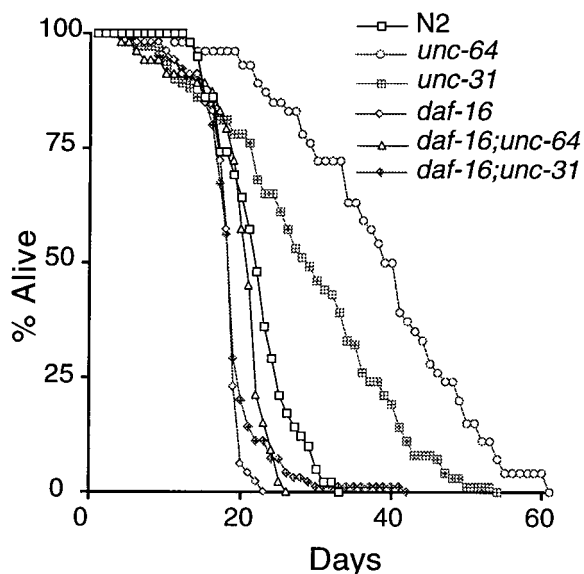


Fig. 2. Mutations in *unc-64* and *unc-31* do not enhance the longevity of *daf-2* mutants. Animals were grown at 15° to allow development past the dauer stage and then were shifted to 20°. *unc-64; unc-31* had a significantly longer life span than either *unc-64* ( $P = 0.0002$ ) or *unc-31* ( $P = 0.0005$ ). The *daf-2 unc-64* double mutant and *daf-2* were not significantly different ( $P = 0.5139$ ). The maximum life span of *daf-2 unc-64* was extended to >100 days by a single animal. We have not investigated whether this has any possible significance. This figure uses the same data set as Table 2.



mutants appear to be the result of a defect in some type of  $\text{Ca}^{2+}$ -regulated secretion that is most sensitive to the elimination of *unc-64* and *unc-31* function.

Insulin release by  $\beta$  cells of the mammalian pancreas is determined by  $\text{Ca}^{2+}$ -regulated secretion. The simplest model for *unc-64* and *unc-31* function in regulating life span and dauer formation is that they are involved in the  $\text{Ca}^{2+}$ -regulated secretion of an insulin-like ligand for the DAF-2 receptor. Although no such ligand has been identified yet, there are a large number of candidate insulin-like genes in the *C. elegans* genome. We propose that this putative insulin is released from within the nervous system. Mutations in the *unc-64* and *unc-31* genes would prevent this release and, hence, would prevent activation of the DAF-2 receptor. An alternative model is that *unc-64* and *unc-31* do not directly mediate secretion of insulin but instead regulate classical neurotransmitter input to the cells that release insulin. Of interest, *daf-2* mosaic analysis shows that the DAF-2 receptor functions in neurons and in other tissues to regulate life span and dauer formation (29). Thus, it appears that the nervous system regulates life span at several steps in this pathway.

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