A novel nuclear receptor/coregulator complex controls *C. elegans* lipid metabolism, larval development, and aging

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Environmental cues transduced by an endocrine network converge on *Caenorhabditis elegans* nuclear receptor DAF-12 to mediate arrest at dauer diapause or continuous larval development. In adults, DAF-12 selects long-lived or short-lived modes. How these organismal choices are molecularly specified is unknown. Here we show that coregulator DIN-1 and DAF-12 physically and genetically interact to instruct organismal fates. Homologous to human corepressor SHARP, DIN-1 comes in long (L) and short (S) isoforms, which are nuclear localized but have distinct functions. DIN-1L has embryonic and larval developmental roles. DIN-1S, along with DAF-12, regulates lipid metabolism, larval stage-specific programs, diapause, and longevity. Epistasis experiments reveal that *din-1S* acts in the dauer pathways downstream of lipophilic hormone, insulin/IGF, and TGFβ signaling, the same point as *daf-12*. We propose that the DIN-1S/DAF-12 complex serves as a molecular switch that implements slow life history alternatives in response to diminished hormonal signals.

*Keywords:* Nuclear receptor; coregulator; aging; dauer; heterochrony

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Nuclear hormone receptors orchestrate diverse aspects of metazoan development, metabolism, and homeostasis, often coordinating programs throughout the body via lipophilic hormones [Mangelsdorf et al. 1995]. Some nuclear receptors, such as retinoic acid and thyroid receptors, turn on target genes in response to hormone, and turn them off in the absence, by assembling coactivator and corepressor complexes, respectively [Glass and Rosenfeld 2000]. Identified coactivators such as SRC-1, GRIP, CBP/p300 and PCAF promote histone acetylation and potentiate transcription. Conversely, corepressors such as NCoR and SMRT, recruit histone deacetylases and catalyze transcriptional silencing. Although intensely studied biochemically, physiological descriptions of such complexes are just beginning [Yao et al. 1998; Jepsen et al. 2000; Xu and Li 2003]. Governed by systemic signals, these complexes are well poised to act as molecular switches that can work at the level of the whole organism, specifying metabolic states, developmental alternatives, or transitions from juvenile to adult forms.

A genetic analysis of the *Caenorhabditis elegans* nuclear hormone receptor DAF-12 reveals the remarkable intricacy of receptor signaling in a simple in vivo model. DAF-12 is homologous to vertebrate vitamin D, pregnane, and androstane receptors [Antebi et al. 1998, 2000]. In harsh environments, DAF-12 promotes dauer diapause, a stress-resistant, long-lived alternate third larval stage [Riddle et al. 1981; Antebi et al. 1998]. In favorable environments, DAF-12 promotes reproductive development, regulating third stage [L3] and later programs within the heterochronic circuit, a regulatory pathway that controls developmental timing [Antebi et al. 1998, 2000]. Moreover, it influences lipid metabolism, fertility, and adult life span [Larsen et al. 1995; Gems et al. 1998; Hsin and Kenyon 1999; Gerisch et al. 2001; Jia et al. 2002]. Alleles fall into at least six classes based on dauer and heterochronic phenotypes, reflecting *daf-12* functional complexity [Antebi et al. 1998]. Notably, DNA-binding domain mutants fail to form dauer larvae [Daf-d], whereas some ligand-binding domain mutants constitutively form dauer larvae [Daf-c].
Many organisms, including mammals, couple environmental and physiological signals, such as nutrient availability to metabolism, development, and life span. For example, dietary restriction can delay puberty and prolong life in rodents (Tatar et al. 2003). The role of daf-12 and functionally related genes in C. elegans dauer formation gives an unprecedented view of how environmental signals transduced by endocrine networks influence similar processes (Riddle and Albert 1997; Finch and Ruvkun 2001). In abundant food, C. elegans transits rapidly through four larval stages (L1–L4) to short-lived reproductive adults (fast life history). Conditions signifying starvation, overcrowding, and thermal stress drive juveniles into long-lived dauer (slow life history). Molecular genetic and cellular studies of dauer formation reveal that specific neurons integrate sensory information and secrete insulin/IGF and TGFβ peptides in favorable environments (Ren et al. 1996; Riddle and Albert 1997; Finch and Ruvkun 2001; Li et al. 2003). Their respective signal transduction pathways ultimately converge on cytochrome P450, DAF-9, which is postulated to produce a lipophilic hormone, possibly a sterol, in endocrine cells (Gerisch et al. 2001; Jia et al. 2002; Gerisch and Antebi 2004; Mak and Ruvkun 2004). Acting through the nuclear receptor DAF-12, this hormone antagonizes dauer, promotes reproductive development, and short life. In unfavorable environments, peptide signaling pathways are suppressed; it is proposed that no hormone or an alternate hormone is made, and DAF-12 promotes dauer formation and long life. In higher metazoans, insulin/IGF signaling regulates sexual maturation, reproduction, and life span, and could do so in part by modulating lipophilic hormones (Tatar et al. 2003).

How are these activities specified? To elucidate how signaling circuits modulate daf-12, we investigated interacting proteins by the yeast two-hybrid method and identified DIN-1. It belongs to a family of large nuclear proteins typified by human SHARP and fly SPEN, which are corepressors for several transcription factors (Wiellette et al. 1999; Chen and Rebay 2000; Kuang et al. 2000; Shi et al. 2001; Oswald et al. 2002). By using genetic epistasis, we show that a small isoform, DIN-1S, works in an in vivo context for nuclear hormone signaling. We propose that as sterol hormone production declines in unfavorable environments, the DIN-1S/DAF-12 complex arrests development and specifies slow life history alternatives. This study reveals that coregulators can instruct alternate organismal fates.

**Results**

**DIN-1 and DAF-12 interact**

Nuclear receptors consist of an N-terminal ligand-independent activation function, a DNA-binding domain (DBD), a hinge region, and a ligand-binding domain (LBD) that binds corepressors, as well as fat soluble hormones and coactivators dependent on a C-terminal transactivation helix called AF-2 (Mangelsdorf et al. 1995). To identify DAF-12 complexes, we sought interacting proteins by the yeast two-hybrid method. With hinge and LBD of DAF-12 [amino acids 281–753] as bait, [Fig. 1B] we screened against an oligo-DT primed C. elegans mixed-stage cDNA library and obtained three independent clones corresponding to the C terminus of F07A11.6, dubbed DIN-1 [DAF-12-interacting protein 1; Fig. 1A, amino acids 1–567].

**DIN-1 structure**

The din-1 locus contains a coding region composed of 23 exons spanning 18.3 kb [Fig. 2A], and a 13-kb predicted promoter. The cDNA structures, determined by RT-PCR and from estent ESTs, revealed at least five isoforms. Exons 3 and 4 were joined in some isoforms, and exons 15 and 19 were alternately spliced. Notably, DIN-1L long isoforms [A–D] lacked exon 19, whereas DIN-1S short isoform uniquely contained it. One DIN-1S product was trans-spliced to SL1 at the exon 19 junction, another 438 bp upstream of exon 19. Other EST clones [yk814c08 and yk38b7] had even longer 5’UTRs [452 and 688 bp, respectively], suggesting din-1S is transcribed as a discrete functional unit. Indeed, the analysis described below shows that din-1L and din-1S have distinct functions, and that din-1S specifically interacted with daf-12.

**DIN-1 is evolutionarily conserved**

DIN-1L belongs to a taxonomically conserved protein family, which includes founding member SPEN, impli-
cated in multiple signaling pathways in flies (Wiellette et al. 1999; Kuang et al. 2000; Lane et al. 2000; Rebay et al. 2000; Lin et al. 2003); human corepressor SHARP (Shi et al. 2001); and mouse MINT (Newberry et al. 1999). DIN-1 and homologs are large nuclear proteins that contain two to four N-terminal RNA recognition motifs (RRMs); a long hinge of low complexity, predicted nuclear localization and AKT phosphorylation sites (RXRXXS/T); and a C-terminal conserved domain known as SID/RD (SMRT interaction domain/repressor domain; Fig. 2B). With respect to SHARP, there is 29% identity within the SID/RD domain and 22% and 26% identity within RRM1 and RRM2. DIN-1S uniquely contains exon 19, comprising the DAF-12 receptor interaction domain (RID), fused only to the SID/RD (Fig. 2A,B). With respect to SHARP, there is 29% identity within the SID/RD domain and 22% and 26% identity within RRM1 and RRM2. DIN-1S uniquely contains exon 19, comprising the DAF-12 receptor interaction domain (RID), fused only to the SID/RD (Fig. 2A,B).

Analysis of interacting regions

To further define interacting regions, we analyzed various DIN-1 peptides for their association with the DAF-12 bait (Fig. 1A). The interaction of short isoform DIN-1S (amino acids 1–567) was delimited to the N-terminal half encoded by exon 19 (amino acids 1–378) and defined by mutation as functionally crucial (see below). Further truncations within this region led to observable (amino acids 54–378), reduced (amino acids 151–378) or no interaction (amino acids 1–156 and 260–378), suggesting the interacting domains may be distributed. In contrast, the SID/RD, common to DIN-1S and DIN-1L showed no interaction (amino acids 379–567). We also examined the association of DIN-1S with several DAF-12 peptides (Fig. 1B). Full-length isoform DAF-12D, with conserved cysteines (magenta), (Bottom) LBD helices (H3–5). Amino acid changes are shown above the sequence, allele names below. (Arrow) Splice mutation, (asterisk) stop codon.

Figure 2. din-1 structure. (A) Cosmids from the din-1 region, gene structure with mutations and din-1S::gfp insertion site (green triangle) are shown. (Bottom) mRNA isoforms containing SL1 trans-splice leader (line), start codon (arrow), stop codon (stop sign), and polyadenylation site (poly A). gfp insertion site of din-1LB is shown. (B) Domain structure of DIN-1 and homologs. (RRM) RNA recognition motif (red); [NLS] Nuclear localization sites (gray); (RID) nuclear receptor interaction domain (blue); [SID/RD] SMRT interaction domain/repression domain (black); [stars] L/IXXL/I/VL/I motifs; (triangles) AKT sites. (C) din-1 mutations. (D) daf-12(rh61) intragenic revertants. (Top) DAF-12 DBD, with conserved cysteines (magenta). (Bottom) LBD helices (H3–5). Amino acid changes are shown above the sequence, allele names below. (Arrow) Splice mutation, (asterisk) stop codon.
ing to isoform B [amino acids 501–753], composed of LBD only. A truncation of the LBD C terminus corresponding to the \( \text{daf-12(rh61)} \) mutant protein [amino acids 1–617] produced visible but reduced interaction. Finally, removal of the LBD N terminus [amino acids 617–753] abolished interaction, revealing that amino acids 501–617 are crucial. Interestingly, this region comprises helices H1–H5, which in vertebrate receptors interact with corepressors NCoR and SMRT [Horlein et al. 1995; Hu and Lazar 1999; Perissi et al. 1999]. Pull-down experiments showed that short isoform DIN-1S specifically associated with DAF-12.:GST in vitro but not with GST alone [Fig. 1C]. Similarly, radiolabeled DAF-12 interacted with DIN-1S.:GST.

din-1S alleles

RNAi against exon 19 of din-1S potently suppressed the Daf-c and heterochronic defects of \( \text{daf-12} \) LBD mutants. We used this observation to obtain bona fide \( \text{din-1} \) alleles by selecting for revertants of \( \text{daf-12(rh61)} \) and \( \text{daf-12(rh274)} \) gonadal migration \( \text{Mig} \) mutants [below]. Such screens yielded five \( \text{din-1} \) alleles: \( \text{dh118}, \text{dh128}, \text{dh149}, \text{dh127}, \text{and} \ \text{dh181}. \) Another allele, \( \text{sa1262} \), suppresses the Daf-c phenotypes of \( \text{nhr-1nhr-2} \) mutants [J. Li, G. Brown, and J. Thomas, pers. comm.]. \( \text{nhr-1} \) and \( \text{nhr-2} \) encode homologs of Niemann-Pick C1 [Sym et al. 2000], a neurodegenerative disease locus implicated in cholesterol trafficking. Remarkably, all six \( \text{din-1} \) alleles clustered around exon 19 [Fig. 2A,C] and selectively affected \( \text{din-1S} \). Since the DAF-12 receptor interaction domain localized to this region [Fig. 1A, amino acids 1–378], association is likely largely disrupted.

Mapping farthest upstream, \( \text{dh149} \) deleted 10 bp, causing a frameshift at D16 that results in a stop codon 45 amino acids downstream [Fig. 2C]. Stop codons \( \text{dh127}, \text{dh181}, \text{dh118/dh128} \) truncated the protein at Q41, Q118, and R303, respectively. Finally, \( \text{sa1262} \) harbored a point mutation in the promoter/5' UTR, 808 nucleotides before exon 19, suggesting that \( \text{din-1S} \) comprises a distinct transcriptional unit.

We also obtained nine \( \text{daf-12(rh61)} \) intragenic revertants from these genetic screens. Interestingly, five mutations affected the LBD between helices 3 and 5 [Fig. 2D], a region crucial for DIN-1 interaction [above], and thus might affect binding. Accordingly, we found that while the \( \text{rh61} \) fragment, DAF-12 [amino acids 1–617], interacted with DIN-1S in the yeast two-hybrid system, a derivative containing the \( \text{dh115} \) [L587F] lesion did not [Fig. 1B, amino acids 1–617']. Finally, four mutations affected the DBD [Fig. 2D], suggesting that loss of DNA binding abolishes the unregulated activity of \( \text{daf-12(rh61)} \) mutant protein.

din-1S regulates dauer formation

Based on the homology of DIN-1 with nuclear coregulators, we hypothesized that DIN-1 and DAF-12 work together in \( \text{C. elegans} \) dauer formation. To test this, we specifically reduced \( \text{din-1S} \) expression by using an exon 19 fragment in RNAi feeding experiments. Alleles isolated in genetic screens gave similar phenotypes. Candidate molecular null alleles \( \text{dh149}, \text{dh127}, \) and \( \text{dh181} \) were partially dauer-formation defective [DAF-d] forming fewer dauer larvae, which abnormally resumed pharyngeal pumping and gonadal development [Table 1]. \( \text{dh181} \) had the most severe dauer formation defects, whereas \( \text{dh128} \) was actually non-Daf.

To place \( \text{din-1S} \) in the dauer pathways, we performed genetic epistasis experiments with various dauer constitutive [Daf-c] mutants. \( \text{din-1S} \) RNAi or \( \text{dh127} \) suppressed Daf-c phenotypes of conditional mutants that impede signaling from Insulin/IGF, TGFß, and cGMP pathways at 25°C [Fig. 3A]. Suppression of \( \text{daf-2/Insulin/IGF} \) receptor was seen with weak alleles \( \text{e1368, e1369, and e41} \), but not strong ones \( \text{e1370 and e1391} \). Similarly, suppression of the \( \text{daf-7/m62} \) null was incomplete.

We next performed genetic epistasis on Daf-c mutants acting further downstream. Mutations in \( \text{daf-9}/\text{cytochrome P450} \) are thought to block production of a lipophilic hormone for DAF-12, whereas mutations in the DAF-12 LBD [e.g., \( \text{rh273, rh274} \)] presumably diminish hormone binding itself [Antebi et al. 2000; Gerisch et al. 2001; Jia et al. 2002]. Notably, \( \text{din-1(dh127)} \) suppressed Daf-c phenotypes of such mutants completely [Fig. 3A]. Moreover, although hypomorphic Mig mutants \( \text{daf-12(rh284)} \) and \( \text{daf-9(rh50)} \) alone are not visibly Daf-c, the \( \text{rh50/rh284} \) double displayed a penetrant synthetic Daf-c phenotype [97%, \( n = 489 \)], similar to the \( \text{daf-9} \) null. \( \text{din-1(dh127)} \) completely suppressed the Daf-c phenotype of this double (0%, \( n = 500 \), Fig. 3A), consistent with action in a singular pathway. Because \( \text{daf-12} \) and \( \text{din-1S} \) nulls behave similarly, suppression of Daf-c mutants could arise from DAF-12 destabilization. However, the \( \text{daf-12:gfp} \) expression pattern was unchanged in \( \text{din-1(dh127)} \) [\( n = 20 \)]. We conclude that \( \text{din-1S} \) acts partially downstream or parallel to insulin/IGF and TGFß signaling, but fully downstream from lipophilic hormonal regulation of nuclear receptor \( \text{daf-12} \).

din-1S influences larval stage-specific programs

The heterochronic loci are stage selectors that regulate \( \text{C. elegans} \) developmental timing. Mutants repeat or delete larval stage-specific programs in different tissues. Alone, \( \text{din-1S} \) alleles or \( \text{din-1S} \) RNAi had no observable heterochrony, but suppressed the delayed heterochronic phenotypes of \( \text{daf-12} \) LBD and \( \text{daf-9} \) hypomorphic mutants. In particular, \( \text{daf-12(rh61)} \) mutants fail to execute L3 seam cell divisions, adult cuticle programs, and gonadal cell migrations [Mig phenotype] on schedule and instead inappropriately repeat earlier larval programs [Fig. 3B,D,F; Table 1, Antebi et al. 1998]. \( \text{daf-9(rh50)} \) mutants display gonadal Mig phenotypes. Significantly, \( \text{din-1S} \) mutants restored L3 stem cell division, adult cuticle formation, gonadal cell migration, and brood size in \( \text{daf-12} \)
such suppression was also seen with daf-12 downstream of a cholesterol sensitive step. Similarly, (Gerisch et al. 2001), suggesting din-1

<table>
<thead>
<tr>
<th>Genotype</th>
<th>% Normal dauer formationa</th>
<th>Daf phenotype</th>
<th>% L3 seamb</th>
<th>% adult seamc</th>
<th>% Reflexed distal tip celld</th>
<th>Brood sizen</th>
</tr>
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<tbody>
<tr>
<td>N2</td>
<td>100, 100</td>
<td>non-Daf</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>303 ± 26, 190 ± 42</td>
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<tr>
<td>din-1(dh149)</td>
<td>19, 0f</td>
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<td>dh127</td>
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<td>100</td>
<td>100</td>
<td>100</td>
<td>235 ± 50</td>
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<tr>
<td>dh181</td>
<td>7, 0f</td>
<td>Daf-d</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>N.D.</td>
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<tr>
<td>dh128</td>
<td>100, 100i</td>
<td>non-Daf</td>
<td>100</td>
<td>100</td>
<td>89k</td>
<td>76 ± 52, 4.6 ± 5.6</td>
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<td>100</td>
<td>100</td>
<td>191 ± 40</td>
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<td>80</td>
<td>0</td>
<td>37 ± 29</td>
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<td>0</td>
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<td>50</td>
<td>99</td>
<td>100</td>
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<tr>
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<td>Daf-d</td>
<td>62</td>
<td>99</td>
<td>100</td>
<td>134 ± 33</td>
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<tr>
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<td>Daf-d</td>
<td>77</td>
<td>99</td>
<td>100</td>
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<tr>
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<td>Daf-d</td>
<td>82</td>
<td>100</td>
<td>100</td>
<td>191 ± 40</td>
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<tr>
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<td>98</td>
<td>100</td>
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<tr>
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<td>80</td>
<td>N.D.</td>
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<td>100</td>
<td>11</td>
<td>N.D.</td>
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<tr>
<td>din-1SRNAi rh50</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>91</td>
<td>N.D.</td>
</tr>
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</table>

*Percentage of dauer larvae that have normal developmental arrest in all tissues and do not pump the pharynx, n ≥ 25 dauers (from starved out plates).

n ≥ 10 animals [110 cells].

n ≥ 25 animals.

n ≥ 25 animals [50 cells], distal tip cells that reach the dorsal side.

n ≥ 5 animals.

Measured at 25°C [phenotypes measured at 20°C except where noted].

11% Mig includes any mismigrated gonadal arm.

N.D. not determined.

12(th61), and gonadal cell migration in daf-9(rh50) [Fig. 3C,E,G; Table 1]. Moreover, din-1(dh127) suppressed the Mig phenotypes of several other daf-12 mutants including rh193, rh285, and rh284 [Supplementary Table S1], which are predicted to disrupt ligand binding or transactivation, suggesting that DIN-1S works with the unliganded receptor. In addition, dh127/rh61 double mutants were similar in their residual heterochronic phenotypes to the din-1(rh61/dh115) intragenic revertant [Table 1], which affects DAF-12 association with DIN-1S [Fig. 1B, amino acids 1–617*.] Finally, din-1 RNAi in the rh61/dh115 background made no difference in phenotype, further supporting the idea that DIN-1 acts principally through the DAF-12 LBD.

Although a ligand for DAF-12 is unidentified, current evidence hints at a derivative of cholesterol, the precursor to many lipophilic hormones. Cholesterol deprivation of wild-type phenocopies Mig and Daf-c defects of daf-9 and daf-12 LBD mutants and enhances hypomorphic alleles (Gerisch et al. 2001), suggesting that loss of hormone production or binding causes these severe phenotypes. Importantly, we found that din-1(dh149) suppressed these induced phenotypes. Whereas 21% and 28% of wild type were Mig and Daf-c (n = 352) on low cholesterol media, 0% of dh149 mutants exhibited either phenotype (n = 73), revealing a critical function downstream of a cholesterol sensitive step. Similarly, such suppression was also seen with daf-12 nulls [Gerisch et al. 2001], suggesting din-1 and daf-12 may work together in this process.

**12 is required for longevity**

Lipophilic hormones may also influence nematode life span. daf-9 Daf-c animals that have recovered to adult live at least 25% longer than wild type, particularly at 15°C (Gerisch et al. 2001; Jia et al. 2002). In addition, they are thermotolerant (B. Gerisch and A. Antebi, unpubl.), often a correlate of longevity [Lithgow et al. 1995]. din-1(dh127) alone had little effect on life span, but fully suppressed daf-9(e1406) longevity (Fig. 3H). Similarly, daf-9 longevity largely depends on daf-12(+) (Gerisch et al. 2001; Jia et al. 2002). These results suggest that in the absence of a daf-9-produced hormone, the DIN-1/DAF-12 complex specifies programs that promote longer life. dh127e1406 animals actually lived shorter than dh127 alone, perhaps reflecting a synthetic phenotype. Indeed, they also displayed impenetrant heterochronic phenotypes in L3 seams [17% mutant, n = 121 cells] not seen in either single mutant, but reminiscent of daf-12 nulls [21% mutant; Antebi et al. 1998].

**DIN-1S has intrinsic repressor activity**

Given the similarity to the SHARP corepressor, we asked whether DIN-1S displayed any repressor activity. To address this, we fused DIN-1S to the yeast GAL4 DBD and assayed transcription from a GAL4 UAS-luciferase reporter in HEK293 cells. As expected, the luciferase reporter displayed a basal level of expression that was transactivated by GAL4 [Fig. 4A, lanes 3,5]. Transactiva-
tion was potently inhibited by the GAL4–DIN-IS fusion protein far below basal levels of transcription (Fig. 4A, lane 6) and was dose dependent (Fig. 4B). Moreover, a GAL-4-DIN-1S (amino acids 1–378) construct lacking the SID/RD domain abolished repression (data not shown). These experiments reveal that DIN-1S possesses intrinsic repressor activity and suggest DIN-1S can recruit human components that mediate transcriptional repression.

din-1L is required for embryonic and larval development

Because long isoforms were mutationally untouched and had no visible RNAi phenotype, we screened for a deletion among mutagenized pools using PCR (McKay and Jones 2002). hd36 deleted 1.54 kb, removing exons 5–6 (RRM2) and creating a frameshift after Y157 that truncates the protein 12 amino acids downstream (Fig. 2A,C). hd36 displayed temperature sensitive phenotypes wholly different from din-1S. At 20°C half the animals were egg-laying defective (Egl) with occasional mispositioned gonadal arms (Fig. 5C–E; Table 1). At 25°C, 18% arrested as embryos (Emb). Those animals that hatched usually displayed variable morphology defects in body and pharynx (Vab; Fig. 5A,B,E). Nearly all animals that reached adult were small (Sma), clear (Clr), slightly uncoordinated, constipated, and virtually sterile (Fig. 5E; Table 1), phenotypes not seen in din-1S mutants. hd36 was non-Daf, failed to suppress the Daf-c phenotype of daf-2(e1368) and had little effect on the ability of din-1S to do so (Fig. 3A). Moreover, hd36 din-1SRNAi animals had no additional discernible phenotype (not shown).

Although linked, din-1S and din-1L behaved essentially as two independent genes. Notably, they complemented one another: din-1S(dh127)/din-1L(hd36) transheterozygotes looked normal [100% non-Clear, non-Small, n = 68] and did not suppress daf-2 Daf-c phenotypes. In all, 62% of the F1 progeny of din-1S/din-1L daf-2(e1368) mothers formed Daf-c dauer larvae at 25°C (n = 872), approximating the expected 75% frequency had hd36 fully complemented dh127.

As another test of separability, we examined genomic constructs for rescue of din-1 phenotypes. First, cosmid T14A11, which encodes the entire locus, rescued both din-1L and din-1S. From a hd36 strain segregating...
T14A11 extrachromosomal array dhEx282, 100% of the animals scored as wild type (non-Clear, non-Small, non-Sterile, n = 50) contained the array, whereas 70% scored as mutants lacked it (n = 50). Thus, hd36 phenotypes arise from a disruption of din-1L, and not from a linked locus. From din-1S(dh127)/daf-2(e1368) dhEx282 animals, 24% (n = 38) of those carrying the transgene showed antisuppression, restoring the daf-2 Dal-c phenotype. Moreover, extrachromosomal array dhEx235, comprising a mere 8.5 kb genomic fragment (exons 17–23), also rescued 39% Dal-c, showing that a minigene encoding din-1S suffices to restore function.

**din-1 influences lipid metabolism**

Prior to diapause, nematodes store lipid in intracellular droplets within the intestine and hydorhids. Daf-c mutants, such as daf-9 and daf-2, inappropriately accumulate excess intestinal fat in favorable conditions, a phenotype suppressed by Daf-d loci daf-12 and daf-16, respectively [Ogg et al. 1997; Gerisch et al. 2001]. Surprisingly, din-1S Daf-d mutants as well as din-1L stored nearly twice the fat as wild type [Fig. 6A,B,E,K], as measured by vital staining with Nile red [Ashrafi et al. 2003]. Moreover, daf-12 nulls suppressed the din-1S phenotype [Fig. 6C,E,G,K], suggesting that din-1S(+) antagonizes daf-12(+) fat storage. Paradoxically, din-1(dh127) also suppressed Nile red accumulation in daf-9 [Fig. 6D,F,K]. Whereas each single mutant accumulated excess fat, din-1(dh127) did not, displaying a synthetic phenotype resembling the daf-12 null [Fig. 6C,H,K]. Thus, din-1(+) antagonizes fat storage under reproductive growth but promotes it during entry to dauer, revealing a key regulatory role.

**din-1 is widely expressed in cell nuclei**

To investigate the din-1 expression pattern, we generated various GFP fusions. For din-1S, we inserted gfp in frame near the beginning of exon 19 in the 8.5 kb minimal genomic rescuing fragment [Fig. 2A]. This construct was functional as measured by phenotypic rescue, and arrays dhEx285 and dhEx343 gave similar expression patterns. din-1S::gfp localized to the nuclei of most cell types. Detected in hydorhids, seam, intestine, and somatic gonad including the distal tip cells [Fig. 7B–G], din-1S was also expressed in neurons, vulval precursors, body wall muscle, and pharynx, all tissues with heterochronic phenotypes or remodeled during dauer. Expression was first detected in a few nuclei by the comma stage of embryogenesis. By hatch, din-1S was widely expressed, albeit weakly. Overall expression in most tissues was detected at various levels into adult and in dauer larvae. Interestingly, in late L1 and L2 larvae din-1S was often (75%, n = 16) highly expressed in the XXX cells [Fig. 7A], specialized neuroendocrine cells proposed to be a site of synthesis for the daf-9-produced hormone [Gerisch et al. 2001; Jia et al. 2002; Ohkura et al. 2003], suggesting control with endocrine circuits themselves. For din-1L, we placed 8 kb of the din-1 upstream region before gfp followed by din-1LB cDNA. Extrachromosomal arrays dhEx191 and dhEx194 were expressed in the nuclei of most cells from embryo to adult [Fig. 7H–J], as well as in dauer larvae. din-1L expressed more strongly in embryo and L1 larvae than din-1S, consistent with an earlier role.

**Discussion**

Nuclear receptor transcriptional complexes are key regulators of organismal biology. Our studies suggest that the
DIN-1, a coregulator of worm diapause

Although alternate isoforms or promoters are not uncommon in gene organization, the resultant products usually have related functions, often expressed in different tissues. Here, the two products have congruent expression patterns, but almost entirely different functions. Their diversification may reveal how coregulator complexes evolve to selectively interact with some transcription factors and not others. *din-1* homologs in other species come in various isoforms [Kuang et al. 2000], though a short isoform is not yet described. Notably, *DIN-1L* homologs are highly variable in length (2407–5554 amino acids). Speculatively, interaction interfaces for different transcription factors could be modularly added or removed rapidly in evolution.

**DIN-1S, a putative nuclear coregulator**

Several lines of evidence strongly suggest that DIN-1S is a coregulator of DAF-12. First, DIN-1S and DAF-12 physically interacted by the yeast two-hybrid method and GST pull down experiments [Fig. 1]. The interacting domain on DAF-12 corresponded to helices 1–5 of the LBD, a region in other nuclear receptors that docks co-regulators [Horlein et al. 1995; Hu and Lazar 1999; Perissi et al. 1999], while the interacting region of DIN-1S was specific to this isoform and contained several putative hydrophobic coregulator helices. Second, genetic experiments confirmed the in vivo relevance of these regions. Suppressors of the *daf-12(rh61) LBD* mutant either fell within the interacting region of DIN-1S [exon 19 RID], or of DAF-12 [helix 3–5], suggesting an in vivo interaction map [Fig. 2A,C,D]. Particularly revealing was DAF-12[L587F] (*dh115*), which has conserved equivalents in PPARγ [L318] and PPARα [L509] that interact directly with the hydrophobic helices of coregulators, as shown by X-ray crystallography [Nolte et al. 1998; Xu et al. 2002]. Direct contacts with DIN-1S are perturbed by *dh115*, since two-hybrid interactions were abolished [Fig. 1B, cf. amino acids 1–617 and 1–617∗]. Accordingly, *dh115rh61* animals grown on *din-1* RNAi were indistinguishable from *dh115rh61* alone [Table 1]. Third, *din-1S* mutants or RNAi potently suppressed nearly all *daf-12* LBD mutants, presumably deficient in ligand binding as well as *daf-9* mutants, deficient in ligand production [Table 1, Fig. 3; Supplementary Fig. S1], suggesting DIN-1S works with the unliganded receptor. Similarly, vertebrate coregulators are typically ligand regulated. Fourth, both *din-1S* and *daf-12* null alleles were Daf-d and suppressed the Daf-c phenotypes of mutants in insulin, TGFβ, cGMP, and sterol signaling [Oswald et al. 2002]. Thus they have similar phenotypes and patterns of epistasis. Finally, both were widely expressed and nuclear localized [Fig. 7].

Another important clue to DIN-1’s potential biochemical activity comes from studies on the closest *Homo sapiens* homolog, SHARP. It functions as a corepressor with CBF-1 in the absence of intracellular Notch-IC [Oswald et al. 2002], as well as with unliganded nuclear receptors RAR and PPARα [Shi et al. 2001, 2002].

**Figure 6.** Lipid deposition phenotypes. (A–J) Nile red accumulation of indicated genotypes [L3 larvae, 25°C, except e1370 and *dh6*, L2d larvae]. Bar, 10 μM. (K) Nile red quantitation in N2 wild type, *daf-12(rh61rb11)*, *daf-16(mgDf50)*, *daf-2(e1370)*, *din-1S(dh149, dh128, dh181, dh127)*, *din-1L(hd36)*, *daf-9(dh6)* and doubles (n ≥ 28).

din-1 encodes functionally distinct isoforms

We found that *din-1* encodes a complex locus with two largely separable functions encoded by short [S] and long [L] genes [Fig. 2]. The major focus of this study was DIN-1S, which functionally interacted with DAF-12 nuclear receptor in dauer formation, stage specification, fat metabolism, and life span [see below]. *din-1L* phenotypes included embryonic lethality and misshapen larvae. Those animals that grew were uncoordinated, small, stored excess fat, and were nearly sterile [Figs. 5, 6]. Widespread expression and pleiotropic phenotypes probably indicate roles in multiple differentiation pathways that deserve future detailed exploration. Indeed, *Drosophila* SPEN works in EGF [Chen and Rebay 2000; Kuang et al. 2000], E2F [Lane et al. 2000], homeobox [Wiellette et al. 1999], and wingless signaling [Lin et al. 2003].
suggesting that interaction with nuclear receptors is phyletically ancient. What DIN-1S and SHARP share in common is a receptor interaction domain and the C-terminal SID/RD domain, which has been shown to co-precipitate SMRT corepressor and five members of the NURD complex, HDAC1, HDAC2, MTA2, MBD3, and RbAp48 causing target gene repression (Shi et al. 2001; Ariyoshi and Schwabe 2003). Importantly, unliganded receptors such as RAR or TR typically act as transcriptional repressors by recruiting such histone deacetylase complexes to promoter sites (Glass and Rosenfeld 2000).

As mentioned earlier, nearly all din-1 phenotypes were revealed with daf-12 mutants predicted to be unable to bind ligand, consistent with a corepressor function. For example, DIN-1 physically interacted with the rh61 mutant protein DAF-12 (amino acids 1–617; Fig. 1B), and din-1S mutants suppressed rh61 heterochrony (Fig. 3B–G). Truncated upstream of the C-terminal AF-2 transactivation helix, the rh61 encoded protein is likely ligand insensitive and predicted to repress transcription, based on analogous lesions in vertebrates (Glass and Rosenfeld 2000).

As mentioned earlier, nearly all din-1 phenotypes were revealed with daf-12 mutants predicted to be unable to bind ligand, consistent with a corepressor function. For example, DIN-1 physically interacted with the rh61 mutant protein DAF-12 (amino acids 1–617; Fig. 1B), and din-1S mutants suppressed rh61 heterochrony (Fig. 3B–G). Truncated upstream of the C-terminal AF-2 transactivation helix, the rh61 encoded protein is likely ligand insensitive and predicted to repress transcription, based on analogous lesions in vertebrates (Glass and Rosenfeld 2000). Similarly, din-1S mutants suppressed the phenotypes of several other daf-12 LBD mutants, including rh273 (Fig. 3A), which affects a predicted ligand contact site and is thought to diminish hormone binding to the receptor, and rh284 (Supplementary Table S1), which affects the AF-2 transactivation helix (Antebi et al. 2000). Moreover, din-1S suppressed daf-9/CYP450 mutants (Fig. 3A,H, Table 1) presumably deficient in DAF-12 hormone production (Gerisch et al. 2001; Jia et al. 2002). Consistent with a postulated role as corepressor, DIN-1S demonstrated intrinsic repressor activity when tethered to GAL4 in mammalian cell culture system (Fig. 4). Arguably, DIN-1S could still function as a coactivator, as it contains several LXXLL motifs typical of coactivators such as SRC-1 (Heery et al. 1997), and even classical corepressors coactivate in some promoter contexts (Tagami et al. 1999).

In either scenario, a simple hypothesis is that DIN-1S and DAF-12 form an active coregulator complex that is inhibited by ligand. In the absence of ligand, this complex could repress reproductive genes, activate dauer-specific genes, or both. This view not only helps clarify daf-12 phenotypic complexity, but helps explain how loss of ligand binding can cause more severe phenotypes than loss of DNA binding (Antebi et al. 1998; Antebi et al. 2000), as in cases of thyroid resistance (Hu and Lazar 2000). Such complexes likely inhibit the activity of other transcription factors resident at those promoters, thus silencing expression even below the basal level. In contrast, DBD mutants can neither transactivate nor repress. Therefore their phenotypes are milder because basal transcription is unimpeded. Further exploration of this mechanism will be facilitated by the identification of target genes and ligand. Below we discuss the physiological role of din-1S in more detail.
din-1S acts in the dauer pathways

For the first time in any organism, we placed a SHARP-like coregulator in an in vivo context for nuclear receptor signaling. In particular, because din-1S was separable from din-1L, we could focus specifically upon this pathway. Our studies showed that din-1S was crucial for dauer formation and variably suppressed Daf-c mutants [Fig. 3A]. Importantly, complete suppression was seen only with daf-9 nulls and daf-12 LBD mutants, suggesting a proximal role within the nuclear receptor branch. Altogether, our findings suggest that din-1S acts partly downstream or parallel to insulin/IGF and TGFβ signaling, but fully downstream from hormonal regulation of the nuclear receptor, a genetic position consistent with the detected DAF-12/DIN-1S physical interaction. Partial suppression of insulin/IGF and TGFβ signaling mutants, however, does not exclude a direct role within these pathways either. Whereas daf-12 is absolutely essential for diapause, din-1S is not, arguing that other complexes could substitute. These could include DAF-3/SMAD and DAF-16/FOXO, which mediate the transcriptional output of TGFβ and insulin/IGF signaling, respectively [Finch and Ruvkun 2001], since their homologs are coregulators of nuclear receptors [Yanagisawa et al. 1999; Zhao et al. 2001; Dowell et al. 2003].

din-1S mediates daf-12 heterochrony

Evidently, hormonal signals also control the timing of developmental events including gonadal distal tip cell outgrowth [Gerisch et al. 2001; Gerisch and Antebi 2004; Mak and Ruvkun 2004]. Mutations that perturb these signals delay or arrest migration, in daf-9(th50) and daf-12 LBD mutants, distal tip cells fail their L3 migratory turns. din-1S mutants potently suppressed these phenotypes [Fig. 3B–G; Table 1], suggesting that din-1 works with daf-12 to regulate larval stage-specific programs. Interestingly, fly TAIMAN, a homolog of the AIB1 coactivator, also influences edysone dependent migrations of ovarian border cells [Bai et al. 2000], suggesting that hormonal control of gonadal outgrowth may be conserved. Delayed heterochrony in L3 and adult epidermal seam cells was also rescued, revealing that din-1S functions in multiple tissues. Accordingly, din-1S was nuclear in most stages and cells [Fig. 7], including those exhibiting heterochronic phenotypes as well as tissues remodelled during dauer formation. Homologs in other species are also nuclear and expressed widely [Newberry et al. 1999; Chen and Rebay 2000; Kuang et al. 2000; Shi et al. 2001].

din-1 perturbs lipid metabolism

While din-1S and daf-12 mutants had similar phenotypes for many processes, din-1S mutants influenced lipid metabolism in an unexpected way. Animals amassed excess lipid as measured by Nile red accumulation [Fig. 6]. This is surprising because daf-12 mutants, as well as other Daf-d loci, have the opposite phenotype. Moreover, excess lipid accumulation in din-1(db127) was suppressed by daf-12 nulls. This implies a novel function for the DIN-1/DAF-12 complex in the presence of the DAF-12 ligand during reproductive growth. Perhaps din-1S(+) prevents daf-12(+) from assembling with competing complexes that promote lipid accumulation. Both din-1 and daf-12 mutants also suppressed excess fat storage of daf-9, suggesting that upon dauer entry, din-1(+) has the opposite function, to promote fat storage. These results indicate a key role of receptor and coregulator in controlling organismal lipid metabolism.

din-1S mediates daf-9 longevity

daf-9 longevity is proposed to arise from a heterochronic re-expression of dauer-like programs in the adult [Gerisch et al. 2001]. din-1S(+) was required for longer life, as daf-9 longevity was suppressed by din-1S mutants [Fig. 3H]. Conceivably, in the absence of a daf-9-produced hormone, the DIN-1S/DAF-12 complex specifies dauer-like programs that promote somatic endurance, stress resistance, and survival. Whereas Insulin/IGF signal transduction is clearly established as a central regulator of metazoan aging, recent work suggests that nuclear receptor signaling and silencing complexes may mediate or modulate some of the effects [Tatar et al. 2003]. Mutations that attenuate the fly edysone receptor [Simon et al. 2003] and histone deacetylase rpd3 [Rogina et al. 2002] result in increased longevity. rpd3 loss augments the NAD-dependent deacetylase, sir2, whose increased activity extends life span in yeast and worms [Guarente 2000; Tissenbaum and Guarente 2001]. Intriguingly, human sir2 works in a corepressor complex with the PPARγ nuclear receptor to promote fat mobilization from white adipocytes [Picard et al. 2004]. Conceivably, sir2 influences longevity in part through PPARγ.

Because din-1Sdaf-9 double mutants were slightly shorter-lived than either single mutant, din-1S is not strictly epistatic. Intriguingly, in other respects, din-1Sdaf-9 animals displayed synthetic phenotypes, such as weak heterochronic defects in L3 seam cells or fat storage phenotypes, not seen in either single mutant, but resembling daf-12 nulls. Presumably daf-12 null mutants can neither activate nor repress transcription. Similarly, the double might be deficient in both daf-9(+) dependent hormonal activation and din-1(+) mediated repression.

din-1S instructs slow life history traits

An emergent model for C. elegans’ life history regulation by the nuclear receptor DAF-12 suggests that it has two activities: DAF-12A, which specifies reproductive development and short life span, and DAF-12B, which specifies dauer development and slow aging [Antebi et al. 1998; Gerisch et al. 2001]. Genetic data show that DAF-12 is essential for diapause but only partly required for reproductive growth. Several lines of evidence suggest
that A and B activities are specified by the presence and absence of a DAF-12 ligand, respectively. First, daf-9 encodes a cytochrome P450 related to steroidogenic and fatty acid hydroxylases that regulates DAF-12 and has phenotypes similar to DAF-12 ligand-binding domain mutants, notably Daf-c and Mig defects [Gerisch et al. 2001; Jia et al. 2002]. Second, cholesterol deprivation phenocopies daf-12 LBD and daf-9 mutants, suggesting the hormone could be a sterol. Third, daf-9/CYP450 acts cell nonautonomously from select endocrine tissues and is regulated by environmental and genetic inputs through a DAF-12 feedback loop [Gerisch and Antebi 2004; Mak and Ruvkun 2004], all features characteristic of an endocrine mechanism.

In this work, we extend this model by showing the coregulator din-1 specifically works with the unliganded receptor to specify daf-12B slow life history traits [Fig. 8]. We propose that when worms sense environmental stress, insulin/IGF, TGFβ, and cGMP pathways are suppressed, resulting in down-regulation of a DAF-9-produced hormone. Without hormone, DAF-12B/DIN-1S coregulator complexes inhibit programs of reproductive development, specify diapause, fat storage, and long life [slow life history]. In favorable environments, sensory cues stimulate insulin/IGF, TGFβ, and cGMP signaling to activate DAF-12, which then produces a putative sterol hormone for DAF-12. With hormone, DAF-12A assembles postulated coactivator complexes to promote rapid reproductive growth and short life, whereas DIN-1S antagonizes fat storage [fast life history]. It is highly adaptive for animals to adjust their physiology attuned to their environment. In particular, the capacity to shift metabolism, delay or arrest development and reproduction, and invest in maintenance and stress resistance to outlast adversity, could consequently delay somatic decline and aging. Endocrine coordination of these traits by lipophilic hormone and Insulin/IGF signaling, and their associated transcriptional complexes, could prove a common feature in metazoan evolution [Tatar et al. 2003].

Materials and methods

Nematode culture, mutant isolation, and phenotypic analysis

Except where noted, nematodes were cultured at 20°C on NGM media inoculated with Escherichia coli strain OP50. Low cholesterol media was prepared as described [Gerisch et al. 2001]. din-1 alleles dbh118, dbh127, dbh128, and dbh149 were isolated after ethyl methane sulfonate (EMS) mutagenesis in F2 screens of 168,000 haploid genomes for reversion of the daf-12(her61) Mig phenotype [Antebi et al. 1998]. din-1(dh181) was isolated in a screen of 112,000 haploid genomes for reversion of daf-12(her74) Mig and slow growth phenotypes. Revertants were outcrossed to +/dpy-10 males. Mig cross males indicated extragenic suppressors, whereas non-Mig cross males indicated X-linked intragenic suppressors. Linkage to dpy-10(II) was established to identify din-1 candidates. bds6 was isolated in PCR-based screens for gene deletions using the poison primer protocol [McKay and Jones 2002]. To identify mutations, genomic DNA from mutants was PCR amplified and sequenced on both strands. Mutants were outcrossed at least three times.

Brood size, L3 and adult seam, dauer formation, and life span assays were carried out as described [Antebi et al. 1998; Gerisch et al. 2001]. Statistical analyses were performed with the Excel 98 Student’s t-test. RNAi feeding experiments were carried out according to published procedures [Timmons et al. 2001]. Nile red accumulation was carried out according to Ashrafi et al. (2003). Stained animals were observed by epifluorescent microscopy using Rhodamine filters on a Zeiss Axioplan microscope. Photographs were taken with a Hamamatsu Orca ER CCD digital camera, using Zeiss Axiosvision software to quantitate pixels, and relative values normalized to N2.

Yeast two-hybrid screens

Yeast two-hybrid screens were performed as described [Wanker et al. 1997] using strain L40ccu. A LexA-DAF-12 hybrid containing daf-12 cDNA fragment corresponding to amino acids 281–753, was cloned into the KpnI site of LexA bait vector, pBTM117. The LexA–DAF-12 construct was used to screen a C. elegans oligo-dT primed cDNA library cloned into the GAL4 activation domain in pACT2 [R. Barstead, Oklahoma Medical Research Center, Oklahoma City]. Independent transformants (10³) were plated on minimal medium lacking histidine and uracil. His+, Ura+ colonies were tested for β-galactosidase activity. Counter selection on canavanine [15 μg/mL] induced loss of the bait plasmid. Positive clones were selected for Amp+ in E. coli and sequenced. For DAF-12/DIN-1 interaction, daf-12 fragments using gene specific primers were subcloned into pBTM117, transformed into L40ccu expressing pACT-DIN-1S-31, and assayed for growth on selective media. Similarly, din-1S fragments were cloned into pACT2 and transformed into L40ccu expressing DAF-12 bait. Fusion proteins in yeast were detected by Western blot using anti-LexA or GAL4 antibody, respectively.

GST pull-down experiments

daf-12A cDNA and din-1S cDNA were cloned into the pGEX4T-1 vector. GST fusion proteins were induced in bacterial cultures in the presence of 10 μM ZnSO₄. Recombinant protein was bound to glutathione-agarose beads [Sigma]. 35S-methionine labeled protein was produced in vitro using the TnT expression kit [Promega], incubated with GST fusions or GST alone, and washed with 20 mM TrisHCl [pH 7.4], 200 mM NaCl, 1 mM EDTA. Proteins were resolved by SDS-page, blotted to nitrocellulose membranes, and followed by autoradiography.
Repressor assays
din-1S cDNA was cloned in frame downstream from GAL4 in pCMX-GAL4 to make pCMX-GAL4–DIN–1S. Various plasmid constructs (Shi et al. 2001) were cotransfected into HEK293 cells grown on 3-cm dishes at the following levels: pCMX-GAL4 (600 ng), pCMX-GAL4–DIN–1S (600 ng), and PMH100X4-TK-LUC (200 ng). pCMX-BGAL (200 ng) was used as a transfection control for normalization. Luciferase and β-galactosidase were assayed with the Enhanced Luciferase Assay Kit (BD Pharmingen) and Gal-Screen System, (Applied Biosystems) respectively, and quantitated by scintillation counting. At least three independent trials were performed. Expression of constructs was confirmed by Western blotting with anti-GAL4 antibody.

cDNA clones
YK501h2, YK297g11, YK132c3, YK96c12, YK38b7, and YK1121c03 din-1 cDNA clones from Y. Kohara [National Institute of Genetics, Mishima, Shizuoka, Japan] were sequenced on both strands. The 5′ ends of the L and S isoforms were determined essentially as described [Antebi et al. 2000]. To obtain full-length din-1LB cDNA overlapping 5′ (ZK20.12055-4845) and 3′ fragments (ZK20.5090-F07A11.29280) were amplified from nematode cDNA and spliced together by their common Xbal site in TOPO-Blunt II, resulting in the plasmid Topo-din-1LB-cDNA.

Constructs and transgenics
For RNAi feeding constructs, a BglIII fragment containing exon 19 [F07A11.32391-33145] was excised from pACT-DIN–1S-31 and ligated into the BglIII site of RNAi expression vector L4400 [Timmons et al. 2001]. To make the din-1L::gfp promoter construct, an 8-kb fragment of the din-1 promoter region [ZK20.20003-12055] flanked by NotI [5′] and Agel [3′] sites was PCR amplified and cloned into gfp vector L3781 [A. Fire, Stanford University, Palo Alto, CA]. To construct the din-1L::gfp translational fusion, the 7.7-kb din-1LB cDNA flanked by Nhel and Apal sites was cloned into din-1L::gfp, placing GFP at the N terminus. For the din-1 genomic construct, an 8.5 kb genomic fragment [ZK20.1081-F07A11.28147] was cloned into TOPO blunt (Topo-din-1L). For the din-1 genomic construct tagged with gfp, a 3.4-kb din-1 promoter region [ZK20.1081-F07A11.33195] flanked by Kpn1 sites was amplified and cloned in front of gfp in vector L3781 [called din-1Sp::gfp]. The 5-kb coding region, [F07A11.33195-28230] flanked by Nhel and Spel sites was amplified and cloned into din-1Sp::gfp to give din-1S::gfp. T14A11 cosmid, gfp, and plasmid constructs were injected at 10–20 ng/µL into the gonads of T14A11 cosmid, din-1L/H11019, and plasmid constructs were injected at 10–20 ng/µL into the gonads of T14A11 cosmid, din-1L/H11019, and plasmid constructs were injected at 10–20 ng/µL into the gonads of T14A11 cosmid, din-1L/H11019.

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