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Antagonistic Smad transcription factors control the dauer/non-dauer switch in *C. elegans*

Donha Park[1], Annette Estevez[2,*] and Donald L. Riddle[1,2,†]

SUMMARY

The *C. elegans daf-8* gene encodes an R-Smad that is expressed in a subset of head neurons, the intestine, gonadal distal tip cells and the excretory cell. We found that DAF-8, which inhibits the DAF-3 Co-Smad, is associated with DAF-3 and the DAF-14 Smad in vivo and in vitro. Overexpression of *daf-8* conferred a dauer-defective phenotype and suppressed constitutive dauer formation in *daf-8* and *daf-14* mutants. In contrast to mammalian systems described thus far, active DAF-3 drives a feedback regulatory loop that represses transcription of *daf-7* (a TGFβ ligand) and *daf-8* by directly binding to their regulatory regions. Hence, DAF-8 and DAF-3 are mutually antagonistic. The feedback repression may reinforce the developmental switch by allowing DAF-3 to freely activate dauer transcription in target tissues, unless sufficiently inhibited by DAF-8 and DAF-14. In the adult, DAF-8 downregulates *lag-2* expression in the distal tip cells, thus promoting germ line meiosis. This function does not involve DAF-3, thereby avoiding the feedback loop that functions in the dauer switch.

KEY WORDS: *C. elegans*, TGFβ signaling, Dauer formation

INTRODUCTION

In a favorable environment, the nematode *C. elegans* develops through four larval stages (L1-L4) to a reproductive adult. However, overcrowding, starvation and high temperatures induce the formation of an alternative third larval stage: the dauer larva (Riddle and Albert, 1997; Hu, 2007). The developmental switch is influenced by environmental cues such as food supply, dauer-inducing pheromone and temperature (Golden and Riddle, 1984a; Golden and Riddle, 1984b), which are detected by amphid neurons (Bargmann and Mori, 1997). Molecular genetic analysis of dauer-constitutive (Daf-c) and dauer-defective (Daf-d) mutants revealed that dauer formation is regulated by a guanylyl cyclase pathway, an insulin-like pathway and a TGFβ-like pathway (Birnby et al., 2000; Kimura et al., 1997; Hu, 2007; Riddle et al., 1981).

TGFβ family members control cell proliferation, differentiation and apoptosis from flies to humans (Massagué and Gomis, 2006). Upon ligand binding, type I and type II transmembrane receptors form a heterotetrameric receptor complex and phosphorylate the C-terminal Ser-X-Ser motif of receptor-associated Smad (R-Smad) transcription factors (Attisano et al., 1993). Phosphorylated R-Smads form homodimers and heterotrimers with a common-mediator Smad (Co-Smad) and are transported into the nucleus to regulate transcription of target genes (Massagué and Gomis, 2006).

In *C. elegans*, TGFβ signaling promotes larval growth and inhibits dauer arrest. The *C. elegans daf-7* gene encodes a member of the TGFβ superfamily that is expressed in amphid ASI neurons. Transcription of *daf-7* is repressed by dauer pheromone (Ren et al., 1996; Schackwitz et al., 1996). The *daf-1* and *daf-4* genes encode type I and type II receptor kinases, respectively, and are required for non-dauer development (Estevez et al., 1993; Georgi et al., 1990). The *daf-14* gene encodes a Smad protein of atypical structure (lacking a DNA-binding domain) that acts redundantly with DAF-8 (Inoue and Thomas, 2000).

The *daf-7*, *daf-1*, *daf-4*, *daf-8* and *daf-14* genes are required for non-dauer development at higher growth temperatures (Golden and Riddle, 1984a; Golden and Riddle, 1984b). Mutations in these genes result in a temperature-sensitive Daf-c phenotype. The *daf-3* and *daf-5* genes encode Smad and Sno/Ski transcription factors, respectively, and are required for dauer formation (da Graca et al., 2004; Patterson et al., 1997). Genetic epistasis tests were used to order these and other *daf* genes in a pathway. The *daf-8* mutation is suppressed by mutations in the downstream genes *daf-3*, *daf-5* and *daf-12*, but not by mutations in *daf-16* or in upstream genes such as *daf-10* (Riddle et al., 1981; Vowels and Thomas, 1992).

The dauer TGFβ pathway in *C. elegans* is unique in that the upstream Smads antagonize DAF-3 instead of activating it (Patterson et al., 1997). In other systems, inhibitory Smads (Smad6 and Smad7) antagonize TGFβ signaling by inhibiting interaction between the receptor complex and R-Smads and/or inhibiting Smad heterotrimer formation (Massagué et al., 2005). Transcription of *Smad6* and *Smad7*, which antagonize BMP signaling, is positively regulated by BMP-induced Runx2 (Wang et al., 2007). *SnoN* (*Skil*) transcription is also upregulated by TGFβ1, demonstrating a negative-feedback regulation of TGFβ signaling by SnoN (Stroschein et al., 1999).

In *C. elegans*, inhibitory Smads have not been characterized. Furthermore, the biochemical functions of the known dauer pathway components are largely inferred from mammalian orthologs, and this is particularly true for the interaction between Smad proteins. This report reveals novel aspects of TGFβ signaling that reflect the biology of the dauer developmental switch and the adaptation of this pathway for modulating developmental and reproductive strategies in response to environmental quality.

[1]Michael Smith Laboratories, University of British Columbia, Vancouver, BC V6T1Z4, Canada. [2]Division of Biological Sciences, University of Missouri, Columbia, MO 65211, USA.

*Present address: Department of Neurology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261, USA
†Author for correspondence (driddle@msl.ubc.ca)

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MATERIALS AND METHODS

Nematode strains

C. elegans strains were cultured with *E. coli* OP50 as the food source according to standard techniques (Brenner, 1974) unless otherwise noted. Worm strains and alleles used in this study are LG I: *daf-8(e1393*, *m85*, *m121*, *m430*, *m475*, *sa233*, *sa234*, *sa343*, *sa345*, *m430*, *m547)*; LG II: *daf-5(e1385*, *m14)*; LG III: *daf-7(e1372)*; LG IV: *daf-1(m40)*, *daf-14(m77)*, *mut-6(st702)*; LG X: *daf-3(e1376*, *m19); mEx174 [daf-8p::daf-8::FLAG*, *myo-2p::gfp]*, *mIs27 [daf-8p::daf-8::gfp*, *rol-6(su1006)]*, *mEx178 [daf-14p::daf-14::FLAG*, *myo-2p::gfp]*, *mIs34[daf-3p::daf-3::FLAG*, *rol-6(su1006)]*, *qIs56[lag-2p::gfp*, *unc-119(+)]*, *daf-14(m77); mIs27*, *daf-8(e1393); mIs27*, *daf-1(m40); mIs27*, *mEx174; mIs27*, *mEx178; mIs27*, *mIs27; mIs34*, *mEx178; mIs34*, *qIs56; mEx174*.

Tc1 mutagenesis and cloning of *daf-8*

Three *daf-8* Tc1 transposon-insertion mutants were identified by screening populations of the mutator strain RW7097 visually at 20°C for the presence of dauer larvae, as previously described (Georgi et al., 1990). Spontaneous Daf-c mutants were genetically complemented with *daf-8* (*e1393* and *m85*). A novel 3.2 kb fragment was identified by genomic Southern blot in transposon-induced *daf-8* alleles (*m430*, *m475* and *m487*), but not in the wild type or in the spontaneous revertant *m430m547*. A flanking genomic fragment was isolated and sequenced to identify the *daf-8* gene. The structure of the genomic region and cDNA is shown in Fig. S1 in the supplementary material. The predicted 546 amino acid product is a Smad protein (Massagué et al., 2005), with a 116 amino acid Mad homology 1 (MH1) domain and a 198 amino acid MH2 domain separated by a proline/serine (PS)-rich region. MH1 is a DNA-binding domain, whereas MH2 is a protein-protein interaction module.

Seven ethylmethane sulfonate (EMS)-induced mutant alleles, as well as three Tc1 insertion alleles, were sequenced (see Fig. S1 in the supplementary material). Brood sizes of *daf-8* mutants were examined at 15°C, 20°C and 25.5°C. At all temperatures, broods were reduced between 10-80% relative to wild type (see Table S1 in the supplementary material).

Transgene construction and transformation

Primer sequences are listed in Table S1 in the supplementary material. A genomic fragment containing the entire coding region of *daf-8* plus 3.3 kb upstream of the ATG was amplified. The *gfp* gene was amplified from *pPD95.75* (provided by A. Fire, Stanford University School of Medicine) and used for recombinant PCR to generate *pDH36* (*daf-8p::daf-8::gfp*), a translational fusion of *gfp* at the end of *daf-8* exon 6. The fusion was verified by sequencing and introduced by microinjection into the adult germ line (45 ng/μl) along within the co-injection marker *pRF4* [*rol-6* (*su1006*)] (9 ng/μl). Details of the generation of FLAG-tagged fusion constructs and transgenic lines for *daf-8*, *daf-3* and *daf-14* will be provided upon request.

Anti-phospho-Histone H3 staining of dissected gonads

Gonad dissections were performed as described (http://www.genetics.wustl.edu/tslab/protocols.html). The dissected gonads were fixed in 3% formaldehyde/K_2HPO_4 (pH 7.2) for 1 hour followed by methanol fixation for 10 minutes. After blocking in TBS-Tween 20 (0.1%)/3% BSA for 20 minutes, the dissected gonads were incubated with anti-phospho-Histone H3 antibody (1:500, Millipore #06-570) for 16 hours at 4°C, followed by incubation for 4 hours with FITC-conjugated secondary antibody (1:250, Abcam #ab6717) at room temperature. DAPI (100 ng/ml) was added to the final washing step to visualize germ cell nuclei.

Immunoblots, in vitro GST pull-down assay and in vivo co-immunoprecipitation

For developmental immunoblots, eggs were prepared by alkaline hypochlorite treatment and synchronized by hatching in M9 buffer. After growth on OP50, animals at each stage were collected in 1×TBS buffer containing protease inhibitor cocktail (Calbiochem). Total lysates were obtained by sonication (Sonifier), and 40 μg of total protein was used for immunoblotting. The blots were probed with anti-GFP antibody (1:2000, Abcam) or anti-Tubulin antibody (1:1000, Sigma).

For the in vitro GST pull-down assay, full-length cDNAs for each Smad protein were isolated and cloned into the pGEX4T-1 and pBluescript SKII vectors. Bacterially expressed GST fusion proteins were isolated with the Bulk GST Purification Module (GE Healthcare, #27-4570-01). The TNT®T7 Coupled Reticulocyte Lysate System (Promega) was used for in vitro transcription and translation of each Smad protein with [^{35}S]methionine. The GST pull-down assay was performed as described (http://www.nature.com/nmeth/journal/v1/n3/full/nmeth1204-275.html).

For in vivo co-immunoprecipitation (co-IP), synchronized worms were washed three times with M9 buffer and protein extracts were prepared in IP buffer (25 mM Tris-Cl pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.5% NP40) containing protease inhibitor cocktail. The lysate was precleared with IgG-agarose (Sigma) and incubated with anti-FLAG-agarose M2 (Sigma) for immunoprecipitation. The immunoprecipitate was washed four to five times with IP wash buffer (25 mM Tris-Cl pH 7.4, 100 mM NaCl) and eluted with FLAG peptide (Sigma). After SDS-PAGE and transfer to PVDF membrane, the blots were probed with anti-FLAG or anti-GFP antibody.

Quantitative RT-PCR (qPCR) and chromatin immunoprecipitation (ChIP)

Mixed-stage worms were harvested from NG agar plates and lysed by sonication. Total RNA (1 μg) from each sample was used for reverse transcription with Superscript II reverse transcriptase (Invitrogen) using oligo(dT) priming. The resulting first-strand cDNA was used for PCR analysis (for primers, see Table S1 in the supplementary material). qPCR was performed using an Applied Biosystems 7500 with BioRad iTaq SYBR Green Supermix with ROX.

ChIP experiments were performed with slight modification of the method described by Ercan et al. (Ercan et al., 2007). Worms were subjected to a 1.5% formaldehyde solution for 30 minutes at room temperature to promote the cross-linking of DNA and proteins. Immunoprecipitation was performed by incubating the lysate with either anti-DAF-3 antibody (1:250, Novus) or IgG at 4°C for 16-18 hours. The precipitates were washed, de-crosslinked and eluted. For each eluate, 5 μl was used for PCR.

Dauer assays and RNAi

Percentage dauer formation was scored visually for each genotype grown at permissive (15°C), intermediate (20°C) and restrictive (25.5°C) temperatures. Feeding RNAi was performed as described (Timmons et al., 2001) on 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG).

Microscopy

A Zeiss Axioscope equipped with a QImaging camera (Retiga 2000R) was used for differential interference contrast (DIC) microscopy and GFP expression analysis. For DiI staining, worms were synchronized by hatching purified eggs into M9 buffer, grown on NG agar plates until the L2 stage, washed in M9 buffer and then DiI stained as described (Blacque et al., 2004).

RESULTS

Isolation and characterization of *C. elegans daf-8* mutations

To characterize targets of the DAF-4/DAF-1 receptor that controls dauer development, the *daf-8* gene was cloned with the aid of a Tc1 transposon insertion mutant (see Fig. S1 in the supplementary material). *daf-8* encodes a 546 amino acid protein that shares strong sequence similarity to R-Smads. All ten mutations that were sequenced were located within the Mad homology 2 (MH2, protein-protein interaction) domain, except for *sa343*, a nonsense mutation in the MH1 (DNA-binding) domain (Table 1; see Fig. S1 in the supplementary material). Missense mutations in *m121*(G502R) and *sa234*(G502E) alter the highly conserved glycine that has been identified as a mutational hot spot in mammalian Smad proteins (Miyaki et al., 1999; Wrana and Attisano, 1996). The corresponding glycine is mutated to arginine in *C. elegans sma-3*(*e491*) (Savage et al., 1996). The MH2 domain is required for receptor-mediated phosphorylation and for

Table 1. Molecular lesions and dauer phenotype of *daf-8* mutants

Allele	Molecular lesion	Region of mutation	Dauer formation (%)		
			15°C (*n*)	20°C (*n*)	25.5°C (*n*)
e1393	S391L	MH2	0.5±0.5 (183)	0.9±0.3 (126)	72.4±2.2 (108)
m85	R416Stop	MH2	3.0±0.9 (157)	1.7±0.8 (179)	100 (133)
m121	G502R	MH2	0 (155)	1.4±0.4 (188)	70±3.7 (113)
m430	Y481(Tc1 insertion)*	MH2	5.1±0.7 (181)	0.5±0.3 (169)	74.7±1.8 (192)
m475	Y481(Tc1 insertion)*	MH2	4.3±1.1 (115)	0.6±0.2 (131)	75.5±1.1 (211)
m487	Y481(Tc1 insertion)*	MH2	1.1±0.8 (180)	1.1±0.6 (116)	41.7±2.7 (144)
sa233	Deletion/insertion†	MH2	3.3±0.8 (171)	0.4±0.3 (176)	83.4±1.7 (188)
sa234	G502E	MH2	6.4±1.3 (197)	1.8±0.9 (169)	85.1±1.6 (218)
sa343‡	Q52Stop	MH1	0.5±0.1 (166)	0.3±0.1 (177)	98.2±0.2 (182)
sa345§	Deletion¶	MH2	0.5±0.2 (201)	0.4±0.2 (194)	38.2±2.3 (197)

Values are the percentage of dauer larvae ± s.e.m. L4 larvae grown at 15°C were placed on NG agar plates with OP50 until egg laying was completed, then removed. The eggs were hatched at permissive (15°C), intermediate (20°C) and restrictive (25.5°C) temperatures to observe the Daf-c phenotype. Progeny reaching the L4 stage after 3-5 days (depending on the temperature) were counted and removed. Remaining progeny were scored as dauer larvae.
n, population size.
*Three independent Tc1 insertions derived from strain RW7097 were found to be at the same site between the T and the A in codon Y481.
†In *sa233,* 226 bp are deleted and replaced by a 14 bp insertion resulting in a premature stop codon 8 bp into the insertion. A truncated 435 amino acid protein is predicted.
‡When placed over the deficiency *nDf23, sa343* displays a partially penetrant egg-L1 lethality at 25.5°C.
§This strain produced 58% unhatched eggs and 2% dead larvae at 25.5°C.
¶The *sa345* deletion removes the 3′ splice acceptor of intron 4 and all but the last 33 nucleotides of exon 5.

molecular interaction with partner molecules (Huse et al., 2001; Wu et al., 2001). Phosphorylation of two C-terminal serines in the MH2 domain of human SMAD2 is necessary for transducing signals from TGFβ (Kawabata et al., 1998; Macias-Silvia et al., 1996). DAF-8 and DAF-14 have the Ser-X-Ser motif, but DAF-3 lacks this C-terminal region (Inoue and Thomas, 2000; Patterson et al., 1997).

Mutations in *daf-8* confer reduced brood size (Table 2) and an egg-laying defect (Trent et al., 1983). In addition, one allele, *sa345*, exhibited 60% larval lethality (Table 1). The temperature-sensitive Daf-c phenotype is most penetrant at 25.5°C (Table 1). Most dauer larvae formed at 25.5°C remained arrested for at least 7 days, although some dauer larvae developed to the L4 stage 12-76 hours after formation. At 15°C and 20°C, the mutants formed some sodium dodecyl sulfate (SDS)-resistant dauer larvae constitutively, but only transiently and at low frequency (A. O. Z. Estevez, PhD thesis, University of Missouri, 1997). The *daf-8* mutants displayed a less severe Daf-c phenotype than *daf-7*, *daf-1* or *daf-4* mutants, suggesting that other genes partially substitute for *daf-8*. A candidate is the Daf-c Smad gene *daf-14*, as its overexpression suppresses a *daf-8* mutation (Inoue and Thomas, 2000).

In order to determine whether overexpression of *daf-8* also substitutes for *daf-14* function, we generated double mutants between *daf-14(m77)* and a functional, *daf-8*-stably-integrated, overexpressing translational *daf-8::gfp* fusion construct. The integrated transgene, *daf-8(mIs27)*, carried the entire coding region of *daf-8*, including 3.3 kb upstream of the start codon, fused to *gfp* at the end of exon 6. Whereas the *daf-14(m77)* single mutant was 96% Daf-c at 25.5°C, the translational *gfp* fusion almost completely suppressed the transient dauer formation of *daf-14(m77)* ($P<0.0001$) in the double mutant. RNAi against *gfp* restored dauer formation up to 88.6% (Table 2). Constitutive dauer formation of *daf-1(m40)*, however, was not suppressed by overexpression of *daf-8::gfp*. The nuclear localization of DAF-8::GFP was absent in the *daf-1* mutants (see Fig. S6 in the supplementary material). These results suggest that the biological activity of the translational DAF-8::GFP fusion is dependent on the type I receptor DAF-1. Taken together, the data suggest that *daf-8* and *daf-14* can substitute for each other.

daf-8 expression pattern

The DAF-8::GFP translational fusion described above was expressed in developing embryos from the pre-comma stage through hatching (Fig. 1A). It was most highly expressed in L1 and gradually decreased into adulthood (Fig. 1I). Expression was strongly downregulated in dauer larvae (Fig. 1H). Overall, the expression pattern of *daf-8* was indistinguishable from that of the type I receptor *daf-1* (Gunther et al., 2000). In larval stages, DAF-8::GFP was expressed throughout development in the ventral nerve cord, intestine, gonadal distal tip cells (DTCs) and subsets of head and tail neurons, most strongly in head neurons ASI and ADL (Fig. 1B-E). These neurons were identified by comparing GFP expression with DiI staining, which stains ciliated neurons in the head (Fig. 1G). In adults, DAF-8::GFP was also detected in the excretory cell (Fig. 1F).

daf-8 function in the adult gonadal distal tip cells

DAF-8::GFP was expressed in adult DTCs (Fig. 1E). *daf-1* (Gunther et al., 2000), *daf-14* and *daf-3* (Inoue and Thomas, 2000; Gunther et al., 2000; Patterson et al., 1997) are also expressed in these cells. This led us to examine whether TGFβ signaling plays a role in DTCs and the germ line. In the *C. elegans* ovaries, the germ line mitotic zone at the distal end is followed by a proximal transition zone, which contains cells in early meiosis (e.g. leptotene and zygotene)

Table 2. Suppression of a *daf-14* mutation by *daf-8* overexpression

Genotype	RNAi	Percentage dauer formation at 25°C (*n*)
daf-8(e1393)	N/A	88.5±3.5 (78)
daf-8(e1393);mIs27	Vector	0 (94)
daf-8(e1393);mIs27	*gfp*	79.6±6.3 (64)
daf-14(m77)	N/A	95.8±3.2 (72)
daf-14(m77);mIs27	Vector	2.3±1.2 (85)
daf-14(m77);mIs27	*gfp*	88.6±4.5 (79)

Numbers are percentage dauer formation (average ± s.e.m.) upon RNAi against vector control or *gfp* gene. L4 larvae (P_0) grown on OP50 were put on either control RNAi plates with HT115 (L4440) or *gfp* RNAi plates with HT115 (*gfp*) to lay eggs, then removed. The F1 progeny were grown to the L4 stage at 15°C. Three F1 L4 larvae were transferred to fresh RNAi plates, then removed as young adults after they had laid 30-40 eggs. Dauer formation in the F2s at 25°C was scored visually. All the genotypes are in the *eri-1(mg366)* background. *mIs27* is a stably integrated *daf-8p::daf-8::gfp* transgene.
n, population size.
N/A, not applicable.

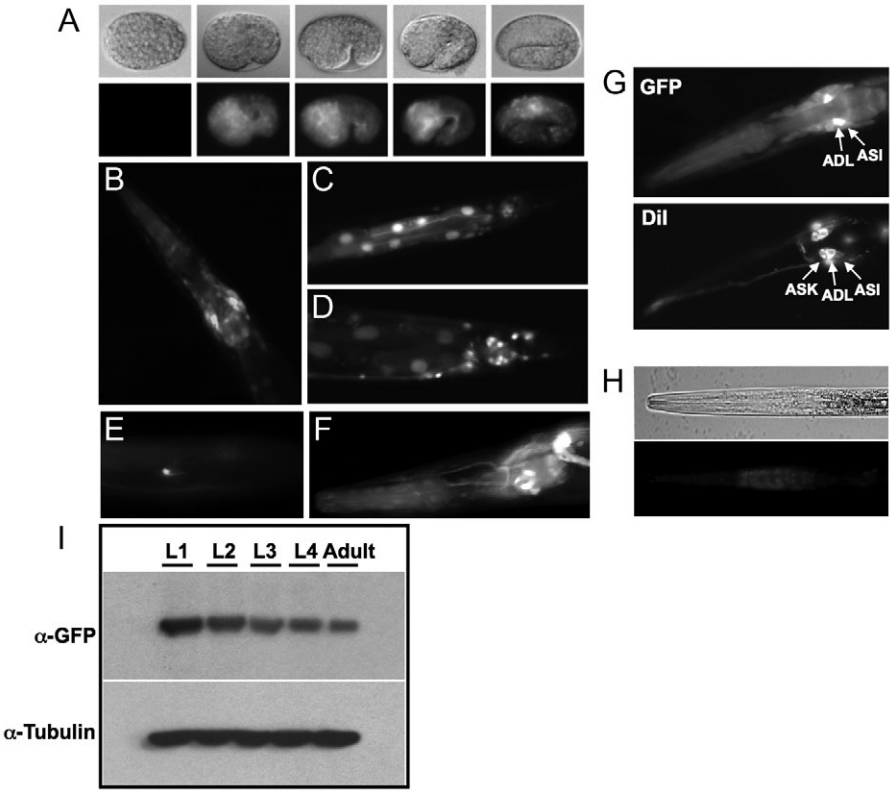

Fig. 1. Embryonic, larval and adult expression of *daf-8p::daf-8::gfp*. (**A**) DIC and fluorescence images of developing *C. elegans* embryos from pre-gastrulation to late pretzel stage. (**B**) Head neurons in an L2 larva. (**C**) Intestine in L2 larva. (**D**) Adult tail neurons. (**E**) Adult gonadal distal tip cell. (**F**) Adult excretory cell. (**G**) Dorsal view of expression in ASI and ADL neurons (arrows in upper panel). The same animal was stained with DiI to show ciliated neurons in the head (lower panel). (**H**) DAF-8::GFP expression is reduced in dauer larvae. (**I**) Immunoblot detecting DAF-8::GFP during larval development. Proteins were extracted from populations synchronized at each stage, electrophoresed, blotted and probed with anti-GFP antibody (upper panel) and with anti-Tubulin antibody (lower panel) as a loading control.

(Crittenden et al., 2006). We examined the gonadal phenotype of *daf-8*, *daf-3*, *daf-5*, *daf-8;daf-3* and *daf-8;daf-5* mutants by DAPI staining. Whereas the mitotic region of *daf-3(e1376)* and *daf-5(e1385)* was indistinguishable from that of the wild type, it was significantly extended in *daf-8(m85)*, *daf-8(m85);daf-3(e1376)* and *daf-8(m85);daf-5(e1385)* mutants (see Fig. S2A in the supplementary material). Two weaker alleles, *daf-8(m121)* and *daf-8(e1393)*, exhibited ~80% of the mitotic zone extension observed in *daf-8(m85)* (data not shown).

We assessed mitotic activity by anti-phospho-Histone H3 (PH3) antibody staining, which labels metaphase and telophase cells in the germ line (Hsu et al., 2000). The wild-type germ line showed two or three antibody-positive cells in each arm of the gonad from 1-day-old adults. *daf-8(m85)* exhibited an increased number of PH3-positive cells (six to seven per gonadal arm, $P<0.001$), and this was not suppressed by *daf-3* (Fig. 2A; see Fig. S2B in the supplementary material). Hence, *daf-8* normally functions to inhibit mitosis and promote the switch to meiosis, but neither *daf-3* nor *daf-5* is required for this (although they are required for the dauer switch). In contrast to *daf-8*, the number of PH3-positive cells in *daf-14(m77)* mutants was comparable to that in the wild-type germ line (2.04±0.3 per gonadal arm, $P>0.05$), and there was no extension of the mitotic zone. The extended mitotic zone in *daf-8* was not affected by *daf-14* in a *daf-8(m85);daf-14(m77)* double mutant (data not shown). However, this phenotype was exhibited by *daf-1(m40)*, which is also expressed in DTCs (see Fig. S2 in the supplementary material).

We tested whether *daf-8* signaling might affect the level of *lag-2* expression in DTCs. The *lag-2* gene encodes a protein of the Delta/Serrate/Lag-2 (DSL) family that functions as a ligand for GLP-1 and LIN-12 Notch-like receptors (Henderson et al., 1997). Expression of *lag-2* in the DTCs is required to maintain mitotic activity at the distal end of the gonad (Henderson et al., 1997). In a wild-type genetic background, expression of stably integrated *lag-2p::gfp* resulted in very strong fluorescence in the DTC cell body, as well as in the cytonemes (DTC projections that embrace the germ line cells). On average, adult transgenic animals carrying extrachromosomal *daf-8p::daf-8::FLAG* exhibited greatly suppressed *lag-2p::gfp* expression in the DTCs (Fig. 2B).

qRT-PCR results confirmed that *lag-2* expression was upregulated in three different *daf-8* loss-of-function mutants and was suppressed by overexpression of *daf-8::FLAG*. However, *lag-2* expression was not significantly changed in two loss-of-function *daf-3* mutants, nor in animals overexpressing *daf-3* (Fig. 2C). These results indicate that *daf-8* signaling inhibits mitotic activity in the germ line by downregulating *lag-2* transcription in DTCs. This bypasses *daf-3* and *daf-5* (see Fig. S2A in the supplementary material), which are downstream of *daf-8* in the dauer pathway.

Interaction between *C. elegans* Smad proteins in vivo and in vitro

Upon activation by the type I receptor, vertebrate R-Smads form homodimers or heterotrimers with a Co-Smad (Howell et al., 1999; Lagna et al., 1996; Zhang et al., 1997). However, little is known about the molecular interactions between Smad proteins in *C. elegans*. To address this in vivo, we constructed translational fusions with either GFP or a FLAG tag at the 3′-end of the Smad genes and generated transgenic lines that overexpress pairs of Smad proteins in various combinations (Fig. 3A). DAF-8 strongly associated with itself, as assessed by in vivo co-immunoprecipitation (co-IP) analyses, and it was also associated with DAF-14 and weakly with DAF-3 (Fig. 3B) in mixed-stage animals. These results do not rule out the possibility that the molecular interactions might be indirect, i.e. through adaptor proteins.

To test direct interaction between the Smads, we performed in vitro GST pull-down analyses. DAF-8 was associated with itself as well as with DAF-14 and DAF-3. DAF-14 was also able to bind to

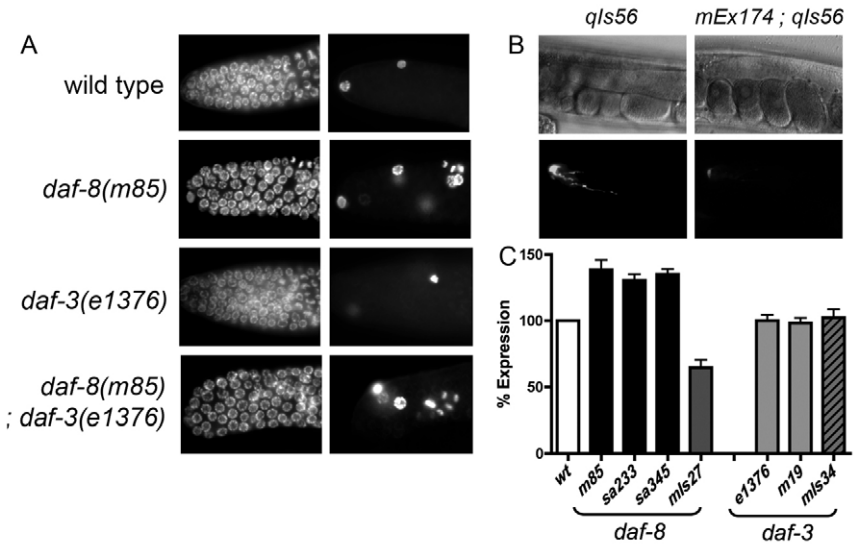

Fig. 2. *daf-8* regulates mitotic activity in the germ line. (**A**) Ovaries from 1-day-old adults were dissected and stained with DAPI (left column), showing the distal tip at left, and with FITC-conjugated anti-phospho-Histone H3 antibody (right column). The extended mitotic zone phenotype and the number of PH3-positive cells per ovary are described in Fig. S2 in the supplementary material. (**B**) *lag-2p::gfp* expression (bottom) is reduced by overexpression of *daf-8p::daf-8::FLAG* (right) in the distal tip cells. (**C**) qRT-PCR for *lag-2* transcripts in *daf-8* and *daf-3* mutant backgrounds, normalized to *act-2* transcripts. *mIs27* and *mIs34* are *daf-8* and *daf-3* overexpressors, respectively. Bars indicate s.e.m.

itself, but binding to DAF-3 was not detected (Fig. 3D) under the conditions we used. However, DAF-14 was associated with DAF-3 in vivo, and this was attenuated in the absence of DAF-8 (Fig. 3C). Although Smads are able to interact in vitro (Funaba and Mathews, 2000), phosphorylation at the Ser-X-Ser motif significantly promotes the interaction. The apparent lack of in vitro binding between DAF-14 and DAF-3 might be because the binding is too weak in vitro or because the phosphorylation of DAF-14 is required to enhance the interaction with DAF-3.

Direct DAF-3 binding to *daf-7* and *daf-8* regulatory regions to repress transcription

While generating transgenic lines in which *daf-8* and *daf-3* are doubly overexpressed, we observed that DAF-8::GFP expression was downregulated by the overexpression of *daf-3* (Fig. 4A). This led us to measure transcript levels for *daf-8* (as well as for *daf-7*, *daf-1*, *daf-4* and *daf-14*) in *daf-3* or *daf-5* mutants by qRT-PCR. *daf-8* and *daf-7* transcripts were upregulated in the two *daf-3* mutant backgrounds tested ($P<0.001$; Fig. 4B). Furthermore, *daf-7* and *daf-8* transcripts were significantly reduced by overexpression of *daf-3* ($P<0.001$; see Fig. S3 in the supplementary material; Fig. 4C). By contrast, levels of *daf-1*, *daf-4* and *daf-14* transcripts were indistinguishable from those of the wild type ($P>0.05$). The difference in the expression was not stage specific as it is known that *daf-1*, *daf-4* and *daf-14* are expressed at all stages (Gunther et al., 2000; Patterson et al., 1997; Inoue and Thomas, 2000). This suggests that feedback repression by DAF-3 specifically targets *daf-7* and *daf-8.* By contrast, *daf-3* transcripts were not affected in *daf-7* or *daf-8* mutant backgrounds, or in *daf-8*-overexpressing animals (see Fig. S4 in the supplementary material; $P>0.05$). In *daf-5* mutants, there were no significant changes in the expression of the genes tested ($P>0.05$; data not shown).

We performed chromatin immunoprecipitation (ChIP) analyses using anti-DAF-3 antibody to examine whether DAF-3 directly binds the regulatory regions of *daf-7* and *daf-8*. Since DAF-3 binding sites had been determined for the *myo-2* promoter (Thatcher et al., 1999), we first searched for putative DAF-3 binding sites in *daf-7* and *daf-8*. Of four putative sites in *daf-7*, DAF-3 was directly associated with one site located 1080 bp upstream of the start codon. DAF-3 also bound two of the three putative binding sites in the *daf-8* region, one 2919 bp upstream of the start codon and another within the first intron, 292 bp downstream of the start codon (Fig. 4D; see Fig. S5 in the supplementary material). Overall, these results indicate that DAF-3 regulates the transcription of *daf-7* and *daf-8* by binding directly to their regulatory regions.

DISCUSSION

We cloned *daf-8* to better characterize targets of the DAF-4/DAF-1 receptors that control dauer development (A. O. Z. Estevez, PhD thesis, University of Missouri, 1997), and to test the genetic model

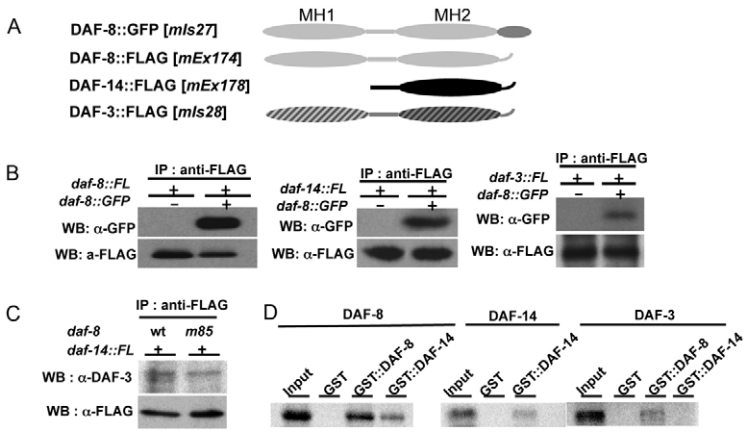

Fig. 3. Smad interactions in vivo and in vitro. (**A**) The Smad constructs used to generate transgenic lines. (**B**) DAF-8 is associated with itself, DAF-14 and DAF-3 in vivo. In vivo co-IP was performed from transgenic animals overexpressing two transgenes in combination. (**C**) In vivo interaction between DAF-14 and DAF-3 in the presence or absence of DAF-8. (**D**) In vitro GST pull-down assay between Smads.

that DAF-8 and DAF-14 R-Smads coordinate to inhibit the DAF-3 Co-Smad (Inoue and Thomas, 2000). In the light of this genetic model, it became important to test whether these three Smads interact directly. We found that the expression of DAF-8 closely overlaps with that of *daf-1* and that overexpression of *daf-8* suppresses the Daf-c phenotype of a *daf-14* mutant. co-IP showed that DAF-8 associates strongly with itself and weakly with DAF-14 and DAF-3 in vivo. DAF-14 is also associated with DAF-3 in a DAF-8-dependent manner. DAF-14 lacks an MH1 (DNA-binding) domain and may function primarily as an enhancer of DAF-3 inhibition. A second novel aspect of dauer signaling is that the downstream Co-Smad DAF-3 negatively regulates transcription of *daf-7* and *daf-8* by binding directly to their regulatory regions (Fig. 5). This represents a novel feedback regulation of TGFβ signaling in *C. elegans*, reflecting the biology of the dauer/non-dauer switch. Once DAF-3 activity is allowed to reach a threshold, the switch to dauer is reinforced by repressing the transcription of key proteins that antagonize DAF-3 function.

It is well established that R-Smads form heterotrimers with Co-Smads (reviewed by Derynck and Zhang, 2003). Interactions between Smad proteins in the dauer pathway have not yet been studied, although DAF-3–DAF-5 and DAF-3–SMA-3 interactions were identified in yeast two-hybrid screens (da Graca et al., 2004; Simonis et al., 2009; Tewari et al., 2004). We performed co-IP experiments using transgenic animals overexpressing pairs of Smad proteins. DAF-8 associated strongly with itself and also with DAF-14 and DAF-*3* in vivo. This was confirmed by in vitro GST pull-down assays that showed that DAF-8 and DAF-14 could form complexes. Although DAF-14–DAF-3 heterodimers were not detected in vitro, DAF-14 was associated with DAF-3 in vivo and this was dependent on DAF-8. It has been proposed that *daf-8* and *daf-14* have redundant function because overexpression of *daf-14* is able to suppress the *daf-8* Daf-c phenotype (Inoue and Thomas, 2000). We observed the reciprocal effect in a *daf-8::gfp(mIs27);daf-14(m77)* double mutant, in which the *daf-14* Daf-c phenotype was completely suppressed. We conclude that suppression resulted from the overexpression of *daf-8* because RNAi against *gfp* restored the Daf-c phenotype. Similarly, we concluded that our DAF-8::GFP construct is biologically functional because the transgene suppressed the *daf-8* Daf-c phenotype and *gfp* RNAi restored this phenotype.

The fact that the overexpression of either Smad (i.e. DAF-8 or DAF-14) suppresses the mutation of the other, suggests that either one can inactivate the DAF-3 Smad, although it is likely that they

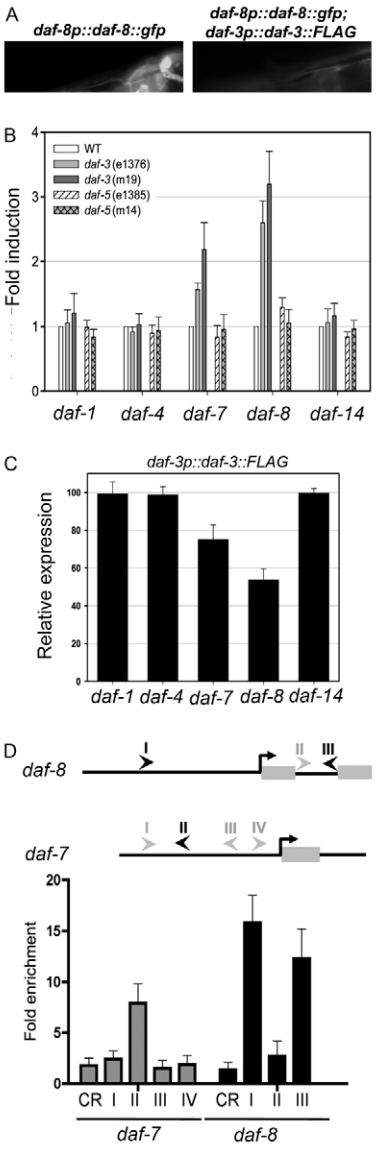

Fig. 4. DAF-3 binds to the regulatory regions of *daf-7* and *daf-8* to repress transcription. (**A**) Overexpression of *daf-3p::daf-3::FLAG* suppresses the expression of *daf-8p::daf-8::gfp*. Head neuron and excretory cell fluorescence is shown. (**B**) qRT-PCR for *daf-1*, *daf-4*, *daf-7*, *daf-8* and *daf-14* transcripts in *daf-3* and *daf-5* mutant backgrounds relative to wild type; normalized to *act-2*. Bars indicate s.e.m. from four replicates. (**C**) qRT-PCR for *daf-1*, *daf-4*, *daf-7*, *daf-8* and *daf-14* transcripts in a *daf-3p::daf-3::FLAG* overexpressor background relative to wild type. (**D**) ChIP with anti-DAF-3 antibody reveals that DAF-3 is directly associated with the regulatory regions of *daf-7* and *daf-8* (see Fig. S5 in the supplementary material). The arrowheads indicate putative DAF-3 binding sites. The black arrowheads are the binding sites detected by chromatin IP. ChIP results were quantitated by qPCR. Bars indicate s.e.m. from three replicates.

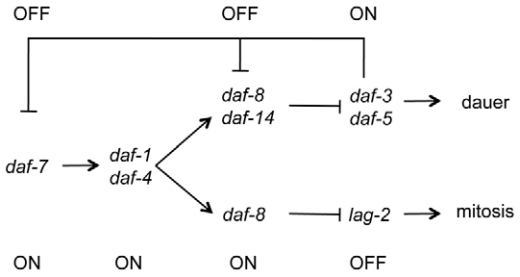

Fig. 5. Working model for DAF-7/TGFβ pathway modulation of dauer formation in larvae and of germ line mitosis in the adult. The DAF-14 and DAF-5 Smads are components of dauer signaling, but are not involved in DAF-3-mediated feedback repression of *daf-7* and *daf-8* transcription. The DAF-7 ligand activates the DAF-4 and DAF-1 receptors, which in turn activate DAF-8. Genetic data showing that DAF-8 inhibits the DAF-3 Co-Smad to prevent dauer formation in larvae is extended here by demonstration of direct protein-protein interactions between DAF-8, DAF-14 and DAF-3. In adults, DAF-8 acts independently of DAF-14 and DAF-3 to repress (directly or indirectly) *lag-2* transcription. The adult pathway for modulation of germ line mitosis does not involve DAF-14 or DAF-3. Although we have not shown directly that DAF-1 signals to DAF-8 to inhibit adult germ line mitosis, it is a likely candidate because DAF-1 is co-expressed with DAF-8 in DTCs, and *daf-1(m40)* shares the extended mitotic zone phenotype with *daf-8* (see Fig. S2A in the supplementary material).

normally act cooperatively. The co-IP results indicated that DAF-14 binding to DAF-3 was DAF-8 dependent. Since DAF-14 lacks a DNA-binding domain, it presumably does not directly regulate transcription, in contrast to DAF-8 and DAF-3. We refer to DAF-14 as an R-Smad because of its apparently redundant function with DAF-8. Both DAF-8 and DAF-14 possess the C-terminal phosphorylation motif. It is possible that phosphorylated DAF-14 assists phosphorylated DAF-8 to inhibit DAF-3 by preventing its association with its co-transcriptional regulator DAF-5 and other binding partners (Tewari et al., 2004). In this model, DAF-14 can inhibit DAF-3 in the absence of DAF-8 only when it is overexpressed.

daf-8::gfp expression (like that of *daf-1*) was detected from pre-comma stage embryos through adulthood. The *daf-8(sa345)* mutant exhibits larval lethality, suggesting a possible role in embryogenesis, as is the case for TGFβ in other animals. For example, loss-of-function mutants in mouse *Smad2* also exhibit embryonic lethality, with defects in anterior-posterior axis formation (Nomura and Li, 1998; Waldrip et al., 1998). However, *sa345* is the only allele with an embryonic arrest phenotype, and it is possible that this deletion mutant is actually neomorphic or antimorphic, or there could be other mutations in this strain that contribute to the embryonic arrest.

Animals overexpressing *daf-8* only rarely exhibited dauer formation upon starvation, and the expression of *daf-8* was greatly downregulated in these rare dauer larvae (Fig. 1H), consistent with its preventive role in dauer formation by transmitting the DAF-7 signal.

The biologically active DAF-8::GFP translational fusion was strongly expressed in two pairs of amphid neurons that we identified as ASI and ADL. Cell ablation analyses have established that ASI neurons inhibit dauer formation (Bargmann and Horvitz, 1991; Schackwitz et al., 1996). Proper expression of the TGFβ ligand DAF-7 is required in ASI to promote reproductive growth (Ren et al., 1996; Schackwitz et al., 1996). ADL neurons are important for avoidance of noxious stimuli (Sambongi et al., 1999), raising the possibility that such stimuli might affect the dauer/non-dauer switch. Expression of *daf-8* largely overlaps with that of the *daf-1* type I receptor in multiple tissues, including head neurons, tail neurons, ventral nerve cord, intestine and DTCs (Gunther et al., 2000), supporting a model in which DAF-8 is phosphorylated by the DAF-1 type I receptor to transduce the DAF-7 signal. DAF-8::GFP was seen in nuclei and in the cytosol. The biological activity of C-terminally tagged DAF-8::GFP is further supported by the lack of DAF-8::GFP nuclear localization in *daf-1* mutants and by its failure to suppress the Daf-c phenotype of *daf-1*. These results suggest that phosphorylation of DAF-8::GFP by DAF-1 not only occurs, but is indeed required for DAF-8::GFP to form Smad complexes.

The dauer pathway is used in the adult to promote egg laying (Trent et al., 1983). Here we conclude that it promotes the germ line switch to meiosis as well. *daf-8* is expressed in the DTCs of the gonad, as are *daf-1* and *daf-3* (Gunther et al., 2000; Patterson et al., 1997). The DTCs play a key role in germ line development by providing a stem cell signal to the distal tip of the gonad (Kimble and White, 1981). Since one of the canonical functions of TGFβ signaling is to suppress cell proliferation (Massagué and Gomis, 2006), we determined whether TGFβ signaling in *C. elegans* might also be involved in the regulation of adult germ line mitotic activity.

Assessment of the germ line mitotic zone by DAPI staining and by staining dissected gonads with anti-PH3 antibody revealed that *daf-8* single mutants had an extended mitotic zone with more PH3-positive cells. *daf-14(m77)* mutants did not exhibit an increased number of anti-PH3-positive cells in the ovaries. This implies a role for DAF-8-mediated TGFβ signaling in the regulation of mitosis in *C. elegans*, as in mammalian counterparts. The mitotic phenotype was not suppressed by mutations in *daf-3* or *daf-5*, which are genetically downstream of *daf-8* in the dauer pathway. This is reminiscent of certain Smad-dependent effects that do not require Smad4 (Sirad et al., 1998; Subramanian et al., 2004; Wisotzkey et al., 1998).

LAG-2 is produced in the DTCs to stimulate germ line mitosis (Henderson et al., 1997). Overexpression of *daf-8* resulted in reduced *lag-2::gfp* reporter expression in the DTC cell bodies as well as in the cytonemes, or cytoplasmic arms. qRT-PCR revealed reduced levels of *lag-2* transcripts in the *daf-8::gfp* overexpressor, and higher levels in three loss-of-function *daf-8* mutants. However, neither *daf-3* mutation nor overexpression affected the level of *lag-2* mRNA, which is consistent with the morphology of the *daf-3* mitotic zone and PH3 staining. Since we have no DAF-8 antibody available to test binding to the *lag-2* promoter, and as DAF-8-binding consensus sites have not yet been identified, we cannot conclude whether its effects on *lag-2* are direct or indirect. However, the data show that DAF-8 signaling suppresses germ line mitotic activity to promote meiosis in favorable environments (Fig. 5). Since this does not require *daf-3* activity, DAF-8 might partner with another Smad in DTCs.

A non-canonical function of Smad proteins was observed in the TGFβ pathway that regulates body size and resistance to pathogenic fungi (Zugasti and Ewbank, 2009). Only the SMA-3 R-Smad was required for the regulation of *cnc-2* expression, and not the SMA-2 R-Smad or SMA-4 Co-Smad. Similarly, a non-canonical Smad pathway might regulate germ line mitotic activity in *C. elegans*. We initially observed that DAF-8::GFP was downregulated by overexpression of DAF-3, so we examined transcript levels for all the Daf-c genes upstream of *daf-3*. Expression of *daf-8* and *daf-7* increased in *daf-3* mutants, but not in *daf-5* mutants. By contrast, DAF-3 overexpression reduced the level of *daf-7* and *daf-8* transcripts. ChIP revealed that DAF-3 was physically associated with two regulatory sites in *daf-8* and one in *daf-7*.

The regulation of *daf-7* and *daf-8* by *daf-3* suggests a possible cell-autonomous function in ASI neurons for dauer formation that promotes a rapid developmental switch in adverse conditions. The regulation of *daf-8* might provide negative feedback in tissues in which *daf-7* is not expressed. This regulatory feedback differs from the negative feedback in mammalian TGFβ signaling, in which TGFβ increases the transcription of genes for the inhibitory Smad6, Smad7 and SnoN proteins (Afrakhte et al., 1998; Stroschein et al., 1999). The inhibitory Smads compete with R-Smads for binding to the type I receptor. No such inhibitory Smad genes have yet been identified in *C. elegans*.

Taken together, the feedback regulation that we have identified in dauer formation suggests a novel regulatory mechanism for TGFβ signaling. Transcription of *daf-7* in the ASI sensory neurons is activated by food and repressed by pheromone. When food is abundant, DAF-7 activates DAF-8 to inhibit the ability of DAF-3 to activate dauer-promoting transcription, and prevents the repression of *daf-8* transcription. However, when DAF-7 is consistently deficient over time, DAF-8 activity falls below a threshold, allowing DAF-3 to throw the developmental switch to dauer by repressing *daf-7* and *daf-8* transcription. Hence, the DAF-8 and DAF-3 Smads are antagonistic to each other at both gene and protein levels.

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Competing interests statement
The authors declare no competing financial interests.

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Table S1. Primers used in this study

	Forward	Reverse
Cloning		
daf-8::gfp	GATAGGTAGGAAAAAGCAACGAAG	GAAAAGTTCTTCTCCTTTACTCATAGTTCTGGATGAACATATACG
daf-8::FLAG	GATAGGTAGGAAAAAGCAACGAAG	TTGGATCCTCAGATCTTATCGTCGTCATCCTTGTAATCAGTTCTGGATGAACATATACG
daf-14::FLAG	GGGCTCGAGCTGTCTGACTTTTCTGGACTGCTCTAC	TTAAGCTTTCAGATCTTATCGTCGTCATCCTTGTAATCTGATCGGGATCCTATTTCCGG
daf-3::FLAG	AAACTCGAGCCGAGGTCATGTGCACTCTCTTG	TTTCTCGAGTCAGATCTTATCGTCGTCATCCTTGTAATCGTCATCACTGACGTTGATTCC
daf-8 cDNA in *pBSIIKS*	CCCGGATCCATGGACGATTTTCCTTCACCAG	TTTGAATTCCTAAGTTCTGGATGAACATATAC
daf-8 cDNA in *pGEX4T-1*	CCCGGATCCGACGATTTTCCTTCACCAGAACC	TTTGAATTCCTAAGTTCTGGATGAACATATAC
daf-14 cDNA in *pBSIIKS*	AAACTCGAGATGTACCCAGATCAAGTTCAC	AAACTCGAGTCATGATCGGGATCCTATTTCCG
daf-14 cDNA in *pGEX4T-1*	AAACTCGAGTACCCAGATCAAGTTCACTTAC	AAACTCGAGTCATGATCGGGATCCTATTTCCG
daf-3 cDNA in *pGEX4T-1*	TTTCTCGAGAAGCTAATAGCAACTTCTCTTC	TTTCTCGAGTTAGTCATCACTGACGTTG
qPCR		
daf-7	AACGAAATTCTCGACCAGCTGA	TGGATCTTTGGCGGTGTAGAAT
daf-1	CAGCAGTCATCCACGTGGCT	TCCGTCTCGCGTAGAGGATCT
daf-4	GGAAAAGTTTTCAAGGCGCAA	CGCACACAAATTCGACGATACTT
daf-8	CGTCTACTGTCTCTCACGAATGTGC	GGAGCTCCCATACAAGTGAGTACAC
daf-3	CCGAACTTGTACGGGTTTCCAACT	TGCCTTGTTGGGACGTATGGTTT
lag-2	TTCCTCTTACTCCTCACCTGCCTCC	GCGAGTGGGACTCTGTGACAATTTC
act-2	GGACTTGTACGCCAACACTG	CACATCTGTTGGAAGGTGGA
ChIP		
daf-7 BD2	GCAAAACGGCCTAACTTCAT	CGAAATTTAGCTTTGCATCTGC
daf-8 BD1	CTTACTCCGTAATGTTGGCATTTCA	TGGCTTCAAAAGACTGGTCAGGT
daf-8 BD3	CTTTAAAAAAGAGCAGAGTTGAGAG	GGCTAATTTGAGAGACACATTCTAA