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Original research article

Genetic markers enable the verification and manipulation of the dauer entry decision

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ABSTRACT

Phenotypic plasticity allows animals to survive in changing environments through the alteration of phenotypes or development. One of the best-studied examples of phenotypic plasticity is dauer larval development in the free-living roundworm *Caenorhabditis elegans*. When faced with hostile environments, *C. elegans* larvae can exit reproductive development and enter the stress-resistant and spore-like dauer larval stage. However, knowledge about how the dauer entry decision is made, and how the different tissues of the animal coordinate to execute transformation into dauer, is limited. This is because identifying animals that make the entry decision, or that fail to coordinately remodel their tissues during dauer development, is time-consuming and labor-intensive. Utilizing our previously reported RNA-seq of animals going through dauer or reproductive development (Lee et al., 2017), we have identified genetic markers for conveniently tracking and manipulating the dauer entry decision. These include *col-183* (which tracks dauer fate in the hypodermis), *ets-10* (neurons and intestine), *nhr-246* (intestine and hypodermis), and *F53F1.4* (reproductive fate in the hypodermis). Using condition shift experiments, we demonstrate that the dauer-specific fluorescent expression of the markers correspond to the commitment event of the dauer entry decision, and therefore label when the decision is made. We show that these markers can be used to manipulate the entry decision by driving the reproduction-promoting gene *daf-9* under the control of the dauer-specific marker *col-183*, through which we could shift animals into non-dauer development. We further demonstrate that the markers can be used to track tissue coordination during the decision. *daf-9*, *daf-15*, and *daf-18* partial dauers exhibit incomplete expression of the *ets-10* marker, with our results indicating that the same gene (*e.g. daf-9* or *daf-18*) can affect dauer development differently in different tissues. Our findings provide molecular tools for studying phenotypic plasticity during a whole animal decision.

1. Introduction

Phenotypic plasticity enables organisms to respond to changing environments through activation of different phenotypes or alternative developmental courses (Kelly et al., 2012). For example, nutritional factors contribute to the development of morphologically distinct castes in some ant species (Schwander et al., 2010), and also influence neuronal plasticity in humans (Nithianantharajah and Hannan, 2006).

Caenorhabditis elegans can go through two different developmental trajectories depending on the conditions of the environment. In favorable environments, they proceed from L1, L2, L3, and L4 larvae stages to reproductive adults. When the animal senses harsh stimuli, including high temperature, low food, and high amounts of pheromone, L1 larvae can enter an alternative pre-dauer stage, L2d, and commit to become a dauer if the unfavorable conditions persist. The dauer entry decision is a whole animal decision that involves remodeling of individual tissues to acquire dauer-specific physiology and behaviors. The specialized physiology of dauers, their thickened cuticle for example, makes them more resistant to environmental insult (Cox et al., 1981; Page and Johnstone, 2007), and their specialized behaviors enable them to disperse to improved environments and resume reproductive development (Hallem et al., 2011; Lee et al., 2011).

Genes involved in dauer development, including insulin and TGF-β signaling genes, have been identified through intense genetic screening (Ren et al., 1996; Schackwitz et al., 1996; Li et al., 2003; Fielenbach and Antebi, 2008). However, our knowledge regarding how the dauer entry decision is made and how the decision is coordinately executed across different tissues is still limited (Androwski et al., 2017). First, it is difficult to identify L2d, the stage when environmental signals are integrated and the dauer-commitment decision is made, because of its lack of

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distinct features (Karp, 2018). Additionally, it can be labor-intensive to look for non-dauer features in dauers that fail to coordinately remodel all of their tissues. SDS sensitivity and fluorescent beads are two available tools for dauer hypodermis and pharynx selection (Cassada and Russell, 1975; Nika et al., 2016), but not for other tissues.

We previously reported gene expression profiles from animals going through dauer or reproductive development (Lee et al., 2017). From this rich dataset, we were able to find genes that are specifically regulated in either of the developmental tracks as potential readouts of the decision. Here, we describe four molecular markers that can track the decision in different tissues and are predictive of the decision. We verified that the markers could also be used to drive gene expression during the dauer entry decision, and to parse incomplete dauer development phenotypes. Our findings provide useful molecular tools for studying phenotypic plasticity during a whole animal decision.

2. Materials and methods

2.1. Animal strains

C. elegans strains were grown using standard protocols with the *Escherichia coli* strain OP50 as a food source (Brenner, 1974). The wild-type strain is N2 (Bristol). Other animal strains are listed in the **KEY RESOURCES TABLE**.

2.2. Transgenic strains

Transcriptional reporter strains. Three of the transcriptional reporters (*col-183p::mcherry*, *ets-10p::gfp* and *nhr-246p::gfp*) were built using fusion PCR (Hobert, 2002). The promoter region of *col-183* was fused to the *mcherry::unc-54* 3′UTR fragment amplified from pGH8 (Addgene). The promoter regions of *ets-10* and *nhr-246* were fused to the *gfp::unc-54* 3′UTR fragment amplified from pPD95_75 (Addgene). *F53F1.4*p*::destabilized yfp* (*dyfp*) was built by cloning the *F53F1.4* promoter region into the pRB3 vector, which contains YFP and PEST destabilized sequence (Hwang et al., 2007). Injection mixtures were prepared at a concentration of 20 ng/μL reporter construct, 50 ng/μL co-injection marker (*unc-119(+)* rescue construct, AWC marker *odr-1p::gfp,* or coelomocyte marker *ofm-1p::rfp*), and 130 ng/μL 1-kb DNA ladder carrier DNA. Transgenic strains were obtained by microinjecting the mixtures into the adult gonads of *unc-119(ed4)* or wild-type animals (Maduro and Pilgrim, 1995). The transgenic animals were further integrated into the genome by X-ray (Mello and Fire, 1995). The fluorescent transcriptional reporter strains that were generated are: PS6726 *unc-119(ed4); syIs264 [col-183p::mcherry*; *unc-119(+)]*; PS8438 *syIs600[col-183p::mcherry; odr-1p::gfp]*; PS7127 *unc-119(ed4); syIs360[ets-10p::gfp; unc-119(+)]*; PS8457 *syIs601[ets-10p::gfp; ofm-1p::rfp]*; PS7921 *unc-119(ed4); syEx1539[nhr-246p::gfp; unc-119(+)]*; PS8083 *syEx1649[nhr-246p::gfp; ofm-1p::rfp]*; PS8437 *syIs599[nhr-246p::gfp; ofm-1p::rfp]*; PS8434 *syIs598 [F53F1.4p::dyfp; odr-1p::gfp]*; PS8435 *syIs602[F53F1.4p::dyfp; odr-1p::gfp]*; PS8436 *syIs603[F53F1.4p::dyfp; odr-1p::gfp]*.

Transcriptional reporters in partial dauer mutant backgrounds. Strains with *ets-10p::gfp* expression in *daf-15(m81)* or *daf-9(e1406)* backgrounds were built by crossing PS7127 *unc-119(ed4); syIs360* with DR732 or AA823. The strains generated are: PS8456 *daf-15(m81) unc-22(s7)/nT1; +/nT1; syIs360*; PS8245 *daf-9(e1406); syIs360; dhEx354[sdf-9::daf-9cDNA::gfp; lin-15(+)]*. Strain with *ets-10p::gfp* expression in the *daf-18(e1375)* background was obtained by microinjecting the injection mixture (20 ng/μL reporter construct, 50 ng/μL *ofm-1p::rfp* coelomocyte co-injection marker, and 130 ng/μL 1-kb DNA ladder carrier DNA) into the adult gonads of CB1375 *daf-18(e1375)*. The strain generated was PS8056 *daf-18(e1375); syEx1647[ets-10p::gfp, ofm-1p::rfp]*. PS8455 *syEx1647[ets-10p::gfp, ofm-1p::rfp]* was built by crossing PS8056 with wild-type animals for expression control.

daf-9 overexpression strain. The *col-183* promoter region was cloned into the pSM vector (a derivative of pPD49.26, gift from Cori Bargmann, Rockefeller University) (Mccarroll, Steven, 2005) that contains *gfp* or *daf-9* cDNA. *daf-9* cDNA sequence was obtained from WormBase and amplified with forward primer 5′-ATGCACTTGGAGAACCGTG-3′ and reverse primer 5′-TTAGTTGATGAGACGATTTCCG-3’. Injection mixtures were prepared at a concentration of 20 ng/μL *col-183p::gfp* or *col-183p::daf-9* cDNA, 50 ng/μL *ofm-1p::rfp* coelomocyte co-injection marker, and 130 ng/μL 1-kb DNA ladder carrier DNA. Transgenic strains were obtained by microinjecting the mixtures into the adult gonads of wild-type animals. The generated transgenic strains are: PS7949 *syEx1628[col-183p::gfp; ofm-1p::rfp]* and PS7931 *syEx1629[col-183p::-daf-9 cDNA; ofm-1p::rfp]*.

2.3. Animal staging

L2d and pheromone-induced dauer. The preparation of crude pheromone and the induction of L2d and dauers on pheromone plates were performed using previously described methods (Golden and Riddle, 1984; Lee et al., 2017; Schroeder and Flatt, 2014). Briefly, crude pheromone plates (NGM-agar with added crude pheromone and no peptone) were used to induce synchronized L2d and dauers. About 85% of wild--type animals entered the dauer stage under the pheromone concentration (2.25% v/v). For each pheromone plate, 20 μL of heat-killed OP50 (8 g/100 mL) was spotted and 12–15 young adult animals were picked onto the plate to lay eggs at 20 °C for 2 h before being removed. The plates were then incubated in 25.5 °C for 24 h (uncommitted L2d), 41 h (dauer-committed L2d), or 48 h (newly-molted dauer). For collecting aged dauers, non-dauers were removed from pheromone plates at hour 48, and the remaining dauers were incubated at 25.5 °C for three extra days.

Starvation-induced dauer. Starvation-induced dauers were picked from 10- to 12-day-old plates. The dauers were identified based on their morphology under dissecting scope, and further verified based on their distinctive dauer alae (Cassada and Russell, 1975) under compound microscope.

Reproductive stages. Embryos were directly picked from plates with many adult animals. L1, L2, L3 and L4 animals were selected based on the developmental timing of wild-type animals (Byerly et al., 1976), and the stages were further confirmed by the animals’ gonadal development (Atwell et al., 2015). Post-dauer L4s were generated by transferring newly-molted pheromone-induced dauers onto plates with food and incubating at 20 °C for 24 h.

2.4. Quantification of fluorescence intensity

The microscope used was ZEISS Imager.Z2, and the camera capture source was an Axiocam 506 mono. The images were first processed using ZEISS Apotome.2 for noise removal, and ZEISS ZEN software was used for quantification. Two regions of interest were drawn for each image: one that outlined the whole body of the animal (Fig. 1) or only the intestine (Fig. 5), and one that outlined a side of the image with no observable fluorescence. The net pixel intensity was calculated by subtracting the mean pixel intensity of the background from the animal or intestine. The maximum pixel intensity that was measured here (11,750 a.u.) was less than the detection limit (16,384 a.u.).

2.5. Verification of dauer markers

Verifying fluorescence in dauers. Dauers induced on pheromone plates were identified by morphology and examined for fluorescence expression. The number of dauers and the proportion that were fluorescent was recorded. Fluorescent animals were further treated with 1% SDS and survival was scored after 30 min to confirm resistance (a key dauer characteristic).

Environmental condition shift of fluorescent animals. Fluorescent expression in the dauer reporter strains was detectable under dissecting microscope staring around 30–33 h after egg laying. At hour 34, we

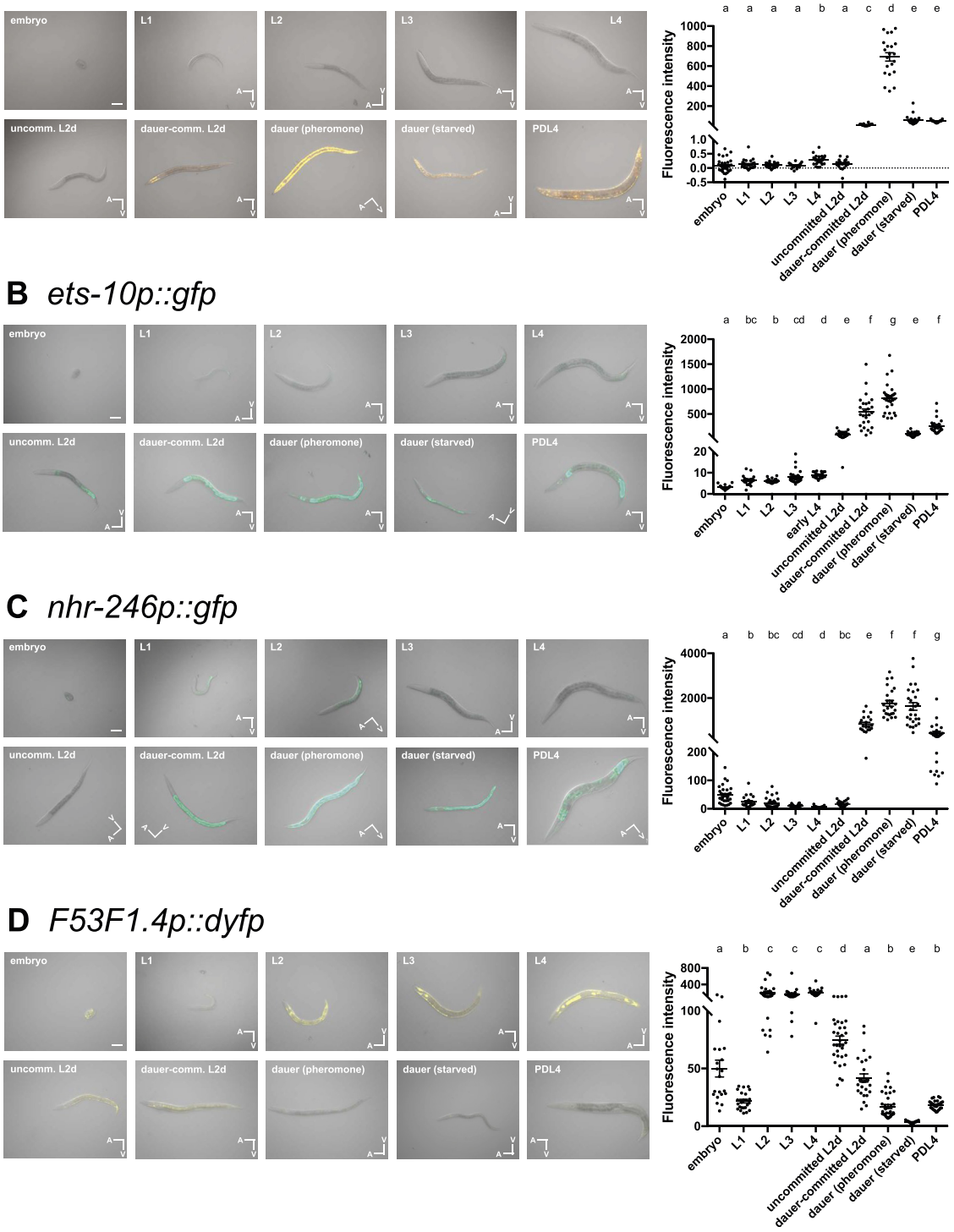


Fig. 1. Genetic reporters with dauer- and reproductive-specific expression patterns. **(A-D)** Representative fluorescence images of **(A)** *col-183p::mcherry,* **(B)** *ets-10p::gfp,* **(C)** *nhr-246p::gfp,* and **(D)** *F53F1.4p::dyfp* expression (left) and quantified expression levels (right) across developmental stages (embryo, L1, L2, L3, L4, uncommitted L2d, dauer-committed L2d, dauer and post-dauer L4). All images were taken from different animals. Animal strains examined are PS6726, PS7127, PS7921, and PS8435. Each dot represents one animal, and at least twenty animals were examined for each developmental stage. Bars indicate the mean and the standard error of the mean (SEM). Statistic: nonparametric permutation test. Letters indicate groups that are significantly different from each other (q-value < 0.01). Uncomm. L2d: uncommitted L2d, 24 h after egg laying (AEL); dauer-comm. L2d: dauer-committed L2d, 41 h AEL; dauer (pheromone): newly-molted dauers generated with crude pheromone, 48 h AEL; dauer (starved): dauers collected from 10- to 12-day-old starved plates; PDL4: post-dauer L4, 72 h AEL; A: anterior; V: ventral. Fluorescence intensities are given in arbitrary units (a.u.). Scale bar: 50 μm.

transferred the fluorescent animals from dauer-inducing pheromone plates to reproduction-inducing plates, which contained high amounts of bacteria but no pheromone, and then incubated the plates at 25.5 °C. 11 h after the transfer, animals were treated with 1% SDS, and the survival rate was scored after 30 min.

Uncommitted L2ds of *F53F1.4p::dyfp* expressing animals were also transferred from dauer-inducing to reproduction-inducing plates, and incubated at 20 °C. The fluorescence expression levels of the animals were measured 0 h and 6 h after the transfer.

2.6. Dauer formation assay

The preparation of crude pheromone and the dauer entry assay were performed using previously described methods (Schroeder and Flatt, 2014; Lee et al., 2017). On the day of the experiment, seven to ten young adults were picked onto each pheromone plate (NGM-agar with added crude pheromone and no peptone) and allowed to lay approximately 50–60 eggs before being removed. 20 µl of heat-killed OP50 was added to the plates as a food source for the un-hatched larvae. After 48 h of incubation at 25.5 °C, dauers and non-dauers were counted on each plate based on their distinct morphologies.

2.7. Statistical analysis

Non-parametric, pairwise permutation tests were performed using the rcompanion (Mangiafico, 2015) and coin (Hothorn et al., 2006) packages in R. A *q*-value cutoff of 0.01 was used.

2.8. Key Resources Table

The animal strains and primers used are summarized in the **KEY RESOURCE TABLE**.

Reagent or resource	Source	Identifier
Bacterial and Virus Strains		
Escherichia coli: Strain OP50	*Caenorhabditis* Genetics Center	OP50
Experimental Models: Organisms/Strains		
C. elegans: Strain N2: Bristol	*Caenorhabditis* Genetics Center	N2
C. elegans: Strain PS6726: *unc-119(ed4); syIs264[col-183p::mcherry; unc-119(+)]*	This paper	PS6726
C. elegans: Strain PS8438: *syIs600[col-183p::mcherry; odr-1p::gfp]*	This paper	PS8438
C. elegans: Strain PS7127: *unc-119(ed4); syIs360[ets-10p::gfp; unc-119(+)]*	This paper	PS7127
C. elegans: Strain 8457: *syIs601[ets-10p::gfp; ofm-1p::rfp]*	This paper	PS8457
C. elegans: Strain PS7921: *unc-119(ed4); syEx1539[nhr-246p::gfp; unc-119(+)]*	This paper	PS7921
C. elegans: Strain PS8083: *syEx1649 [nhr-246p::gfp; ofm-1p::rfp]*	This paper	PS8083
C. elegans: Strain PS8437: *syIs599[nhr-246p::gfp; ofm-1p::rfp]*	This paper	PS8437
C. elegans: Strain PS8434: *syIs598 [F53F1.4p::dyfp; odr-1p::gfp]*	This paper	PS8434
C. elegans: Strain PS8435: *syIs602 [F53F1.4p::dyfp; odr-1p::gfp]*	This paper	PS8435
C. elegans: Strain PS8436: *syIs603 [F53F1.4p::dyfp; odr-1p::gfp]*	This paper	PS8436
C. elegans: Strain PS7949: *syEx1628 [col-183p::gfp; ofm-1p::rfp]*	This paper	PS7949
C. elegans: Strain PS7931: *syEx1629 [col-183p::daf-9 cDNA; ofm-1p::rfp]*	This paper	PS7931
C. elegans: Strain AA823: *daf-9(e1406) dhEx354[sdf-9::daf-9cDNA::gfp lin-15+]*	Gerisch and Antebi (2004)	AA823
C. elegans: Strain PS8245: *daf-9(e1406); syIs360[ets-10p::gfp; unc-119(+)]; dhEx354[sdf-9::daf-9cDNA::gfp lin-15+]*	This paper	PS8245
C. elegans: Strain DR732: *daf-15(m81) unc-22(s7)/nT1 IV; +/nT1*	*Caenorhabditis* Genetics Center	DR732
C. elegans: Strain PS8456: *daf-15(m81) unc-22(s7)/nT1;; +/nT1; syIs360[ets-10p::gfp; unc-119(+)]*	This paper	PS8456
C. elegans: Strain CB1375: *daf-18(e1375)*	*Caenorhabditis* Genetics Center	CB1375
C. elegans: Strain PS8056: *daf-18(e1375); syEx1647[ets-10p::gfp, ofm-1p::rfp]*	This paper	PS8056

(*continued on next page*)

(*continued*)

Reagent or resource	Source	Identifier
C. elegans: Strain PS8455: *syEx1647 [ets-10p::gfp, ofm-1p::rfp]*	This paper	PS8455
Oligonucleotides		
mcherry::unc-54 3′UTR forward: 5′-GCTTAATGGTCTCAAAGGGTGA-3′	This paper	*mcherry::unc-54* 3′UTR forward
mcherry::unc-54 3′UTR outer reverse: 5′-GTAAAACGACGGCCAGTGAA-3′	This paper	*mcherry::unc-54* 3′UTR outer reverse
mcherry::unc-54 3′UTR inner reverse: 5′-ACGACGGCCAGTGAATTATC-3′	This paper	*mcherry::unc-54* 3′UTR inner reverse
gfp::unc-54 3′UTR forward: 5′-AGCTTGCATGCCTGCAGGTCGACT-3′	This paper	*gfp::unc-54* 3′UTR forward
gfp::unc-54 3′UTR outer reverse: 5′-TCATGAGAGGCCCAGACGTGCG-3′	This paper	*gfp::unc-54* 3′UTR outer reverse
gfp::unc-54 3′UTR inner reverse: 5′-TGCGAAGAAATAAAAATTGCGGTC-3′	This paper	*gfp::unc-54* 3′UTR inner reverse
col-183p forward: 5′-AATCGCAAACCTTCAACGAAGAG-3′	This paper	col-183p forward
col-183p mcherry fusion reverse: 5′-TCACCCTTTGAGACCATTAA GCGGTTGACTGGTTGCTGTTGCT-3′	This paper	col-183p *mcherry* fusion reverse
ets-10p forward: 5′-TGCGATGAATGAGAAAGCTG-3′	This paper	*ets-10p* forward
ets-10p gfp fusion reverse: 5′-AGTCGACCTGCAGGCATGCAAG CTGTTTGTCAGCTAGTTTGCGG-3′	This paper	*ets-10p gfp* fusion reverse
nhr-246p forward: 5′-CACCGATGCGTTGTTATAGG-3′	This paper	*nhr-246p* forward
nhr-246p gfp fusion reverse: 5′-AGTCGACCTGCAGGCATGCAAGCT ATTGTTGAAATTGA AAATTATTTTGAA-3′	This paper	*nhr-246p gfp f*usion reverse
FseI-*F53F1.4p* forward: 5′-AATTAAGGCCGGCCGGC CGAGAATCACAAAACTC-3′	This paper	FseI-*F53F1.4p* forward
BamHI-*F53F1.4p* reverse: 5′-AATTAAGGATCCGTTGAAA ATGTTGAAAGTCAAAAGAG-3′	This paper	BamHI-*F53F1.4p* reverse
daf-9 cDNA forward: 5′-ATGCACTTGGAGAACCGTG-3′	This paper	*daf-9* cDNA forward
daf-9 cDNA reverse: 5′-TTAGTTGATGAGACGATTTCCG-3′	This paper	*daf-9* cDNA reverse
Recombinant DNA		
col-183p::mcherry	This paper	*col-183p::mcherry*
ets-10p::gfp	This paper	*ets-10p::gfp*
nhr-246p::gfp	This paper	*nhr-246p::gfp*
F53F1.4p::dyfp	This paper	*F53F1.4p::dyfp*
col-183p::gfp	This paper	*col-183p::gfp*
col-183p::daf-9 cDNA	This paper	*col-183p::daf-9 cDNA*
Software and Algorithms		
GraphPad Prism 7	GraphPad	http://www.graphpad.com
ApoTome.2	Carl Zeiss	https://www.zeiss.com/microscopy/us/products/imaging-systems/apotome-2-for-biology.html
ZEISS ZEN	Carl Zeiss	https://www.zeiss.com/microscopy/us/products/microscope-software/zen-lite.html
R	The R Foundation	https://www.r-project.org
rcompanion (R package)	CRAN	http://rcompanion.org/
Coin (R package)	CRAN	http://coin.r-forge.r-project.org/
Other		
Zeiss Imager Z2 microscope	Carl Zeiss	https://www.zeiss.com/microscopy/us/products/light-microscopes/axio-imager-2-for-biology.html
Axiocam 506 Mono camera	Carl Zeiss	https://www.zeiss.com/microscopy/us/products/microscope-cameras/axiocam-506-mono.html
Stemi SV 11 Apo Stereoscope	Carl Zeiss	NA
Fire-i 501b	UniBrain	https://www.unibrain.com/products/fire-i-digital-camera/

3. Results

3.1. Dauer and reproductive markers demonstrate stage-specific expression patterns

Unfavorable conditions promote L1 larvae to develop into pre-dauer L2d. Depending on whether the environment improves and enough dafachronic acid (DA) growth hormone gets amplified in the animal's body, L2d larvae can progress to either reproductive or dauer development. In our previous study, we controlled the animals' binary developmental choice by withholding or adding synthetic DA at 24 h post hatch (hph) to *daf-9(dh6)* mutants, which lack DA, and we profiled the transcriptional changes from animals going through dauer (L2d, dauer-committed L2d, and dauer) or reproductive development (L3-committed L2d and L4) (Lee et al., 2017). To find useful markers for dauer, we selected candidate genes based on the following criteria: (i) Genes that have high

expression specifically during dauer or reproductive development; (ii) Genes that are expressed in large tissues, including collagen genes, for convenient observation under low magnification; (iii) Genes that might shed light on dauer biology, including transcription factors and unknown genes.

139 of 164 genes in the collagen (*col*) family were detected and differentially expressed in the RNA-seq dataset. Among those, five collagen genes (*col-2*, *col-37*, *col-85*, *col-40* and *col-183*) have the highest transcripts per million (tpm) counts in dauer-committed L2d, while having low counts in other stages (Supplementary Figs. 1 and 4, Supplementary Table 1). Indeed, *col-2* and *col-40* have previously been reported to have specific expression in dauer (Kramer et al., 1985; Lee et al., 2017). We noticed that, unlike wild-type dauer, the dauers expressing *col-85p::mcherry* were abnormally sensitive to SDS treatment, possibly caused by promoter quenching or toxicity. Therefore, we chose to focus on the *col-183p::mcherry* transcriptional reporter for the following analyses despite its lower read counts during dauer development.

We detected 270 transcription factor genes that are differentially expressed during dauer and reproductive development. We clustered those genes by their expression profiles, looked for dauer marker candidates, and found 119 that fit our criteria (Supplementary Fig. 2 and Supplementary Table 1). We decided to focus on two of the transcription factors, *ets-10* and *nhr-246*, whose tpm counts increase only during dauer development and are at their highest level at the dauer-commitment time point (Supplementary Fig. 4).

In addition to dauer-specific genes, we also looked for genes that are downregulated specifically in dauer. *F53F1.4* has the highest tpm read counts during reproductive development, and its reporter strain is the healthiest among five tested candidates (Supplementary Figs. 3–4 and Supplementary Table 1). We picked *F53F1.4* for further reproductive development-specific expression analysis.

The following section describes the expression of each transcriptional reporter:

3.1.1. *col-183*

Non-dauer: Expression was not observed in any tissues. *col-183p::mcherry* fluorescence is undetectable under the dissecting scope in embryos to L4 (mean intensity ranges from 0.086 arbitrary unit (a.u.) in L3 to 0.28 a.u. in L4, n = 20–34) (Fig. 1A).

Dauer: Expression occurs specifically in the hypodermis (Fig. 2A). Expression is undetectable under the dissecting scope in uncommitted L2d (mean = 0.15 a.u., n = 26). Expression increases in the hypodermis 81-fold in dauer-committed L2d (mean = 12.17 a.u., n = 26), 4,612-fold in pheromone-induced dauers (mean = 691.8 a.u., n = 21), and 372-fold in starvation-induced dauers (mean = 55.79 a.u., n = 25) (Figs. 1A and 2A). The hypodermal expression remains but the intensity is significantly

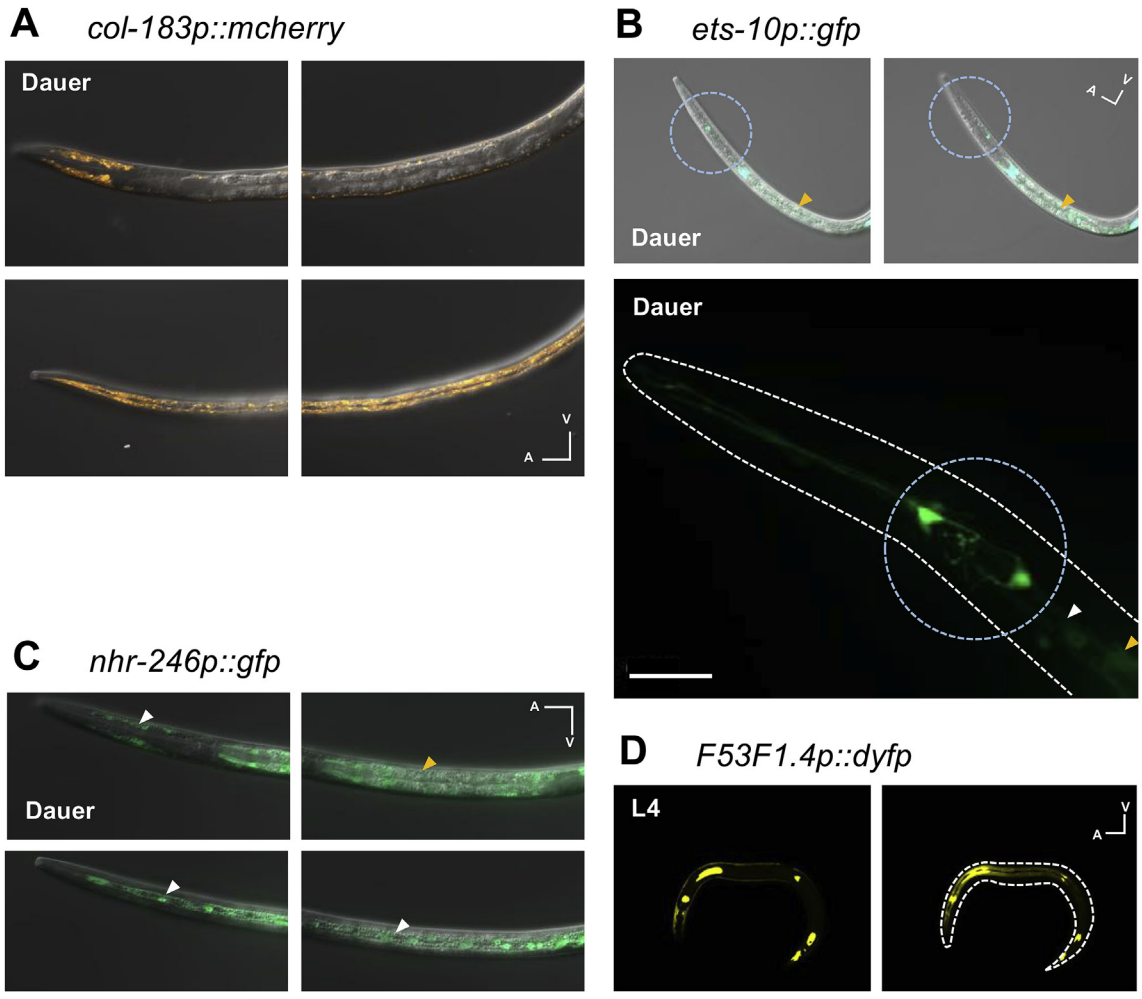


Fig. 2. Spatial expression patterns of the genetic reporters. **(A)** *col-183p::mcherry* expression in dauer. The photos were taken from the same animal on different focal planes (upper and lower panels). **(B)** *ets-10p::gfp* expression in dauer. The upper panel photos were taken from the same animal on different focal planes, and the lower panel photo was from a different animal. The blue circle highlights the neuronal cells in the head. The orange and white arrowheads point to intestine and hypodermis, respectively. **(C)** *nhr-246p::gfp* expression in dauer. The photos were taken from the same animal on different focal planes (upper and lower panels). **(D)** *F53F1.4p::dyfp* expression in L4. The photos were taken from the same animal on different focal planes (left and right). Animal strains examined are PS6726, PS7127, PS7921, and PS8435. A: anterior; V: ventral. Scale bars: 20 μm **(A–C)** and 50 μm **(D)**.

reduced in aged dauers (mean = 190.7 a.u., n = 21) and post-dauer L4s (mean = 47.98 a.u., n = 23) (Supplementary Fig. 5 and Fig. 1A).

3.1.2. *ets-10*

Non-dauer: Expression was only observed in the uterine and spermathecal cells of early-mid-L4 to adults (Fig. S6). *ets-10p::gfp* expression is undetectable under dissecting scope in embryos to early-L4 (mean intensity ranges from 3.05 a.u. in embryos to 8.83 a.u. in early L4, n = 22–26) (Fig. 1B).

Dauer: Expression was observed in the intestine, five neurons, and faintly in the hypodermis (Fig. 2B). Intestinal expression starts from the posterior in uncommitted L2d (mean = 92.83 a.u., n = 28) and spreads throughout the entire intestine after dauer-commitment (mean = 537.9 a.u., n = 26) (Fig. 1B). Intestinal expression peaks in intensity during dauer (mean = 816.6 a.u., n = 31), dims in starved (mean = 99.17 a.u., n = 30) and aged dauers (mean = 491.53 a.u., n = 24), and disappears from the center of the intestine in post-dauer L4s (mean = 259.4 a.u., n = 22) (Fig. 1B and Supplementary Fig. 5).

Neuronal expression is detectable in 58% of the dauer-committed L2d (n = 15/26), and exists in all dauers (n = 31). The neurons consist of one pair immediately anterior to the terminal bulb of the pharynx, with processes that end in the nerve ring, and one triplet approximately one cell body diameter posterior to the metacorpus, with processes that end in the nose. Based on their positions and morphologies, the best candidates for the neuron identities include: ADF, ADL, AFD, AIA, AIB, ASG, ASH, ASI, ASK, AWA, AWB, AWC, BAG, IL1, IL2, and OLQ. Using co-localization markers, we eliminated AIB, AWC, BAG, IL1, IL2, and OLQ as the *ets-10*-expressing neurons. The precise identities of the neurons remain unknown.

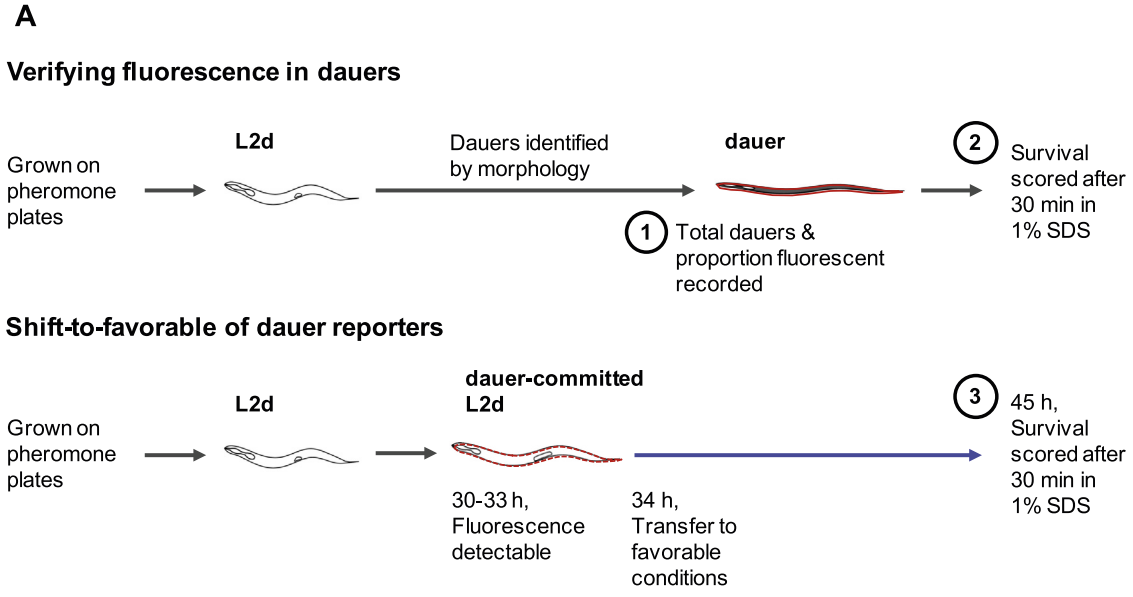
3.1.3. *nhr-246*

Non-dauer: Expression was only observed in the embryo and in the intestine of L1 and L2 animals (mean intensity ranges from 20.63 a.u. in L2 to 48.43 a.u. in embryos, n = 25–32) (Fig. 1C and Supplementary Fig. 7). *nhr-246p::gfp* expression is undetectable under dissecting scope in L3 and L4 (mean = 2.82–4.44 a.u., n = 21–22).

Dauer: Expression was observed in the hypodermis and intestine (Fig. 2C). Expression is undetectable under the dissecting scope in uncommitted L2d (mean = 16.2 a.u., n = 26). Intestinal expression increases after dauer-commitment (mean = 843.7 a.u., n = 21) and peaks in intensity in pheromone-induced dauers (mean = 1771 a.u., n = 24) and starvation-induced dauers (mean = 1628 a.u., n = 26) (Fig. 1C). The intestinal expression remains, but the intensity is significantly reduced in aged dauers (mean = 740 a.u., n = 27) and disappears from the center of the intestine in post-dauer L4s (mean = 439.8 a.u., n = 26) (Supplementary Fig. 5 and Fig. 1C). Hypodermal expression is detectable in 14% of the dauer-committed L2d (n = 3/21) and exists in all dauers (n = 26).

Similar gene expression dynamics were observed in an independent

A



B

1	Fluo-rescent	Non-fluorescent	Total dauers
col-183p::mcherry	247	0	247
ets-10p::gfp	155	0	155
nhr-246p::gfp	176	0	176

2	SDS-resistant	SDS-non-resistant	Total animals
col-183p::mcherry	224	1	225
ets-10p::gfp	159	0	159
nhr-246p::gfp	156	1	157

3	SDS-resistant	SDS-non-resistant	Total animals
col-183p::mcherry	29	0	29
ets-10p::gfp	26	0	26
nhr-246p::gfp	34	2	36

Fig. 3. Dauer reporters are expressed in dauers and dauer-committed L2d. **(A)** Experimental design for verifying reporter expression in dauers, and confirming expression in dauer-committed L2d. Assays are numbered. Red, *mCherry* fluorescence; blue, favorable conditions. **(B)** Data corresponding to the assays in (A). Data were pooled from 2 to 3 independent trials. Animal strains examined are PS6726, PS7127, and PS7921.

transgenic line (Supplementary Fig. 8).

3.1.4. F53F1.4

Non-dauer: Expression occurs in the embryo and specifically in the hypodermis during L1 through L4 (Figs. 1D and 2D). The intensity is the lowest in L1 (mean = 21.75, n = 23) and stays high in L2 to L4 (mean intensity ranges from 169.6 to 203.3, n = 22–27).

Dauer: Expression was observed in the hypodermis, and the level becomes significantly reduced during dauer development (Fig. 1D). Compared with the expression intensity in reproductive L2 (mean = 203.3 a.u., n = 26), the expression level reduces to 37% in uncommitted L2d (mean = 74.64 a.u., n = 33), 21% after dauer commitment (mean = 41.75 a.u., n = 26), 8% in pheromone-induced dauer (mean = 17.09 a.u., n = 35), and 2% in starvation-induced dauer (mean = 3.65 a.u., n = 25). *F53F1.4p::dyfp* expression remains low in aged dauers (mean = 11.09 a.u., n = 22) and post-dauer L4s (mean = 18.49 a.u., n = 34) (Fig. 1D and Supplementary Fig. 5).

The gene expression dynamics of the markers fits well with the read count changes during dauer development (Lee et al., 2017) (Supplementary Fig. 4, left). It also aligns well with the expression data from 246 RNA-seq datasets spanning a wide range of *C. elegans* life stages (Gerstein et al., 2010) (Supplementary Fig. 4, right). In summary, we have developed three dauer markers (*col-183p::mcherry*, *ets-10p::gfp*, and *nhr-246p::gfp*) that have increased expression in distinct tissues during dauer. We also developed a reproductive development marker (*F53F1.4p::dyfp*) that marks non-dauers from dauers.

3.2. col-183, ets-10 and nhr-246 label the dauer commitment decision

Because the dauer markers have high expression levels during dauer-commitment, we expected that their fluorescence would be useful for selecting dauer-committed L2d. If the markers do label dauers and animals that are committed to dauer, then: (i) all dauers will have fluorescence expression (Fig. 3A, 1–2) and (ii) fluorescent L2d larvae will still become dauer even if the environment improves (Fig. 3A, 3). We found that the fluorescence markers were turned on in all of the dauers examined (100% for all three marker strains, n = 155–247) (Figs. 3B, 1), and almost all fluorescence animals are SDS-resistant (99.4–100%, n = 157–225) (Figs. 3B, 2), suggesting that the expression of these reporters does not produce SDS-sensitive partial dauers. Moreover, we transferred L2d animals from unfavorable to favorable conditions as soon as fluorescence was detected under the dissecting microscope (30–33 h after egg laying, Supplementary Fig. 9), and we observed that 94%–100% of the animals still entered dauer despite the shift (*col-183p::mcherry* 100%, n = 29; *ets-10p::gfp* 100%, n = 26; *nhr-246p::gfp* 94%, n = 36) (Fig. 3B, 3). These data suggest that *col-183* and *ets-10* label the dauer commitment decision, and *nhr-246* labels the decision at or slightly before commitment.

We also tested if the reproductive marker *F53F1.4p::dyfp* labels L2d larvae that have committed to L3 by transferring uncommitted L2ds to favorable conditions, and measuring the fluorescence intensity 6 h after the shift. Compared to uncommitted L2d (mean = 74.74 a.u., n = 33), the unfavorable-to-favorable shift increased the *F53F1.4p::dyfp* fluorescence

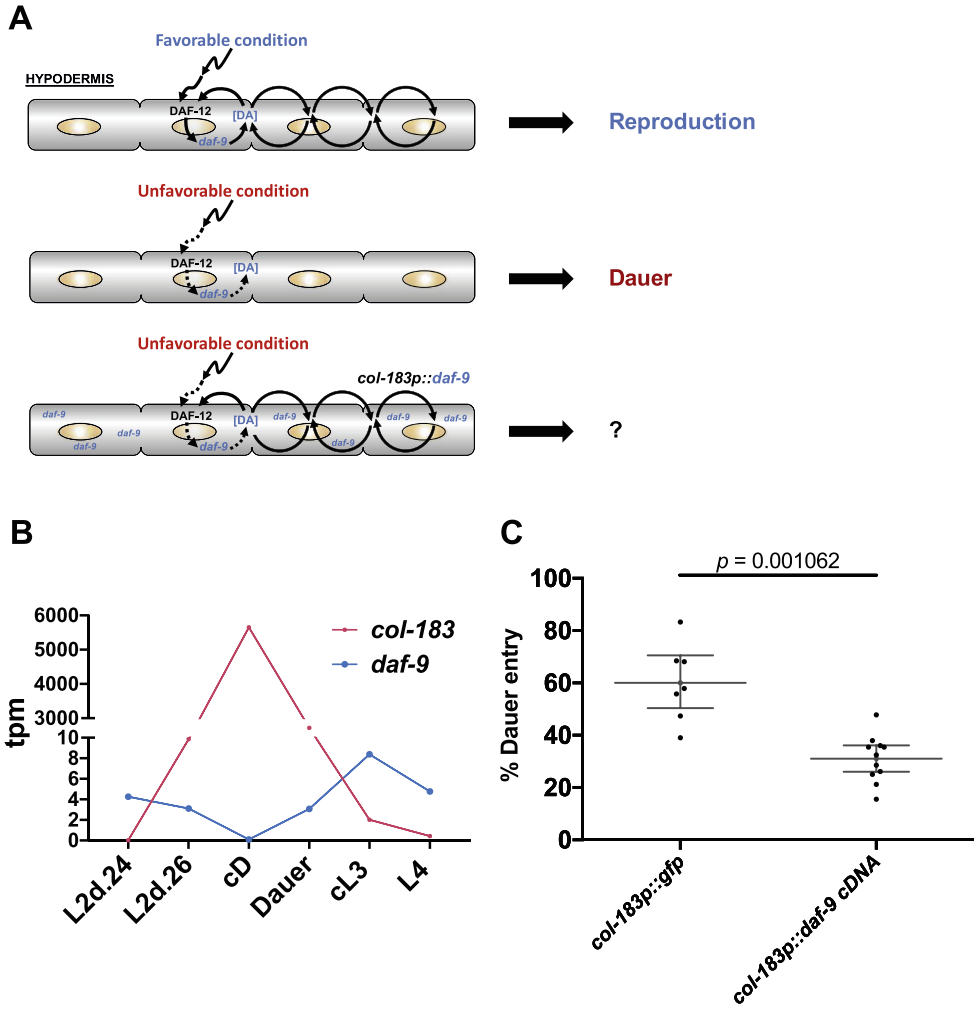


Fig. 4. Overexpressing *daf-9* in the hypodermis during dauer-commitment promotes the reproduction decision. **(A)** Experimental rationale for investigating the effect of *daf-9* overexpression in the hypodermis during dauer-commitment. **(B)** Average read counts of the *col-183* and *daf-9* gene across developmental stages. **(C)** Dauer entry assay on animals with the *col-183* promoter driving expression of *gfp* or *daf-9* cDNA. Animal strains examined are PS7949 and PS7931. Bars indicate the bootstrapped mean and 99% confidence intervals. Each dot is one trial, and the data were collected from at least three different days. Statistics: nonparametric permutation test. DA, dafachronic acid; tpm, transcripts per million.

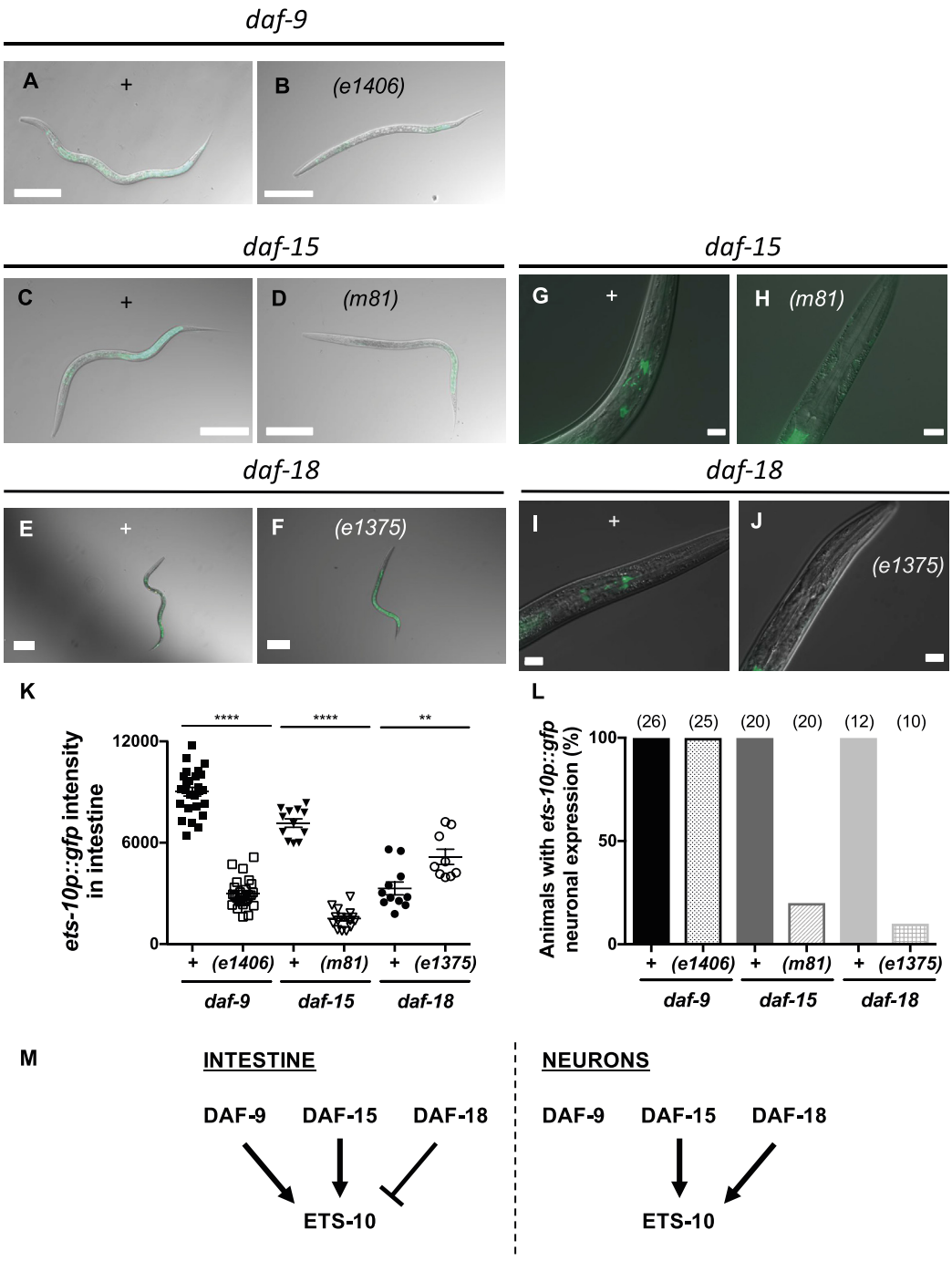


Fig. 5. Partial dauers misexpress dauer markers. **(A–J)** *ets-10p::gfp* expression in wild-type **(A, C, E, G and I)**, *daf-9(e1406)* **(B)**, *daf-15(m81)* **(D and H)**, and *daf-18(e1375)* **(F and J)** dauers. **(K)** Quantification of *ets-10p::gfp* intestinal fluorescence intensity in wild-type, *daf-9(e1406),* and *daf-15(m81)* dauers. Each dot represents one animal. The error bars show the SEM. Statistic: nonparametric permutation test. $^{**}p < 0.01$; $^{****}p < 0.0001$ **(L)** The percentage of wild-type, *daf-9(e1406)*, *daf-15(m81),* and *daf-18(e1375)* dauers with *ets-10p::gfp* neuronal expression. The number in parenthesis indicates the number of animals examined. Data were pooled from 2 independent trials. **(M)** A proposed model for how DAF-9, DAF-15, and DAF-18 influence *ets-10* expression in the intestine and neurons, based on quantitative (intestine) and qualitative (neuronal) data. The animal strains examined are PS7127 **(A, C and G)**, PS8245 **(B)**, PS8456 **(D and H)**, PS8455 **(E and I)**, and PS8056 **(F and J)**. Fluorescence intensities are given in arbitrary units (a.u.). Scale bar: 100 μm **(A–F)** and 10 μm **(G–J)**.

expression by 1.5-fold (mean = 109.4 a.u., n = 20) (Supplementary Fig. 10). Because this increase is mild, it may be difficult to observe under the dissecting microscope for convenient use.

3.3. The promoters of the dauer markers can be used to manipulate the dauer decision

Reproductive development in *C. elegans* requires the synthesis of DA, the product of DAF-9/cytochrome P450 (Gerisch and Antebi, 2004; Mak and Ruvkun, 2004). The timing of *daf-9* expression and the amplification of DA in the hypodermis has been shown to coincide with the critical period of time when L2d animals decide to go through reproductive development instead of dauer (Schaedel et al., 2012). However, it is not known whether ectopically expressing *daf-9* during dauer-commitment can alter the developmental trajectory (Fig. 4A). We therefore used the *col-183* promoter to overexpress *daf-9* in the hypodermis during dauer-commitment when *daf-9* would otherwise be expressed at its lowest level (Fig. 4B). We then examined the animals' decision between dauer and reproductive development under dauer-inducing conditions. We observed that animals with *daf-9* overexpression were 0.5 times as likely to become dauers compared to those with control *gfp* (*col-183p::daf-9* bootstrap mean = 31%, n = 336; *col-183p::gfp* bootstrap mean = 60%, n = 262) (Fig. 4C). These data suggest the promoters of the dauer markers can be used to drive ectopic gene expression during dauer-commitment, and that *daf-9* hypodermal expression can shift animal development from dauer to adulthood.

3.4. The dauer markers can be used to study coordination between tissues

The dauer entry decision is a whole-animal decision, with all the tissues coordinating dauer development programs. Previous studies have identified partial dauers, where one or more of the tissues fail to coordinate and therefore exhibit non-dauer features. Known partial dauer phenotypes include an un-constricted pharynx; continued pharyngeal pumping; indistinct dauer alae; and L2/L3-like neurons, intestine, or excretory gland morphologies. For example, *daf-9(e1406)*/cytochrome P450 dauers have a non-dauer intestine, cuticle, pharynx, and neurons (Albert and Riddle, 1988); *daf-15(m81)*/RAPTOR dauers fail to remodel the cuticle, pharynx, neurons, intestine, and excretory gland (Albert and Riddle, 1988); and *daf-18(e1375)*/PTEN dauers have an unremodeled, still-pumping pharynx and an intestine that is neither fully dauer nor L3 (Vowels and Thomas, 1992).

Because identifying partial dauers relies on close examination of each animal's morphology, it can be time-consuming and requires experience. We therefore utilized the dauer-specific *ets-10p::gfp* expression in neurons and intestine to pinpoint partial dauer phenotypes.

In *daf-9(e1406)* dauers, we confirmed their partial dauer phenotype in the intestine: we observed a 3-fold decrease in *ets-10p::gfp* expression in the intestine as compared to wild-type dauers (mean intensity in wild type = 9,017 a.u., n = 26; mean intensity in *daf-9(e1406)* = 2,998 a.u., n = 25) (Fig. 5A-B and 5K), providing a clear indication of the non-dauer feature of *daf-9(e1406)* intestines. We also detected *ets-10p::gfp* expression in neurons in all *daf-9(e1406)* dauers (n = 25) (Fig. 5L).

We were able to confirm the intestinal partial dauer phenotype of *daf-15(m81)* animals as well: we observed a 4-fold reduction in *ets-10p::gfp* intestinal expression as compared to wild type (wild type = 7,166 a.u., n = 12; *daf-15(m81)* = 1,512 a.u., n = 16) (Fig. 5C-D and 5K). Additionally, we confirmed the neuronal partial dauer phenotype of *daf-15(m81)*, as neuronal *ets-10p::gfp* fluorescence was present in all wild-type animals (n = 20), but was undetectable (16 out of 20 animals) or dimly expressed (4 out of 20) in *daf-15(m81)* (Fig. 5G-H and 5L).

In *daf-18(e1375)*, we observed a slight increase in *ets-10p::gfp* intestinal expression (wild type = 3,299 a.u., n = 11; *daf-18(e1375)* = 5,169 a.u., n = 9) (Fig. 5E-F and 5K), and the disappearance of neuronal expression in most of the animals (9 out of 10) (Fig. 5I-J and 5L). These results not only confirmed the partial dauer characteristic of *daf-18(e1375)* intestine but also revealed the previously unknown non-dauer characteristic of *daf-18(e1375)* neurons.

From our results, we have identified *ets-10p::gfp* as a tool for studying the execution of the dauer decision in different tissues. We propose a model for how *ets-10* expression is differentially regulated in the dauer intestine and neurons by DAF-9, DAF-15 and DAF-18, based on quantitative (intestine) and qualitative (neuronal) data (Fig. 5M). In the dauer intestine, DAF-15 and DAF-9 promote *ets-10* expression and DAF-18 inhibits *ets-10*; both DAF-15 and DAF-18, but not DAF-9, positively regulate *ets-10* expression in dauer neurons. This model suggests that the same signaling pathway (*e.g.* downstream DAF-9/cytochrome P450) could have distinct effects on the differentiation of different tissues in dauer.

4. Discussion

We have described four genetic markers that label dauer or non-dauer animals, which can be used for conveniently assaying the dauer entry decision. We demonstrated that the dauer markers in fact mark the dauer-commitment decision using condition-shift experiments. Beyond fluorescence labeling, we were able to use the promoter region to manipulate the commitment decision and tease apart the tissue-specific defects of partial dauer mutants.

We picked members of the hypodermis-expressed collagen gene family as one of our dauer marker candidates because they fit our criteria of being expressed at high levels and in a large tissue. In addition, they offered the opportunity to learn more about the role of hypodermal *daf-9* expression in the developmental decision. When animals commit to reproductive development, *daf-9* functions by promoting a positive feedback amplification loop in the hypodermis to lock in the decision (Schaedel et al., 2012). Even under dauer-inducing conditions, when we introduced *daf-9* expression under the control of *col-183* promoter, we were able to shift the animal's decision toward reproduction. We speculate that the biological function of *col-183* is to shape the stress-resistance and impermeability of dauer cuticle starting from the commitment decision (Blaxter, 1993; Cox et al., 1981).

We also looked at the transcription factor gene class for additional marker candidates. We found that both *ets-10* and *nhr-246* demonstrated dauer-specific expression patterns during dauer-commitment, suggesting their function in execution and maintenance of the dauer program. For instance, the expression of *ets-10* and *nhr-246* in intestine might help establish the specialized intestine structure and metabolism of dauers. We speculate that they participate in remodeling the dauer intestine or switching metabolism from the TCA cycle to long-term lipid metabolism (Braeckman, 2009; Popham and Webster, 1979).

Several genes have been reported to be up-regulated in dauer, including *col-2* in hypodermis (Kramer et al., 1985), *lag-2* in IL2 neurons (Ouellet et al., 2008), *str-2* in the ASI neurons (Peckol et al., 2001), *flp-4* and *flp-8* in touch cells (Kim and Li, 2004), and *inx-6* in the AIB neurons (Bhattacharya et al., 2019). However, whether or not their increased expression correlates with the dauer-commitment decision remains unknown. Notably, *lag-2*, *flp-4*, *flp-8* and *inx-6* have gradual increases in RNA-seq read counts during dauer development; in comparison, *col-183*, *ets-10, nhr-246* and *col-2* expressions peak at the dauer-committment time point and drop in dauer (Lee et al., 2017).

The full coordination of tissue physiology and function is likely important for dauer survival (Albert and Riddle, 1988; Meléndez et al., 2003). Using these markers, we can study how tissue-coordination is achieved during dauer development. Partial dauers represent breaks in tissue-coordination, and by using the markers we can read out their phenotypes on a molecular level. Using *ets-10* markers, we were able to not only recapitulate known partial dauer phenotypes in *daf-9*, *daf-15* and *daf-18*, but also raise the possibility that DA and insulin signaling pathways are combined in distinct ways to regulate *ets-10* expression in different tissues. It would be intriguing to figure out how different tissues might use different *cis*-regulatory elements and signaling receptors to interpret the same signal to meet their specialized needs.

We have described three dauer-specific markers and one reproductive-specific marker selected from our previously published dauer RNA-seq time course (Lee et al., 2017). We have demonstrated that these markers are useful for tracking the dauer-committment decision, driving gene expression during dauer-committment, and for teasing apart partial dauer phenotypes tissue by tissue. 117 transcription factor genes and 6 collagen genes also fit the selection criteria we used to pick our markers. This selection opens up the exciting potential of using these genes for further tracking, manipulating, and parsing the dauer entry decision.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ydbio.2019.06.009.

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