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# *daf-42* is an evolutionarily young gene essential for dauer development in *Caenorhabditis elegans*

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Short running head: *daf-42* is essential in *C. elegans* diapause

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**Abstract**

Under adverse environmental conditions, nematodes arrest into dauer, an alternative developmental stage for diapause. Dauer endures unfavorable environments and interacts with host animals to access favorable environments, thus playing a critical role in survival. Here, we report that in *Caenorhabditis elegans*, *daf-42* is essential for development into the dauer stage, as the null mutant of *daf-42* exhibited a "no viable dauer" phenotype in which no viable dauers were obtained in any dauer-inducing conditions. Long-term time lapse microscopy of synchonized larvae revealed that *daf-42* is involved in developmental changes from the pre-dauer L2d stage to the dauer stage. *daf-42* encodes large, disordered proteins of various sizes that are expressed in and secreted from the seam cells within a narrow time window shortly before the molt into dauer stage. Transcriptome analysis showed that the transcription of genes involved in larval physiology and dauer metabolism are highly affected by the *daf-42* mutation. Contrary to the notion that essential genes that control the life and death of an organism may well be conserved across diverse species, *daf-42* is an evolutionarily young gene conserved only in the *Caenorhabditis* genus. Our study shows that dauer formation is a vital process that is controlled not only by conserved genes but also by newly emerged genes, providing important insights into evolutionary mechanisms.

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## Introduction

Animals adapt various strategies to survive an unpredictable, turbulent environment in the wild. One of these strategies is diapause, a form of dormancy in which the animal enters genetically programmed developmental arrest (HAND *et al.* 2016). Animals enter diapause in response to conditions that are indicative of environmental changes in the future. For example, insects enter diapause at various stages of life, such as embryo, pupa and adult, in response to changes in photoperiod and temperature (DINIZ *et al.* 2017; TOUGERON 2019). Some species of fish, such as killifish or kmar, enter embryonic arrest to survive dry seasons (WOURMS 1972; MESAK *et al.* 2015).

The nematode *Caenorhabditis elegans* has an alternative non-feeding diapause stage known as dauer (CASSADA AND RUSSELL 1975). Under favorable environments rich in nutrients, *C. elegans* quickly develops into reproducing adults through larval stages L1 to L4 in 3 days. However, young L1 *C. elegans* larvae can integrate information from the external environment and develop into the pre-dauer L2d stage and then enter dauer arrest under adverse conditions, such as high population density, high temperature or low food (GOLDEN AND RIDDLE 1984). Dauer worms are stress-resistant, specialized for long-term survival and can live for up to several months until they encounter a favorable environment and resume development into the adult stage and reproduce. The dauer stage is tightly linked to the "boom-and-bust" life cycle of *C. elegans*, in which the worms grow and reproduce with explosive speed in favorable environments and disperse when the resources are exhausted (FREZAL AND FELIX 2015). At the "bust stage", the young larvae develop into the dauer stage in to survive starvation and disperse into other locations, possibly by associating with carriers, such as slugs or isopods (LEE *et al.*

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2011). Because *C. elegans* is mostly found in dauer stage in the wild, the dauer stage is thought to be essential for species survival. (BARRIERE AND FELIX 2005).

The dauer stage is not unique to *C. elegans*, but a widely conserved feature in nematodes, including parasitic nematodes. The infective juvenile (iL3) of parasitic nematodes is analogous to the dauer stage in that both are non-feeding, developmentally arrested third larval stages that resume development when the worms find suitable environmental conditions (CROOK 2014). Parasitic nematodes invade or exit host animals at the iL3 stage, and the dauer stage may be an intermediate step for the evolution of parasitism in nematodes, because dauer and iL3 stages are associated with other animals for dispersal and survival (HOTEZ *et al.* 1993; AHMED *et al.* 2013).

Dauer formation in *C. elegans* has been extensively studied as a genetic model of developmental plasticity. Conserved signaling factors, including the *daf-2*/insulin-like signaling and *daf-7*/TGF-β signaling pathways, relay information on the nutritional state and external environment and converge on steroid hormone signaling to regulate dauer development (HU 2007; FIELENBACH AND ANTEBI 2008). Downregulation of these signaling components leads to the development of the diapause stage. More than 30 genes that have been identified from these studies regulate the developmental switch between adult or dauer stages, and mutants of these genes have altered developmental trajectories. However, downstream factors that directly participate in dauer development after the developmental decision have remained elusive.

Essential genes refer to genes that are necessary for survival of an organism or a cell. Since alterations in these genes cause lethality or other difficulties that severely hinder survival, essential genes are conventionally thought to be evolutionarily conserved factors that are present across diverse species. However, recent studies indicate that newly evolved, young genes also play vital biological roles (CHEN *et al.* 2010; DING *et al.* 2010; CHARRIER *et al.* 2012; DIETZ *et al.*

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2021). In this study, we discovered that *daf-42*, a previously uninvestigated genus-specific gene, is essential for dauer development in *C. elegans*. Null mutants of *daf-42* display lethality in a stage-specific manner at dauer entry. Genetic studies have revealed that DAF-42 acts downstream of the developmental decision process between diapause and reproductive stages and is critical in the short window of time immediately before molting into the dauer stage. We also investigate the phylogenetic conservation of this gene across nematode species.

## Materials and Methods

### Molecular Biology

Plasmids were generated and modified using classical restriction enzyme-based subcloning methods and Q5 site-directed mutagenesis (E0554/New England Biolabs), respectively. The description of plasmids and their generation is available in Supplementary Table 1.

### Worm maintenance and strains

*C. elegans* worms were grown in standard conditions (Brenner 1974), except for the strains in *daf-2(e1370)* and *daf-7(ok3125)* mutant backgrounds, which were raised at 15°C. The following strains were used:

Wild-type strain: N2, CB4856

Single mutant: CB1370 (*daf-2(e1370)*), LJ2701 (*daf-42(ys54)*), RB2302 (*daf-7(ok3125)*), DR2281 (*daf-9(m450)*)

Double mutant: LJ2703 (*daf-2(e1370); daf-42(ys54)*), LJ2770 (*daf-7(ok3125)); daf-42(ys54)*, LJ2771 (*daf-9(+/m450); daf-42(ys54)*), LJ2741 (*daf-2(e1370);daf-42(ys55)*), LJ2742 (*daf-2(e1370);daf-42(ys58)*)

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Transgenic strain:

Transgenic strains used in this study are listed in Supplementary Table 2.

**Generation of transgenic lines**

Transgenes were introduced into worms by injecting purified plasmid DNA into the gonads of young adult hermaphrodite worms as described (MELLO *et al.* 1991). Plasmid concentrations are listed in Supplementary Table 2.

**Dauer induction**

N2 wild-type worms were raised in pheromone plates at 25°C to induce dauer stage. Approximately seven young adult worms were incubated at 25°C on pheromone plates seeded with OP50. Dauer worms on the plate were observed on day 5. The pheromone plates were prepared using synthetic dauer pheromones—ascaroside C7 (ascaroside 3, daumone 1), ascaroside C6 (ascaroside 1, daumone 2) and ascaroside C9 (ascaroside 2, daumone 3) (JEONG *et al.* 2005)— 10μM of which were added to growth media (NGM) without peptone.

Worms in *daf-2(e1370)*, *daf-7(ok3125)* and *daf-9(m540)* mutant backgrounds were raised in regular NGM plates seeded with OP50 at 25°C, as pheromone plates are unnecessary for these strains to induce the dauer stage.

**Transmission electron microscopy**

*C. elegans* animals were overlaid with 20% bovine serum albumin in M9 buffer and frozen under high-pressure using a Leica EM HPM100 system (Leica, Austria). Animals were transferred to the freeze substitution apparatus (Leica EM AFS, Austria) in liquid nitrogen into a solution

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containing 2% osmium tetroxide and 1% water in acetone. The samples were maintained at –90ºC for 72 h, slowly warmed to –20ºC (5 degree per hour), maintained for 24 h and slowly warmed to 0ºC (6 degree per hour). The samples were washed three times with cold acetone at 0ºC, transferred to room temperature, infiltrated with Embed-812 resin series by 20% increments in acetone (each step was 1 h) and embedded in Embed-812 (EMS, USA). After polymerization of the resin at 60ºC for 36 h, sections were cut with a diamond knife on an ULTRACUT UC7 ultramicrotome (Leica, Austria) every 40 μm along the worm body. The 70-nm-thick sections were then mounted on formvar/carbon-coated grids, and were stained with 4% uranyl acetate for 10 min and lead citrate for 10 min. The samples were observed using a Tecnai G2 Spirit Twin transmission electron microscope (Thermo Fisher scientific, USA).

**Dead L2d development and temperature-shift assay**

Synchronized embryos were obtained by placing day 2 adult worms ($n$=10–20) on a fresh NGM plate with OP50 for 2 hours at 15°C. The adults and the plates with newly laid embryos (eggs) were placed at 25°C for the desired time period to observe the developmental outcomes. L2d, dauer and dead L2d worms were distinguished based on their appearance and behavior, such as thin and long body, pointy head, lethargic behavior and rapid movement in response to touch using platinum pick. To calculate the ratio of each developmental stage, we divided the number of worms in each stage by the total number of worms visible on the plate. Some dauer worms were lost as they escaped the plate; these numbers were not included in the number of dauer worms or the total number of worms.

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For the temperature-shift assay, synchronized embryos were obtained. Then, plates were kept at 15°C and moved to 25°C at desired time points. The developmental outcomes were observed at 120 hours after the eggs were laid (HAE).

**Microscopy**

A stereo microscope (Zeiss Stemi 2000-C), fluorescence stereo microscope (Leica M205 FA) and confocal microscope (ZEISS LSM700) were used to observe *C. elegans*. Softwares LAS X (Leica) and Zen (black edition, Carl Zeiss) were used to obtain digital images using Leica M205 FA and Zeiss LSM700, respectively. For confocal imaging, worms were harvested with M9 buffer and placed on 3% agar pads with 3mM levamisole.

**Mutation mapping through mapping-by-sequencing**

To identify the causative mutation for the lethal phenotype, we followed the mapping-by-sequencing strategy (DOITSIDOU *et al.* 2010; DOITSIDOU *et al.* 2016). *daf-2(e1370); daf-42(ys54)* mutant was mated with CB4856 to obtain 48 F2 hybrid strains that developed into dead L2d at 25°C. The worm strains were pooled, and genomic DNA was extracted with a Qiagen Gentra Puregene Tissue Kit using "Purification of DNA from nematodes" protocol. Macrogen Inc. (South Korea) prepared the library and sequenced the pooled genomic DNA using the Illumina Hiseq 4000 100PE platform. Approximately 5 Gb of sequencing data were obtained.

Using the whole-genome sequencing data, the approximate location of the *ys54* mutation was identified using the MimodD pipeline (http://mimodd.readthedocs.io/en/latest/index.html, DOI: 10.5281/zenodo.1189838). Using this pipeline, WGS reads were aligned to the WS220 reference genome, and the density of single nucleotide polymorphisms (SNPs) from CB4856

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were calculated and visualized. A list of mutations that could be *ys54* was obtained from the 5 Mb to 11 Mb region of chromosome IV, which showed an extremely low density of SNPs from CB4856.

**Search for secreted, large and disordered proteins.**

Data on *C. elegans* proteins, including signal peptide prediction, length and disorder content were obtained from MobiDB (https://mobidb.bio.unipd.it). Proteins that have predicted signal peptides, length above 1,000 residues and over 50% disorder content predicted by MobiDB-lite were filtered. MobiDB-lite, version 3.10.0, was used to predict positions of disordered regions in DAF-42 homologs.

**Generation of new alleles of *daf*-42 using CRISPR/Cas9**

A co-conversion strategy using CRISPR/Cas9 was employed to generate new *daf-42* mutants (ARRIBERE *et al.* 2014; WARD 2015). 40 ng/µL of each plasmids containing CRISPR/Cas9 and sgRNA for *daf-42*—pJL1821. pJL1823, pJL1824—were injected into gonads of N2 worms with 20 ng/ul of pJA42 and 20 ng/µL of AF-JA-53 (injection marker containing *rol-6(su1006)* and repair template to generate *rol-6(su1006)*, respectively) (ARRIBERE *et al.* 2014). Injected worms were raised at 25°C, and the F1 offsprings with roller phenotype were singled out for propagation and checked for mutations at *daf-42* locus using T7E1 assay to select heterozygote mutants. Then, non-roller F2 offsprings of the selected heterozygote F1 worms were screened for homozygous mutation at *daf-42* using T7E1 assay.

**Scoring of transgenic animals**

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Synchronized larvae were obtained by placing ten day 2 adult worms on a fresh NGM plate at 15°C for 2 hours and then incubated at 25°C for dauer induction. To observe phenotypes of transgenic animals, transgenic L2d worms were first selected at 48 HAE under a fluorescence stereo microscope (Leica M205 FA). The selected worms were raised in NGM plates with OP50 for desired times until they were observed for scoring of the dead L2d phenotype.

To score expression status, the transgenic worms that express *daf-42p::signal peptide::mCherry* and *grd-10p::gfp* transgenes were observed under a fluorescence stereo microscope (Leica M205 FA). At 48 HAE, each transgenic worm was transferred to a fresh, labeled NGM plate with OP50 for observation. The expression pattern of *daf-42p::signal peptide::mCherry* was observed for each worm every four hours until 72 HAE. Dauer worms tend to move out of the *E. coli* food lawn, and sometimes escape the agar NGM media. Cases in which dauer larvae were not found, were recorded as "worm not found".

**RNA sequencing and analysis**

Synchronized *daf-2* and *daf-2; daf-42* embryos were obtained by placing 20 adult worms each on six NGM plates with OP50 for 2 hours at 15°C and then incubated at 25°C for dauer induction. At 52 HAE and 60 HAE, the worms were harvested with M9 and washed with distilled water five times. The, 250 µL of TRIzol was added to 50 µL of washed worm harvest, and the worm samples were freeze-thawed 10–15 times for disruption. Harvest *daf-2; daf-42* at 60 HAE included both live and dead L2d worms. RNA was extracted through chloroform—isopropanol precipitation. To minimize variation, each set of *daf-2* and *daf-2; daf-42* at 52 and 60 HAE was grown, harvested and disrupted simultaneously. Triplicates of each set were made.

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Theragen Bio Inc. (South Korea) prepared a library with TruSeq Stranded mRNA Sample Prep Kit and RNA sequencing on the Illumina NovaSeq 6000 150-bp paired-end platform. A total of 63–90 million reads were obtained from each sample. The RNA-seq data were analyzed using Kallisto and Sleuth (BRAY *et al.* 2016; PIMENTEL *et al.* 2017).

**Protein alignment**

Protein sequences from 202 bioprojects of 163 nematodes and platyhelminthes were obtained from WormBase Parasite 16 (https://parasite.wormbase.org/ftp.html) (HOWE *et al.* 2015; HOWE *et al.* 2017) and aligned with the DAF-42 m protein sequence using DIAMOND in the ultra-sensitive mode (BUCHFINK *et al.* 2021). For every species, one protein with the highest bit score was selected as the *daf-42* homolog.

To analyze if a homolog has two sites of alignment at the N- and C-terminus, two overlapping fragments of DAF-42 m (1–1500 aa and 1001–2402 amino acid residues) were aligned to protein sequence of *daf-42* homologs in *Caenorhabditis* species, under same procedure with DIAMOND.

Protein dot plot was generated using EMBOSS Dotmatcher (https://www.ebi.ac.uk/Tools/seqstats/emboss_dotmatcher/), under window size 10, threshold 23 and matrix BLOSUM62 (MADEIRA *et al.* 2022). Protein sequences of *C. elegans* DAF-42 m and its best-matching homologs in *Caenorhabditis* species were used as input.

**Results**

**Identification of a mutant with stage-specific lethal phenotype during dauer entry**

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In harsh environments, young *C. elegans* L1 larvae choose to develop into pre-dauer L2d and then arrest into dauer stage until conditions become favorable for reproduction (Fig. 1a). Interestingly, during a mutant screen for dauer-stage behavior (LEE *et al.* 2017a), we discovered that the mutant strain RB2489 developed normally into reproducing adults but failed to develop into the dauer stage and showed a "no viable dauer" phenotype. Larvae grown in a dauer-forming condition (high concentration of dauer pheromone, high temperature and little food) developed into corpses (Supplementary Fig. 1, a and b). These dead worms did not resume growth after transfer into the adult-inducing normal condition, even after one or two days. The dead worms were immobile and trapped in their own cuticles in a straight or slightly curved posture, unlike the S-shaped wavy posture of live worms. This completely penetrant, stage-specific lethal phenotype was unlike the dauer-constitutive (Daf-c) and dauer-defective (Daf-d) phenotypes of known mutants of dauer formation (*daf)* genes, which control the developmental decisions between diapause and reproductive growth; *daf-c* and *daf-d* mutant larvae develop into dauer arrest in absence of pheromones and into reproductive stages even in the presence of pheromones, respectively (RIDDLE *et al.* 1981; FIELENBACH AND ANTEBI 2008). Outcrossing with wild-type N2 revealed that the phenotype was independent of the *ins-15(ok3444)* mutation of strain RB2459 (CONSORTIUM 2012). Therefore, we named the causative gene and mutation *daf-42(ys54)*.

The *daf-42(ys54)* mutation also caused the same phenotype in the *daf-2(e1370)* mutant background (Fig. 1, b and c), a temperature-sensitive mutant that constitutively develops into the dauer stage in high temperature (25°C) even in the presence of food and absence of dauer pheromones (GOTTLIEB AND RUVKUN 1994). This indicates that in addition to changes in rearing conditions, the "no viable dauer" phenotype can be elicited by genetic manipulations that induce

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development into the dauer stage. We continued to study *daf-42* in the *daf-2(e1370)* mutant background as it was easier to induce dauer stage and the induced dauer larvae were relatively more uniform in size and development than the larvae with the wild-type background.

To investigate the mutant phenotype, we collected synchronized embryos and observed their development at 25 °C and various time points. *daf-2* control worms developed into the dauer stage after 60 hours after eggs were laid (HAE), and approximately one-half of the population developed into dauer by 72 HAE. The *daf-2; daf-42* mutant worms started developing the defective phenotype at 54 HAE, and nearly 80% of the population developed the phenotype by 72 HAE, which correspond to the end of the L2d stage (Fig. 1, d and e, Supplementary Fig. 2, a and b). Overall, the developmental defect phenotype of *daf-2; daf-42* mutant larvae appears 8–12 hours prior to the completion of dauer development in control *daf-2* worms.

The *daf-2; daf-42* mutant was no different from the *daf-2* control at 48 HAE; however, at 72 HAE and 96 HAE, *daf-2; daf-42* mutant worms had developmental defects, such as degraded pharyngeal muscle and shortened head (Fig. 1f and Supplementary Fig 2, c-f). Although the worms were alive and could move their heads within the cuticle at this point, they were unable to molt out of the old cuticle and develop into the dauer stage afterwards. Electron microscopy analysis of worm cross sections revealed that at 72 HAE, *daf-2; daf-42* mutant larvae had damage not only around the head, but also at the mid-body and tissues were loose, unlike the packed organization in *daf-2* (Supplementary Fig. 3). At 96 HAE, the *daf-2* control worm fully developed into dauer stage, showing dauer cuticle, alae and constricted body size (WOLKOW AND HALL 2011); however, the *daf-2; daf-42* mutant had damaged head region, ill-developed body and has not escaped the L2d cuticle (Fig. 1g, Supplementary Fig. 3). Moreover, in *daf-2; daf-42* mutant, dauer alae were not formed, and the striated layer in the dauer cuticle terminated at the

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seam cell area. These results indicate that *daf-42(ys54)* mutation causes various developmental defects during the L2d-to-dauer transition and that *daf-42* is essential gene during L2d for development into the dauer stage.

To identify when the developmental defect phenotype occurs, we examined the development of individual worms during the L2d-to-dauer transition. In *C. elegans*, molting can be separated into the lethargus phase and ecdysis. In the lethargus phase, worms slow down physical movement and become lethargic. In ecdysis, the worms resume physical movement and remove their old cuticle (LAZETIC AND FAY 2017). This pattern was visible in *daf-2* control worms, which showed 4–12 hours of lethargus period followed by ecdysis and maturation into the dauer stage (Fig. 1h, top, Supplementary Fig. 4). During ecdysis, we could see a thinner worm inside the L2d cuticle, which indicates that the worm went through radial body constriction at this point (Supplementary Fig. 4c). In contrast, *daf-2; daf-42* mutant worms do enter the lethargus phase but does not show radial constriction and ecdysis. Instead, the larvae develop the developmental defect phenotype (Fig. 1h, bottom, Supplementary Fig. 4g). This indicates that the *daf-42* mutant enters the L2d-to-dauer transition but is unable to develop into the dauer stage. Together, these results show that the *daf-42* mutant shows a developmental defect phenotype specifically at the L2d-to-dauer transition, which leads to lethality under dauer-forming conditions.

**_daf-42_ acts in dauer development after developmental commitment to diapause**

Various genetic pathways regulate dauer formation. We have previously shown that *daf-2; daf-42* double mutant worms exhibit a “no viable dauer” phenotype. Moreover, we analyzed the *daf-42* mutant phenotype in the context of *daf-7* and *daf-9*, which are upstream regulators of tgf-

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ß and nuclear hormone signaling pathways, respectively (REN *et al.* 1996; GERISCH AND ANTEBI 2004). *daf-7(ok3125)* is a temperature-sensitive daf-c mutant that constitutively develops into a dauer at 25°C. We found that the *daf-7(ok3125); daf-42(ys54)* double mutant exhibits the lethal phenotype at 25°C (Fig. 2, a and b). *daf-9(m540)* causes dauer formation unconditionally, followed by dauer exit and growth into reproductive adults after 1–2 days (JIA *et al.* 2002). We were unable to secure a *daf-42(ys54); daf-9(m540)* double mutant strain because worms committed to forming dauer became lethal (Fig. 2c). Further examination of 1,210 offspring from a *daf-42(ys54); daf-9(+/m540)* heterozygote mutant revealed 299 worms with the lethal phenotype, which follows the Mendelian inheritance and confirms that the *daf-42(ys54); daf-9(m540)* double mutant also exhibits the "no viable dauer" phenotype. Our results indicate that *daf-42* functions after the decision process, which is regulated by dauer formation genes *daf-2*, *daf-7* and *daf-9*, and suggest that *daf-42* is a distinct type of daf gene that functions in dauer development during the L2d-to-dauer transition after the decision to enter the dauer stage has been made.

The decision to develop into pre-dauer L2d is made at L1 if the worm is in unfavorable condition. In other words, exposure to favorable conditions before late L1 can still induce diapause, on the condition that the larva is in an unfavorable condition by late L1 (CASSADA AND RUSSELL 1975; GOLDEN AND RIDDLE 1984; SCHAEDEL *et al.* 2012). To investigate whether the lethal phenotype is affected by the experience of favorable conditions, we performed a shift-to-unfavorable assay by shifting the growth temperature of synchronized embryos from 15°C to 25°C progressively (Fig. 2d). *daf-2* control worms transferred to 25°C after 54-72 HAE started to be unresponsive to 25°C and developed into reproductive stages, indicating developmental decision at late L1 (Fig. 2, e and f, black bars).

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We hypothesized that if the "no viable dauer" phenotype occurs specifically the L2d-to-dauer transition after the decision into diapause, then the proportion of dauer worms in *daf-2* will be close to that of dead L2d worms in *daf-2; daf-42*. Similar to our hypothesis, the proportion of larvae entering reproductive fate and diapause fate in *daf-2; daf-42* matched that of *daf-2*, except that *daf-2; daf-42* mutants became dead L2d worms instead of dauer worms (Fig. 2, e and f, red bars). This shows that the dead L2d phenotype is unaffected by exposure to favorable environment before late L1 and suggests that *daf-42* facilitates physiological development that starts after the developmental decision to enter dauer during L2d.

**_daf-42_ is a previously uninvestigated gene that encodes large, disordered proteins**

To identify the causative mutation, we employed mapping-by-sequencing—a method that combines single nucleotide polymorphism (SNP) mapping with whole-genome sequencing (DOITSIDOU *et al.* 2010; DOITSIDOU *et al.* 2016). Mapping-by-sequencing followed by extensive outcrossing revealed a nonsense mutation in exon 1 of ORF Y40C5A.3, located in the middle of chromosome IV, as *daf-42(ys54)* (Fig. 3a, Supplementary Fig. 5, a and b, and Supplementary Table 3). Null mutations introduced into exon 1 of *daf-42* using CRISPR/Cas9, *ys55* and *ys58*, reproduced the lethal phenotype of *daf-42(ys54)*, suggesting that the disruption *daf-42* is responsible for the "no viable dauer" phenotype (Fig. 3, a–c). This was further confirmed by the complete rescue of the lethal phenotype in transgenic *daf-2; daf-42* mutants carrying a fosmid from the *C. elegans* genomic DNA library or a newly constructed plasmid containing the wild-type allele of this gene (Fig. 3, d and e). Thus, ORF Y40C5A.3 is *daf-42*.

To our knowledge, *daf-42* has not been investigated to date. *daf-42* encodes large proteins, ranging from 1,101 to 2,402 amino acid residues across 17 isoforms (Supplementary

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Fig. 5c) (HARRIS *et al.* 2020). The largest isoform DAF-42 m contains all exons and is the 269th-largest protein among 26,548 proteins in the *C. elegans* proteome (OKIMOTO *et al.* 1992; CONSORTIUM 1998). Structural analyses, including the analysis by AlphaFold 2, indicated that DAF-42 m consists primarily of unstructured regions (Supplementary Fig. 5d) (NECCI *et al.* 2017; JUMPER *et al.* 2021; PIOVESAN *et al.* 2021). The first 15 amino acid residues were predicted to be a signal peptide, suggesting that the protein is not likely cytosolic (Supplementary Fig. 5d). To predict the functions of *daf-42,* we searched for large proteins that are predicted to be disordered and have a signal peptide. Interestingly, only seven proteins in *C. elegans* had these characteristics, four of which are incorporated into the cuticle or the extracellular matrix (Supplementary Table 4). This implies that *daf-42* may also play similar structural roles in these tissues.

**_daf-42_ encodes secreted proteins that are essential during a narrow time window during dauer entry**

To identify the role of *daf-42* in dauer development, we examined the site of *daf-42* expression. Interestingly, studies on the *C. elegans* transcriptome suggested that *daf-42* is specifically expressed during dauer entry and is not expressed at other developmental stages (Fig. 4a) (LEE *et al.* 2017b; HARRIS *et al.* 2020). Consistently, transgenic worms expressing mCherry under the *daf-42* promoter showed expression in seam cells between 48 and 60 HAE (Fig. 4b), which corresponds to the time of appearance of the dead L2d phenotype (Fig. 1d). Seam cells are specialized syncytial hypodermic cells along the length of the animal that synthesize cuticle components, such as collagen, and are important for epidermal elongation and molting (SINGH AND SULSTON 1978; THEIN *et al.* 2003). The expression of wild-type *daf-42* in seam cells

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partially rescued the phenotype of the *daf-2; daf-42* mutant, indicating that expression of *daf-42* in seam cells is essential for dauer development (Fig. 4c, Supplementary Fig. 6). The partial rescue of the phenotype using a constitutive seam cell promoter may suggest that expression in a narrow time window is important.

We generated transgenic worms carrying the putative signal peptide fused to mCherry under the *daf-42* promoter (*daf-42p::signal peptide::mCherry*) to investigate the functionality of the predicted signal peptide (Supplementary Fig. 7a). Addition of a signal peptide to the N-terminus of mCherry drove its expression at the periphery of the worm body at 60 HAE, in stark contrast to the previous reporter assay without the signal peptide (Fig. 4d, Supplementary Fig. 7b). The mCherry signal was slightly visible in coelomocytes in some worms at 48 HAE, was more visible in the worm periphery at 60 HAE, and formed puncta through the worm body at 72 HAE as the worms developed into the dauer stage. Analysis of mCherry expression every 4 hours between 48 and 72 HAE revealed that in all cases, smooth expression in the periphery preceded puncta formation (Fig. 4e, Supplementary Fig. 7c). These results suggest that DAF-42 is secreted from seam cells to peripheral body at late L2d before before entering the dauer stage.

Interestingly, the time of expression of *daf-42p::signal peptide::mCherry* coincided with the appearance of the developmental defect in *daf-2; daf-42* mutants. For example, *daf-2; daf-42* mutant larvae started to exhibit the phenotype at 54 HAE (Fig. 1d); similarly, transgenic *daf-2* worms started to express mCherry as early as 52 HAE (Fig. 4e). 31.5% of transgenic *daf-42* worms had *daf-42* promoter activity at 56 HAE, and a similar percentage of *daf-2; daf-42* mutant worms had the developmental defect phenotype at 58 and 60 HAE (Fig. 1e, Fig. 4e). Thus, the absence of *daf-42* in a narrow time window during dauer entry results in developmental defects and suggests that *daf-42* plays a crucial role in dauer development shortly after its expression.

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We analyzed localization of DAF-42 protein using a translational reporter line expressing *daf-42* fused to gfp (*daf-42::gfp)*. At 60 HAE, we observed expression in the seam cell, hypodermis and at the surface of the worm (Fig. 4f). At 72 HAE, ecdysis had begun, some worms had detached their head or tail from the old L2d cuticle (Fig. 4g, arrowheads), and the DAF-42::GFP signal did not remain in the L2d cuticle but was localized with the dauer worm inside the L2d cuticle (Fig. 4g). Together, our results show that *daf-42* is expressed during the L2d-to-dauer transition in seam cells and is secreted towards the surface of the dauer worm, such as the hypodermis or cuticle.

**Absence of *daf-42* interferes with transcriptomic changes during dauer entry**

To examine the transcriptional changes accompanied by the absence of DAF-42 during dauer entry, we performed RNA-sequencing on *daf-2* and *daf-2; daf-42* worms at 52 and 60 HAE, the time at which 0% and ~40% of *daf-2; daf-42* mutants form dead L2d at 25°C, respectively (Fig. 1e). Although the use of the *daf-2* background have unknown effects on the transcriptome, we sought to focus on the difference between the control and mutant strains that is elicited by the presence or absence of *daf-42* in the common genetic background of the *daf-2* mutation. Principal component analysis (PCA) showed that while the transcriptome of the *daf-2; daf-42* mutant is similar to that of the *daf-2* control at 52 HAE, the shift of transcriptome made by the *daf-2* control at 60 HAE has not been completely made by the *daf-2; daf-42* mutant (Fig. 5a). Expression of *daf-42* is reduced in the *daf-2; daf-42* double mutant at both 52 and 60 HAE, likely due to nonsense-mediated decay (Fig. 5b, Supplementary Fig. 8a). The difference in transcriptomes at 60 HAE suggests that the absence of *daf-42* hardly affects the transcriptome at 52 HAE, but significantly affects transcriptional changes that occur over the next 8 hours. To

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429 determine the status of dauer commitment in *daf*-2 worms at 52 and 60 HAE, we examined
430 genes that can be used to mark dauer commitment within the L2d stage (SHIH *et al.* 2019). We
431 found that the expression of *col-183*, which is highly expressed in L2d after dauer commitment,
432 is greatly increased in *daf-2* control larvae at 60 HAE compared to those at 52 HAE (Fig. 5c,
433 Supplementary Fig. 8b). Expression of *col-183* is not altered in *daf-2; daf-42* mutant larvae,
434 implying that dauer commitment is likely to remain intact in the *daf-2; daf-42* mutant and that
435 the action of *daf-42* follows after dauer commitment in L2d.
436 How did the absence of *daf-42* affect the transcriptome? To answer this question, we
437 identified and analyzed differentially expressed genes (DEGs) at 60 HAE. At 60 HAE, 992 genes
438 were significantly upregulated (>2-fold), and 662 genes were significantly downregulated (< 0.5-
439 fold) in the *daf-2; daf-42* mutant compared with the *daf-2* control (Fig. 8d, Supplementary Table
440 5). Phenotype enrichment analysis of the DEGs between *daf-2* and *daf-2; daf-42* at 60 HAE
441 highlighted that the absence of *daf*-42 significantly alters the expression of genes that affect
442 larval physiology and dauer metabolism (Fig. 5e). While the transcriptome of live L2d worms
443 and dead L2d worms of *daf-2; daf-42* mutant may be different, the gene ontology enrichment
444 analysis indicated upregulation of genes related to the immune system and defense response (Fig.
445 5f), which likely reflects the consequence of developmental disruption caused by the *daf-42*
446 mutation.
447
448 **_daf-42_ is an evolutionarily young and essential gene**
449 The results so far indicate that *daf-42* is an essential gene for the development of dauers in *C.*
450 *elegans*, which is considered to be a stage critical for its survival in the wild. We speculated that
451 *daf-42* would be well-conserved across diverse species and sought to find homologs of *daf-42*.

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Contrary to our expectations, a search for DAF-42 homologs on NCBI BlastP revealed no homologs outside nematodes. We searched for DAF-42 homologs in the protein sequences of the 163 nematode species listed in Wormbase Parasite 16 (PARKINSON *et al.* 2004; HOWE *et al.* 2015; HOWE *et al.* 2017; BUCHFINK *et al.* 2021). Strikingly, highly conserved homologs of DAF-42 m, with bit scores above 100, were found only in *Caenorhabditis* species (Fig. 6a, Supplementary Table 6). This implies that despite its essential role in dauer development, *daf-42* is an evolutionarily young, genus-specific gene.

Alignment results to *C. elegans* DAF-42 m protein sequence indicated large changes in DAF-42 sequences within *Caenorhabditis* species (Supplementary Table 7). Not only did the four outgroup species have only short conserved regions, but also the *C. brenneri* homolog lacked a region that corresponds to the first half of *C. elegans* DAF-42 m. A comparison of the dot plot between *daf-42* homologs of *C. briggsae* and *C. inopinata* with that of *C. elegans* indicated that the regions in the middle do not align with each other (Supplementary Fig. 9). To confirm this, we divided the DAF-42m sequence into two overlapping halves—regions containing 1–1,500 and 1001–2,402 amino acid residues—and performed protein alignment. We were able to produce similar results (Fig. 6B, Supplementary Table 7). This is particularly interesting because *C. inopinata* phylogenetically closest to *C. elegans* (KANZAKI *et al.* 2018). Protein dot plots showed that compared to other dauer development-related genes, such as *daf-2* or *daf-7*, *daf-42* underwent more dynamic changes within the genus (Supplemental Fig. 9). In addition, the analysis of protein sequences of *daf-42* homologs in *Caenorhabditis species* for signal peptide also indicated that 6 out of 20 species have lost signal peptide (Fig. 6b). Although all homologs are predicted to contain large proportions of disordered regions, their distributions vary (Supplementary Fig.10, a and b). Despite these changes, amino acid compositions between

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these homologs remain similar (Supplementary Fig. 10c). These results indicate that while *daf-42* is an essential gene for dauer development and is conserved only in the *Caenorhabditis* genus, the gene underwent significant changes even within the genus.

## Discussion

In this study, we have newly identified a genus-specific gene that has an essential role in dauer development in *C. elegans*. We found that *daf-42* encodes large, unstructured proteins that are secreted from hypodermal cells in late L2d during the molt into the dauer stage. Notably, although *daf-42* is essential during dauer development—a feature conserved across nematode species—phylogenetic analysis revealed that the *daf-42* is mainly conserved only within the genus *Caenorhabditis*, implicating that *daf-42* is a recently evolved gene that plays an essential role in the survival of the species.

Studies on dauer formation have identified over 30 daf genes that regulate the decision between diapause and reproductive development in late L1 and L2d stages. The environmental and nutritional conditions surrounding the larvae are conveyed by signaling pathways, including *daf-2*/insulin-like, cyclic GMP and *daf-7*/TGF-beta pathways. These pathways converge on steroid hormone signaling mediated by the nuclear hormone receptor *daf-12* and dafachronic acid (THOMAS *et al.* 1993; GOTTLIEB AND RUVKUN 1994; ANTEBI *et al.* 2000; FIELENBACH AND ANTEBI 2008). Although *daf* mutants are known to exhibit daf-c and daf-d phenotypes (FIELENBACH AND ANTEBI 2008), the *daf-42* mutant displays a completely penetrant lethal phenotype in worms that develop into the dauer stage. As *daf-42* mutation does not affect the developmental decisions of *daf-2*, *daf-7* and *daf-9* mutants (Fig. 1c and Fig. 2, a-c), we hypothesize that *daf-42* belongs to a different category of daf gene that is involved in

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498 physiological changes downstream of developmental decisions. The genetic components that
499 mediate the developmental process after the binary decision are unknown, and *daf-42* provides
500 an entry point for further research in this area.

501 Although the detrimental effect of the *daf-42* mutation in dauer development is clear, the
502 molecular function of DAF-42 protein is unclear. Our results indicate that DAF-42 is a large,
503 disordered protein expressed at late L2d in the seam cells and is secreted towards the surface of
504 the worm. Intrinsically disordered regions are flexible parts of a protein that may provide binding
505 locations and are known to function in various ways, from structural roles to roles in cellular
506 signaling (PEYSSELON *et al.* 2011; VAN DER LEE *et al.* 2014; WRIGHT AND DYSON 2015).
507 Hypodermal cells, including seam cells, express and secrete various proteins to promote proper
508 cuticle formation and molting. Although the cuticle and proteases are part of the secreted
509 proteins, DAF-42 is expected to be neither because of its enormous size and lack of homology
510 (FRAND *et al.* 2005; CHISHOLM AND XU 2012). A previous study reported that the absence of
511 seam cells hinders body contraction during the dauer stage (SINGH AND SULSTON 1978), which
512 may be relevant in the lack of body contraction and dauer alae in dead L2d *daf-42* mutants. In
513 this context, DAF-42 may be a component of the extracellular matrix or play a signaling role
514 specialized for the dauer stage, that function alone or as a scaffold with its binding partners,
515 which are yet to be identified.

516 The dauer stage in free-living nematodes is analogous to iL3 in parasitic nematodes in
517 that both are third larval stages with altered physiology and are developmentally arrested until
518 they find a suitable environment to resume development and reproduce (HOTEZ *et al.* 1993; HU
519 2007; CROOK 2014). These stages are a crucial part of the life cycle; *C. elegans* is found in the
520 dauer stage in the wild, where resources are often scarce (FREZAL AND FELIX 2015), and iL3

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stage plays a special role in host invasion in many parasitic nemaotdes. Studies have reported that *daf-12*, a nuclear receptor that binds dafachronic acid to regulate the decision between dauer and reproductive development programs in *C. elegans* (ANTEBI *et al.* 2000), is conserved in other nematode species. This function is suspected to be evolutionarily conserved, as the loss of *daf-12* impairs dauer and iL3 development in nematodes in Clade V and IV (OGAWA *et al.* 2009; DULOVIC AND STREIT 2019). *daf-12* homologs in several clade IV and clade III parasitic nematodes were activated by dafachronic acid and other steroid derivatives that affect iL3 development in these species (WANG *et al.* 2009; AYOADE *et al.* 2020; LONG *et al.* 2020). By contrast, *daf-42* is different from previous studies in that the gene is conserved only in the genus *Caenorhabditis*, although we may have missed good-matching homolog in other species due to poor quality of protein database. We speculate that nematode species utilize conserved mechanisms involving steroid hormone and *daf-12* in the decision process between diapause and reproductive development, but the mechanisms that mediate physiological changes during the transition into the dauer or iL3 stage may have diverged across various nematode species. A recent study showed that the transcriptome of dauer stage and hypodermis includes more evolutionarily young genes than those of other stages and other tissues in *C. elegans*, respectively (MA AND ZHENG 2023). These results indicate that that dauer stage and hypodermis may have been subjected to evolutionary innovation. In this context, *daf-42* may be a part of this innovation that allowed *Caenorhabditis* species to invent the unique features of their dauer stages.

In contrast to the conventional notion that essential genes are evolutionarily old and conserved, studies in the last decade have revealed that young genes also play vital biological roles in diverse species. In *Drosophila*, silencing of young genes that arose in a subgroup of the

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544 genus causes critical phenotypes, such as failure in pupal development and sterility (CHEN *et al.*
545 2010; DING *et al.* 2010; LONG *et al.* 2013). In *Caenorhabditis*, telomere-binding protein genes
546 *tebp*, which is a genus-specific gene enriched mostly in the Elegans subgroup, caused sterility
547 when mutated (DIETZ *et al.* 2021). Human-specific genes that arose by recent duplication are
548 involved in brain development (CHARRIER *et al.* 2012). Our findings add another example of
549 young and essential genes.

550 How did *daf-42* become so important in dauer development? A recent study in
551 *Drosophila* shows that new genes may gain essential function by interacting with other essential
552 genes in the developmental process (LEE *et al.* 2019). Similarly, *daf-42* may be controlled by or
553 bind to more conserved factors to regulate dauer development. Although we are yet to
554 understand the evolutionary origin and role of *daf-42*, we speculate that dauer development in
555 nematodes may be another example of this phenomenon, as *daf-42* is an evolutionarily young
556 gene that plays an essential role in dauer development.

557

558 **Data Availability Statement**

559 Strains and plasmids are available upon request. RNA-sequencing data of *daf-2(e1370)* and *daf-*
560 *2(e1370); daf-42(ys54)* are submitted to NCBI GEO and are available under accession number
561 GSE230353.

562

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**Conflicts of Interest**

The authors have no conflict of interests.

**Author Contributions**

N.K. discovered the "no viable dauer" phenotype. D.S.L., J.K. and J.L. designed the experiments. D.S.L., J.K. and W.K. performed the experiments. S.-H.L. performed electron microscopy. D.S.L., J.K. and D.L. analyzed the data. D.S.L. and J.L. wrote the manuscript.

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## Figure Legends

**Figure 1. The *daf-42(ys54)* mutant displays a lethal phenotype uner dauer-inducing conditions.**

(a) Life cycle of *Caenorhabditis elegans*. Young larvae in harsh environments develop into L2d and then to dauer larvae, instead of quick growth into reproductive adults in favorable environments.

(b) *daf-2(e1370)* worms develop into reproductive adults at 15°C (left) and into dauer larvae at 25°C (right).

(c) *daf-2(e1370); daf-42(ys54)* worms develop into adults at 15°C (left) but fail to develop into dauer stage at 25°C (right).

(d, e) Percent of worms that develop into dauer stage in *daf-2* worms (black) and that fail to develop into the dauer stage in *daf-2; daf-42(ys54)* worms (red) at 25°C during 48 to 144 hours after the eggs were laid (HAE) (d) and 48 to 60 HAE (e). n > 350 for (d) and > 560 for (e) in three trials. Data show mean ± SEM.

(f) Transgenic *daf-2(e1370)* worms (left) and *daf-2(e1370); daf-42(ys54)* worms (right above, right below) expressing gfp in the pharyngeal muscle (*myo-2p::gfp*) when grown at 25°C for 72 HAE.

(g) Electron microscopy images of cross sections of *daf-2(e1370)* worms (left) and *daf-2(e1370);daf-42(ys54)* worms (right) in the mid-body when grown at 25°C for 96 HAE. White arrows indicate positions of dauer alae, which has formed in *daf-2(e1370)* control (left), but not in *daf-2(e1370); daf-42(ys54)* mutant (right).

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(h) Development of *daf-2(e1370)* (above) and *daf-2(e1370); daf-2(ys54)* (below) at 25°C from 48 to 72 HAE. Each row represents a single worm. After the lethargus period (light green), *daf-2(e1370)* larvae undergo radial constriction of the body and ecdysis (dark green) followed by complete maturation into the dauer stage (black). However, *daf-2(e1370); daf-42(ys54)* larvae show developmental defects (red) after the lethargus period. n = 60 in three trials

**Figure 2. *daf-42* functions downstream of dauer commitment**

(a) Representative images of the development of *daf-7(ok3125)* into dauer stage (above) and *daf-7(ok3125); daf-42(ys54)* into dead L2d (below) at 25°C at 96 HAE.

(b) The *daf-7(ok3125)* mutant develops into the dauer stage at 25°C, whereas a similar percentage of the *daf-7(ok3125); daf-42(ys54)* double mutant develops into dead L2d in the same condition. Data show mean ± SEM. n > 118 in three trials.

(c) Representative images of the development of *daf-9(m540)* into the dauer stage (above) and *daf-42(ys54); daf-9(m540)* into dead L2d (below) at 25°C at 96 HAE.

(d) Schematic representation of the temperature-shift assay. Eggs were collected for 2 hours and grown at 15°C until they were transferred to 25°C at certain time points (shift time). The results on how the worms grew were taken at 120 HAE.

(e, f) Temperature shift assays from L4-inducing condition (15°C) to dauer-inducing condition (25°C) for *daf-2(e1370)* and *daf-2(e1370); daf-42(ys54)* with shift times every 24 hours between 2 HAE and 120 HAE (e) and every 6 hours between 48 and 72 HAE (f). Similar percentages of dauer development in *daf-2* and dead L2d formation in *daf-2; daf-42* show that the lethal phenotype at dauer entry caused by the *daf-42* mutation is downstream of commitment into the

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789 dauer stage development. (+) indicates wild-type allele and (-) indicates mutant allele. Data show
790 mean ± SEM. n > 500 for (e) and > 130 for (f) in three trials.
791
792 **Figure 3. *ys54* is a nonsense mutation in *daf-42*—a previously uninvestigated gene in the**
793 **middle of Chromosome IV.**
794 (a) Gene structure of *daf-42 m*, the isoform of *daf-42* that contains all exons. Black blocks
795 indicate exons, and black lines indicate introns. Mutant alleles *ys54, ys55, ys58*, which are on
796 exon 1, are indicated below.
797 (b) Representative microscopy images of dauer development in *daf-2(e1370)* (left above), *daf-*
798 *2(e1370); daf-42(ys54)* (right above), *daf-2(e1370); daf-42(ys55)* (left below) and *daf-2(e1370);*
799 *daf-42(ys58)* (right below).
800 (c) Development of *daf-2(e1370)* control worms and *daf-2(e1370); daf-42* mutant worms at
801 25°C. *daf-2* develops into the dauer stage; however, all three null alleles of *daf-42* (*ys54*, *ys55*
802 and *ys58*) cause development into dead L2d larvae. Data show mean ± SEM. n > 70 in three
803 trials.
804 (d, e) "No viable dauer" phenotype of *daf-2; daf-42(ys54)* larvae is rescued by transgenic fosmid
805 WRM0626cE11 (d) and plasmid (e) that contain the gene *daf-42*. Representative pictures of the
806 worm with rescued phenotype are shown in the picture below the graph. (+) indicates presence
807 and (-) indicates the absence of each transgene. Data show mean ± SEM. n > 68 in three trials.
808

809 **Figure 4. DAF-42 is secreted from hypodermis and acts in a narrow time window during**
810 **L2d-to-dauer transition.**

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(a) Expression level of *daf-42* in dauer-entering larvae and L4-developing larvae. *daf-42* is expressed specifically at dauer entry. Transcriptome data is from Lee et. al. (2017). Data show the mean ± SEM of the triplicates.

(b) Representative images of *daf-2* expressing *daf-42p::mCherry* at 48 HAE (left), 60 HAE (middle) and 72 HAE (right) under dauer-inducing conditions (25°C). Expression pattern of *daf-42p::mCherry* colocalize with seam cell marker *grd-10p::gfp*.

(c) Ectopic expression of wild-type *daf-42* under the seam cell promoter *grd-10p* partially rescues the dead L2d phenotype of the *daf-42(ys54)* mutant. In contrast, the expression of the same transgene in body wall muscles using the *myo-3* promoter did not rescue the lethal phenotype. (+) indicates presence and (–) indicates absence of each transgene. Data show mean ± SEM. n > 80 in three trials.

(d) Expression of mCherry under *daf-42* promoter and signal peptide (*daf-42p::signal peptide::mCherry*) in *daf-2(e1370)* background at 48 HAE (left), 60 HAE (middle) and 72 HAE (right) under dauer-inducing conditions (25°C).

(e) Quantification of *daf-42p::sp::mCherry* expression between 48 and 60 HAE. Expression occurs between 52 and 64 HAE. Each row indicates a single larva. n = 53 in 3 trials.

(f) Expression of *daf-42p::daf-42::gfp* in *daf-2(e1370)* larvae shows localization of DAF-42 in seam cells, hypodermis and worm surface at 60 HAE (f) and 72 HAE (g). White box in the left indicates the region with the enlarged image shown in the right. White arrowheads indicate places where detachment of dauer-forming worms from old L2d cuticles can be seen at 72 HAE.

**Figure 5. The absence of DAF-42 affects the expression of genes that affect dauer physiology.**

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834 (a) Principal component analysis (PCA) of the expression profile of *daf-2(e1370)* and *daf-*
835 *2(e1370); daf-42(ys54)* strains at 52 HAE and 60 HAE, using transcripts per million (TPM) as
836 the unit of gene expression.
837 (b, c) Average expression levels of *daf-42* (b) and *col-183* (c) at 52 and 60 HAE in *daf-2* control
838 and *daf-2; daf-42* mutant worms.
839 (d, e) Phenotype enrichment analysis (d) and gene ontology enrichment analysis (e) on
840 differentially expressed genes in *daf-2; daf-42* worms compared with *daf-2* worms at 60 HAE.
841 The number of genes detected in each category is on the right side of each bar, and the colored
842 vertical line in each bar represents $\log_2$-fold difference of each gene within the category from
843 *daf-2; daf-42* mutant compared with the *daf-2* control.
844
845 **Figure 6. Homologs of DAF-42 in other nematode species.**
846 (a) Heat map of bit scores of proteins best-aligned with DAF-42 m from 202 protein database of
847 163 species in WormBase ParaSite 16. Species within each clade are sorted in alphabetical order
848 of their species name.
849 (b) Diagram of best-matching DAF-42 m homologs in 21 *Caenorhabditis* species. The length of
850 each bar indicates the length of each homolog, and the region that aligns to *C. elegans* DAF-42 is
851 colored. Pink indicates regions that align to 1–1500 amino acid residues of DAF-42 m, and green
852 indicates regions that align with 1,001–2,402 amino acid residues of DAF-42 m. Region that
853 aligns continuously to most length of DAF-42m is marked in both colors. Numbers indicate bit
854 scores of the alignments between DAF-42 m and each homolog; black number indicates the bit
855 score of the alignment between the whole sequences of DAF-42m and the homolog; pink and
856 green numbers indicate the bit score of the alignment of 1–1,500 and 1000–2402 amino acid

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857 residues of DAF-42 m with the whole sequence of the homolog, respectively. Homologs

858 predicted to have signal peptides are indicated with red circles with the letter “S”.

859

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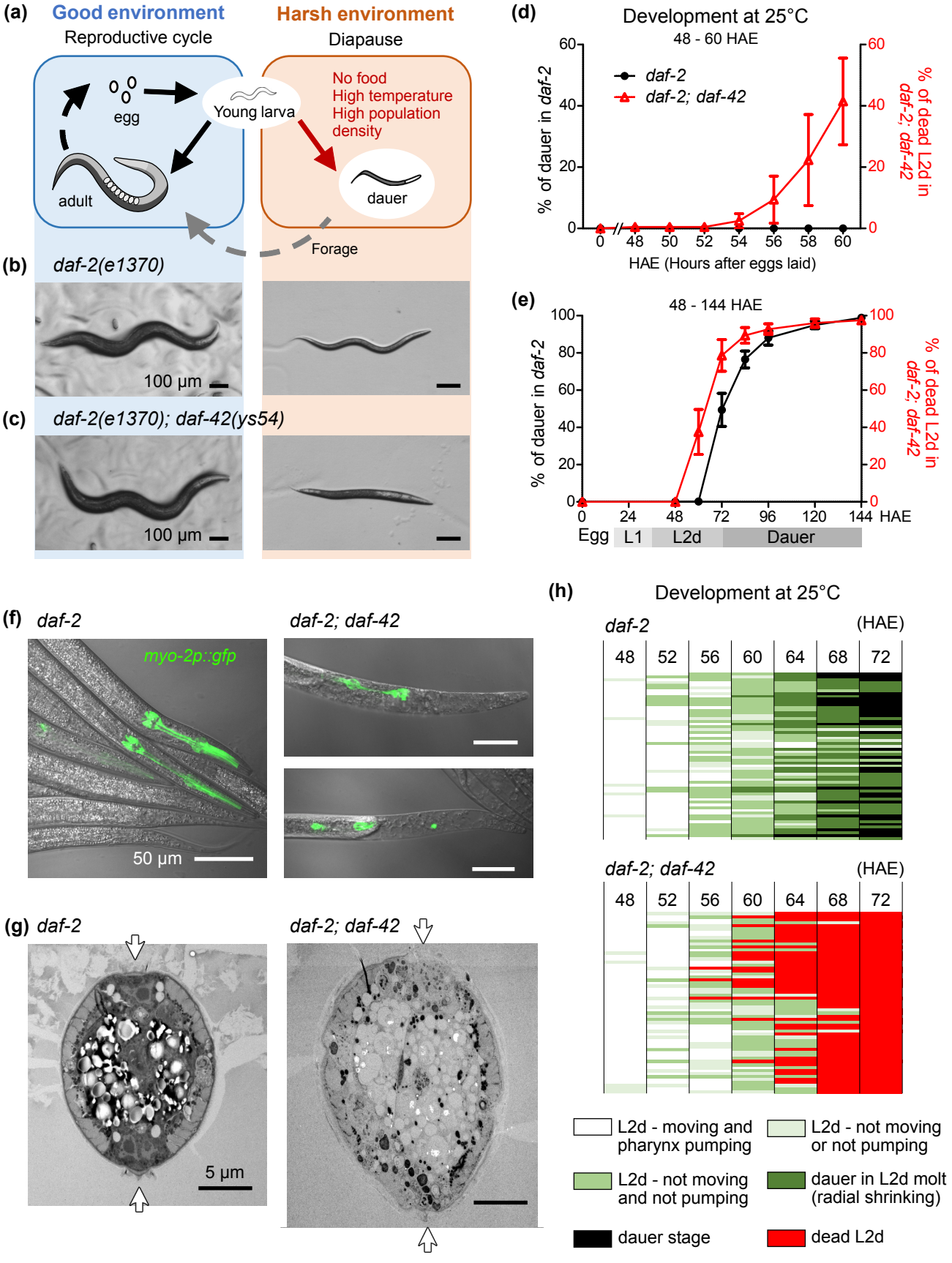

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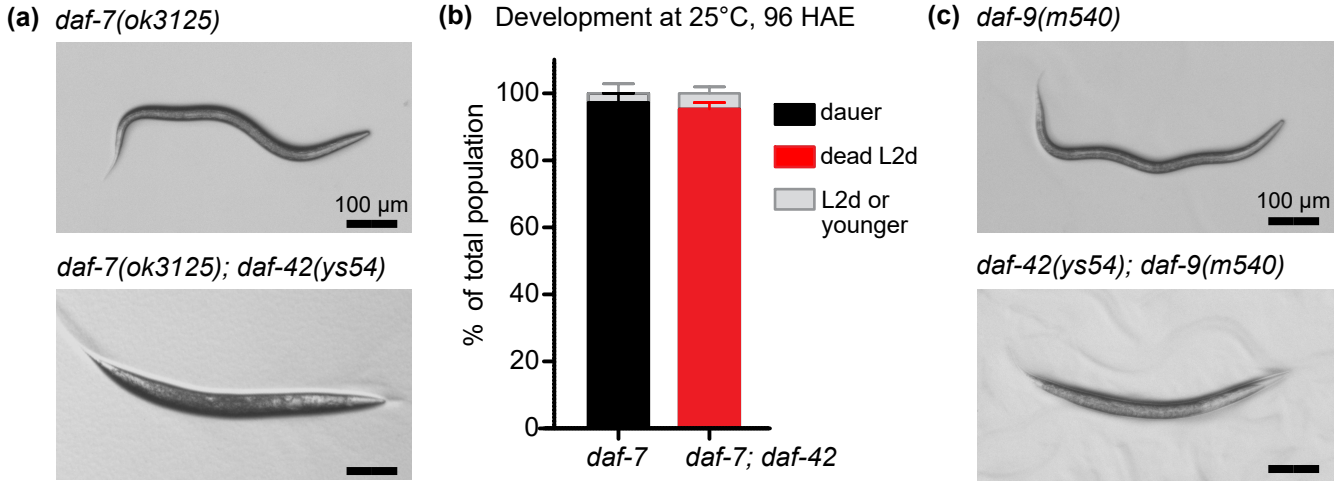


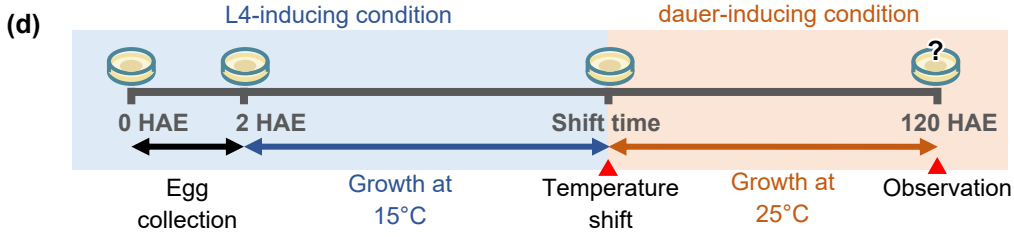


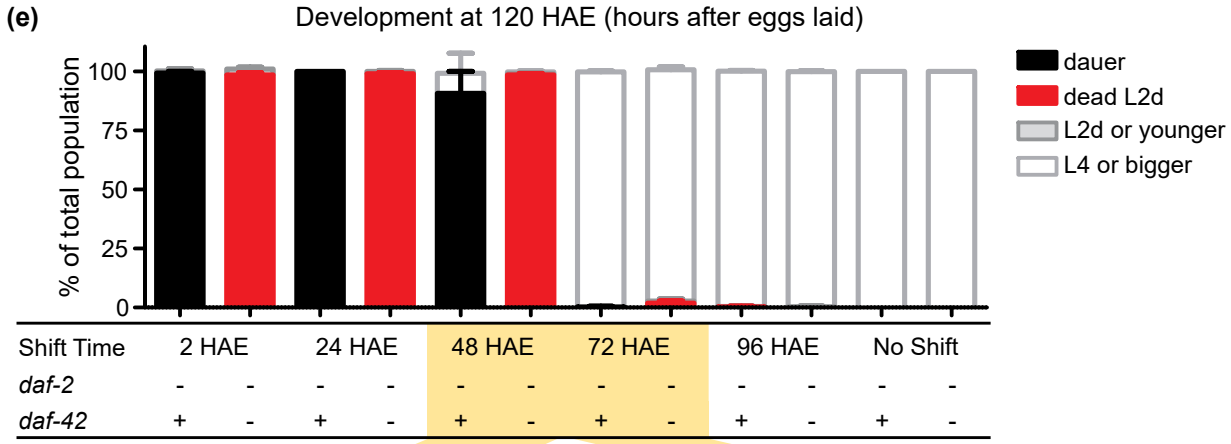


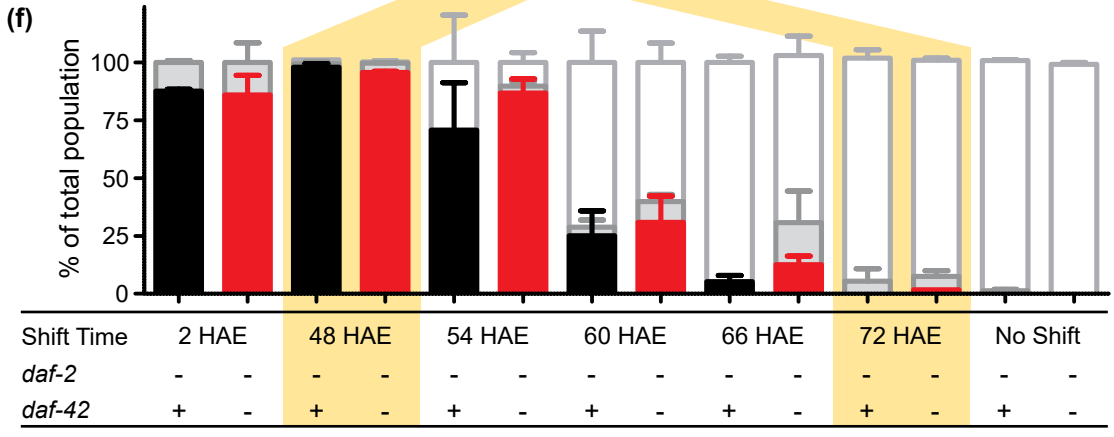

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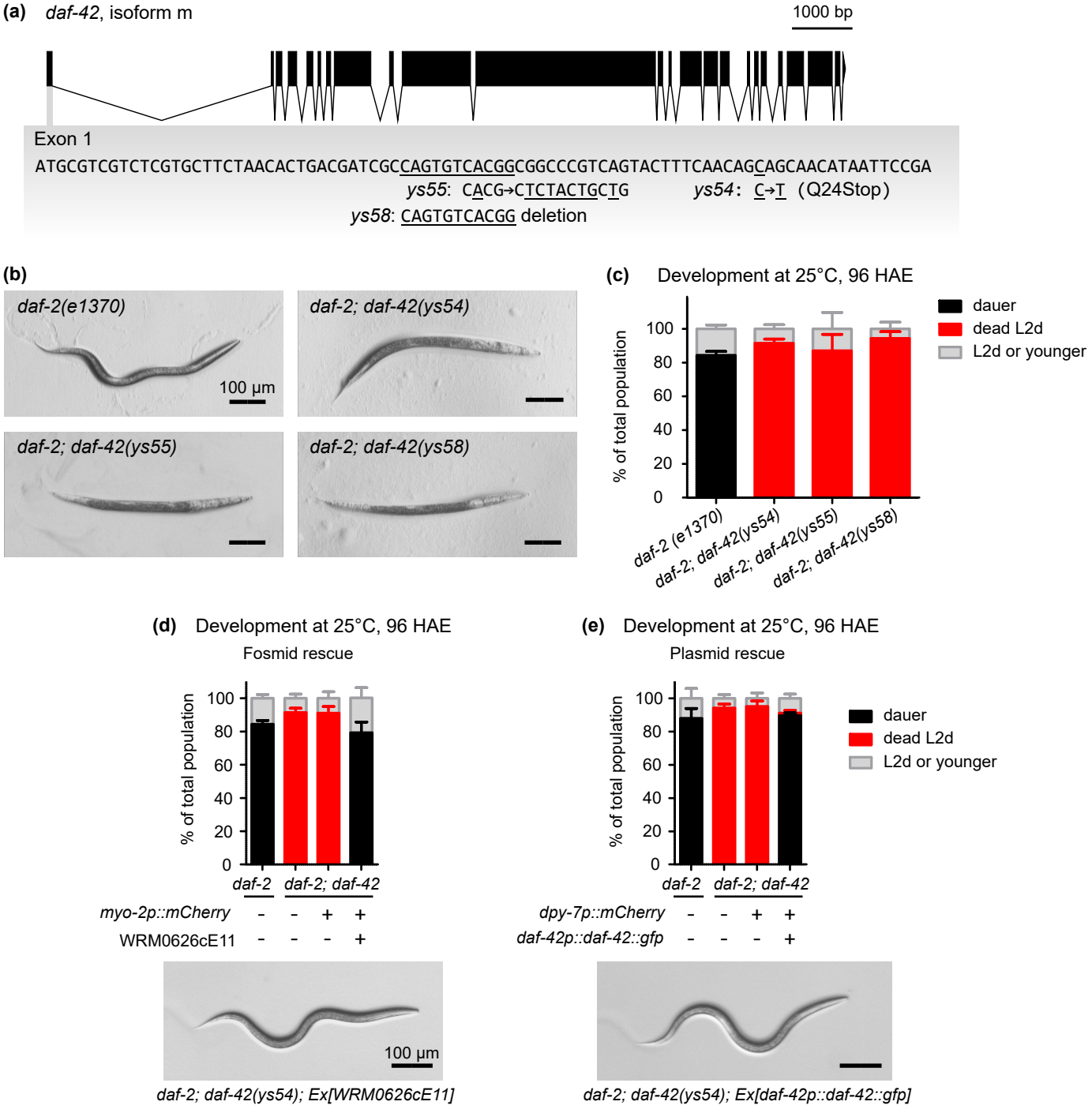

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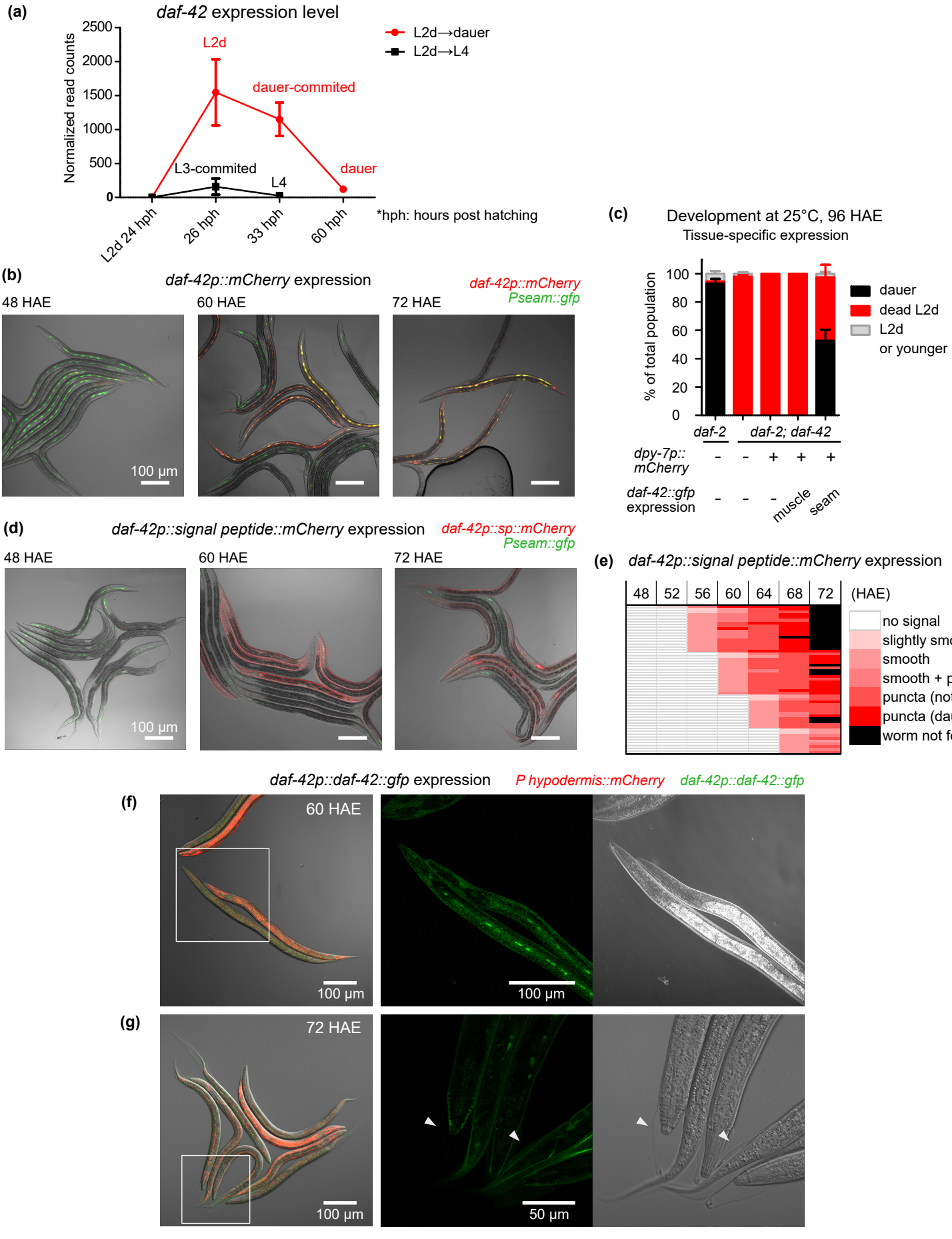

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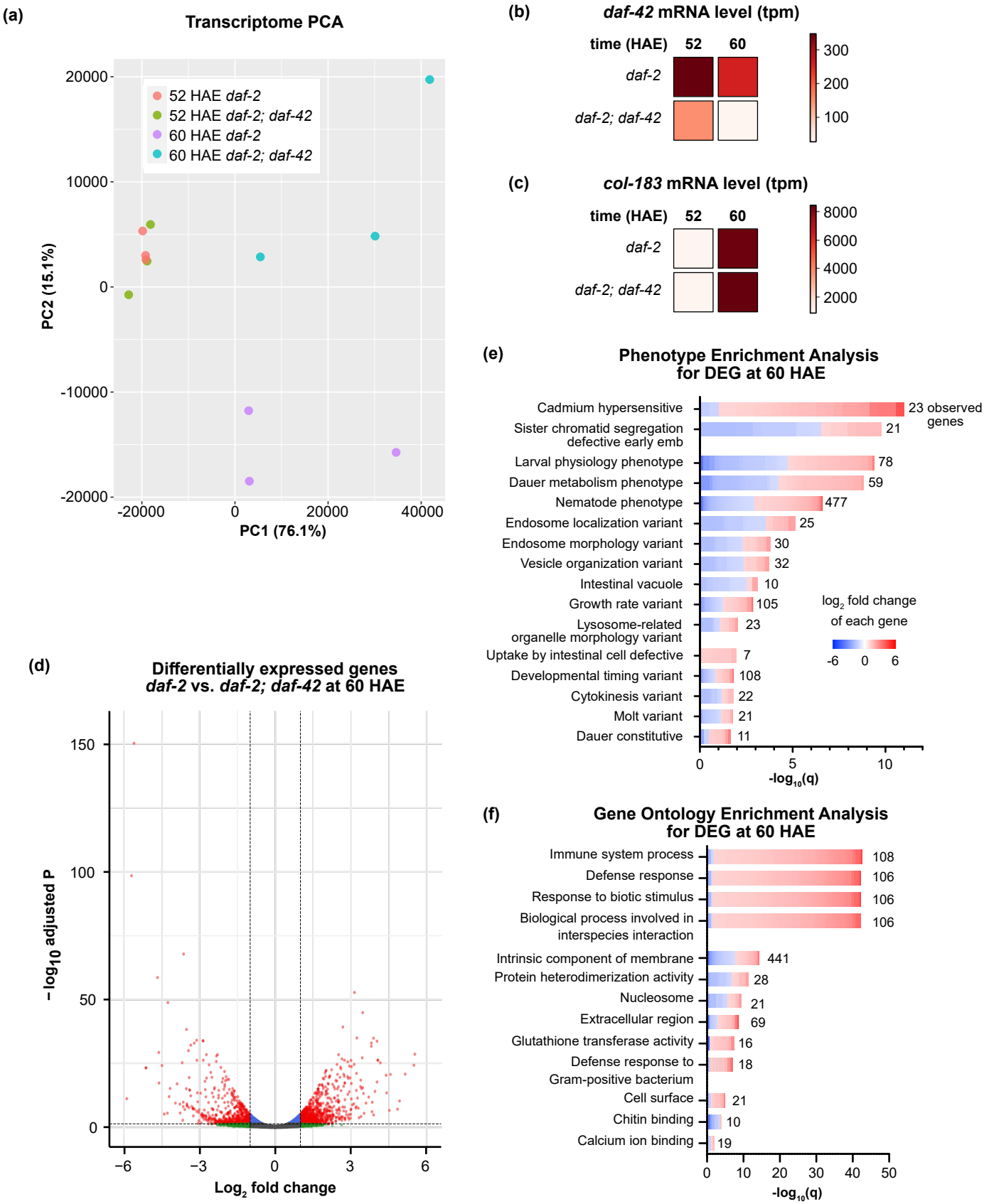

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