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The *C. elegans* developmental timing protein LIN-42 regulates diapause in response to environmental cues

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SUMMARY

Environmental conditions can have a major impact on developmental progression in animals. For example, when *C. elegans* larvae encounter harsh conditions they can reversibly halt the passage of developmental time by forming a long-lived dauer larva at the end of the second larval stage. Here, we show that the *period* homolog *lin-42*, known to control developmental time, also acts as a component of a switch that mediates dauer entry. Loss of *lin-42* function renders animals hypersensitive to dauer formation under stressful conditions, whereas misexpression of *lin-42* in the pre-dauer stage inhibits dauer formation, indicating that *lin-42* acts as a negative regulator of this life history decision. These phenotypes place LIN-42 in opposition to the ligand-free form of the nuclear receptor DAF-12, which indirectly senses environmental conditions and helps to integrate external cues into developmental decisions. Mutations that impair DAF-12 ligand binding are exquisitely sensitive to the absence of *lin-42*, whereas overexpression of LIN-42 can suppress the dauer constitutive phenotype of a ligand-insensitive *daf-12* mutant, suggesting that LIN-42 and DAF-12 are intimate partners in controlling the decision to become a dauer larva. The functional outputs of Period family proteins and nuclear receptors also converge in other organisms, suggesting that the relationship between *lin-42* and *daf-12* represents an ancient genetic framework for responding to environmental stimuli.

KEY WORDS: *daf-12*, Dauer formation, Developmental timing, Heterochronic gene, *lin-42*, *Caenorhabditis elegans*

INTRODUCTION

Animals monitor a variety of environmental cues, including seasonal variation in day length as well as more unpredictable and stress-inducing fluctuations in temperature and nutrient availability, and they respond to these cues by making adaptive changes in metabolism, physiology and behavior. The stress responses of juveniles are further complicated relative to those of adults, as they must integrate stress-induced changes into developmental programs (Rougvie, 2005; Edgar, 2006). Under sufficient stress, animals can stall temporal progression of development and delay sexual maturation until conditions improve, a complex process requiring synchronous changes in multiple developmental pathways.

The influence of environmental conditions on developmental timing has become an important area of investigation in humans, where, for example, studies have correlated adolescent obesity with earlier onset of puberty (Kaplowitz, 2008). To understand the impact stress-related cues have on developmental programs, several groups have applied genetic methods to model organisms. Such studies have revealed that multiple conserved signaling pathways (e.g. insulin, TGFβ, TOR and nuclear receptor signaling pathways) are used to coordinate developmental progression with external stimuli through modulation of body size, nutrient storage and the timing of developmental events (for reviews, see Edgar, 2006; Hu, 2007).

One of the best-understood developmental responses to environmental stress occurs in the nematode *C. elegans*. Under optimal growth conditions, *C. elegans* develops through four larval stages (L1-L4) prior to achieving reproductive competence as an adult. Development through these larval stages is coordinated temporally by the genes of the heterochronic pathway, which ensure that developmental events occur at the correct time (Resnick et al., 2010; Moss, 2007). If L1 larvae encounter an unfavorable environment (e.g. food shortage, overpopulation, high temperature), inputs from insulin, TGFβ and hormonal signaling pathways (Fig. 1A-C) suspend reproductive development after the L2 stage, and the animal forms a dauer larva, an alternative third larval stage specialized for long-term survival and dispersal (Cassada and Russell, 1975; Hu, 2007). These signaling pathways monitor environmental status and relay that information to mediate the decision to enter the dauer pathway. Once the decision to form a dauer is made, not only must the genetic pathways required for the L2-to-dauer transition be activated, but the timing mechanisms that promote continued developmental progression through the L2-to-L3 transition must be suspended. Therefore, the onset of dauer formation provides a unique opportunity to study the interaction between stress-responsive pathways and developmental timing mechanisms. Although a number of proteins have been identified that regulate one program or the other, the nuclear receptor (NR) DAF-12 provides key inputs to both (Antebi et al., 1998; Antebi et al., 2000).

DAF-12 acts as a molecular switch during development, indirectly sensing environmental conditions in order to make appropriate life-stage decisions (Fig. 1C) (Magner and Antebi, 2008). Under normal growth conditions, dietary cholesterol is converted into steroid hormones through a series of enzymatic steps that include modifications by the Rieske-like oxygenase DAF-36 and the cytochrome P450 DAF-9 (Gerisch et al., 2001; Jia et al., 2002; Motola et al., 2006; Rottiers and Antebi, 2006). The resulting hormones (known as dafachronic acids) bind the ligand-binding domain (LBD) of DAF-12 (Motola et al., 2006), and the complex then promotes the L2-to-L3 transition and inhibits dauer formation. Under poor growth conditions, hormone levels drop and

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ligand-free DAF-12 interacts with the SHARP co-repressor homolog DIN-1 (Ludewig et al., 2004; Motola et al., 2006), yielding a complex with the opposite activities: DAF-12–DIN-1 promotes dauer formation and blocks reproductive development through the L3 stage. In addition to their dauer phenotypes, *daf-12* mutants display timing defects in several tissues during continuous development (Antebi et al., 1998). Although there are multiple phenotypic classes of *daf-12* mutants, a general theme is that L2 stage-specific events are reiterated during L3, resulting in a retarded phenotype.

Here we report our discovery that the heterochronic gene *lin-42* acts with *daf-12* at the critical juncture where stress-responsive and developmental timing pathways intersect. In dauer formation, LIN-42 acts in opposition to ligand-free DAF-12 by promoting continuous development through the activity of the ligand-bound form of DAF-12. LIN-42 activity is essential for reproductive development under conditions of mild stress; its activity provides robustness to the system, ensuring that development continues without interruption. By contrast, when environmental conditions mandate dauer formation for survival, the LIN-42 temporal expression pattern is significantly altered to allow for dauer entry.

lin-42 and *daf-12* are also uniquely linked during continuous development as they time a similarly broad set of events, including gonad morphogenesis (Antebi et al., 1998; Tennessen et al., 2006), a process in which other heterochronic genes apparently have no role. In contrast to *daf-12*, loss of *lin-42* activity causes precocious execution of later-stage events (Jeon et al., 1999; Tennessen et al., 2006).

It is noteworthy that *lin-42* is the *C. elegans* homolog of the circadian rhythm gene *period* (Jeon et al., 1999) because metabolic homeostasis and stress responses are coordinated with the circadian clock in other organisms (Hardin et al., 1990; Yang et al., 2006; Kohsaka et al., 2007). Our work reveals an intimate relationship between LIN-42 and DAF-12 activities during dauer formation, and provides important insight into how Period-like proteins might control metabolic homeostasis by regulating the activity of nuclear receptors.

MATERIALS AND METHODS

Nematode growth and strain construction

Nematode growth was carried out as described previously (Sulston and Hodgkin, 1988) using nematode growth media (NGM) supplemented with 5 μg/ml cholesterol except where noted. All strains containing *daf-7* or *daf-2* alleles were maintained at 15°C.

Strains were constructed using standard methods; the more complex manipulations are outlined here. *lin-42(lf); daf-12(lbd)/+* strains were constructed by crossing *lin-42*/+ males to *daf-12* hermaphrodites and identifying F2 progeny that were semi-Dpy [indicating homozygosity of *lin-42(ve11)*] and that segregated animals with the *daf-12* gonad migration (Mig) phenotype. *lin-42(lf); daf-12(lbd)/+* animals segregated one quarter *lin-42; daf-12* progeny that had a synthetic constitutive dauer formation (SynDaf) phenotype and were sterile as adults. To test *lin-42(+)*-mediated rescue of the SynDaf phenotype, *lin-42(lf); daf-12(lbd)/+* animals were injected with the constructs indicated, and transgenic double homozygotes were selected by picking fertile GFP^+ Mig animals.

The *lin-42(ve11); din-1(dh149); daf-12(rh285)* triple mutant was constructed by crossing *din-1/+* males to *lin-42(ve11); daf-12(rh285)/+* hermaphrodites. An F1 animal segregating all three genotypes was identified, and its homozygous *daf-12(rh285)* progeny were singly picked based on their Mig phenotype. F3 animals homozygous for *din-1(dh149)* were then selected based on their non-Mig phenotype, and their progeny were screened for Dpy animals, indicating the presence of *lin-42(ve11)*. The resulting triple-mutant strain was confirmed by sequencing.

To construct strains containing *daf-16(mu86)*, the deletion allele was followed via PCR using the primers MF396 and MF425 (5′-TGTCTCTCAATCGGCCACCA-3′ and 5′-TGCAAGAAGTGGATTC-TGAGCA-3′). *daf-5(e1386)* was followed via its *Sty*I polymorphism (da Graca et al., 2004) after amplification with primers JMT131 and JMT159 (5′-GGTGTCTCCATAGCGGGTCTCTTCG-3′ and 5′-CAGTTCCG-AGCAGTCATGG-3′).

Transformation, rescue and expression studies

C. elegans transformations were performed as described previously (Mello et al., 1991) using either *sur-5::gfp* (Yochem et al., 1998) or *str-1::gfp* (Troemel et al., 1997) as co-injection markers. The transgenes used for rescuing *lin-42(lf)* were described previously (Tennessen et al., 2006). P*dpy-7::lin-42C::gfp::unc-54* (pJT97) contains 1271 bp of the *dpy-7* promoter driving expression of a *lin-42C* cDNA fused to *gfp*, and was injected at 2.5 ng/μl. P*dpy-7::lin-42C::gfp::unc-54* causes some larval lethality, the penetrance of which varies between transgenic lines. These animals were not counted in our assays.

LIN-42 immunofluorescence was performed as described previously (Tennessen et al., 2006) using MH27 staining as a positive control. Imaging and quantification of *daf-9::gfp* were performed as described previously (Gerisch et al., 2001).

Quantitative RT-PCR

Larvae were synchronized using the hypochlorite method of egg selection and RNA was prepared using Trizol Reagent (Invitrogen) with the inclusion of ~500 μm baked glass beads (G-8772; Sigma) to aid cuticle disruption. Contaminant DNA was removed using the DNA-free Kit (Ambion), and cDNA was generated using random hexamers and Transcriptor Reverse Transcriptase following the manufacturer's protocols (Roche). Real-time (RT) PCR was carried out using FastStart SYBR Green (Roche) and a Realplex 2 (Eppendorf). *lin-42* message levels (primers AR205 and AR262: 5′-CCACTGACCCGAGAAGCAC-3′ and 5′-GAGTTGGTGCCACTTGTCGG-3′) were quantitated relative to those of *ama-1* (primers AD78 and AD79: 5′-AGGAGATTAAACGCATGTCAG-3′ and 5′-CATGTCATGCATCTTCCAC-3′). Analysis was carried out using the comparative C_T method, comparing each time point to the mean value for the wild-type mid-L1 stage (Schmittgen and Livak, 2008). Four independent time courses were carried out for *daf-7* and two for wild-type populations. At each time point for a given time course, three populations of worms were harvested independently and processed to make three separate cDNA pools. Each cDNA pool was then analyzed in triplicate by quantitative (q) RT-PCR to determine *lin-42* and *ama-1* expression levels. Not all time points were assayed in each data set.

Dauer formation assays

Dauer formation was quantified by allowing individual hermaphrodites to lay eggs for 12-16 hours at the indicated temperature on plates seeded with *E. coli* OP50. To analyze mutants that do not form a functional vulva, gravid adults were placed in M9, cut with a hypodermic needle, and the released eggs were transferred to plates. Two days later, each brood was scored for dauer formation. Progeny of at least ten hermaphrodites (split between at least two trials performed on different days) were scored for each strain. For cholesterol deprivation experiments, hermaphrodites were placed on sterol-depleted media (NGM made with agarose instead of agar, and lacking peptone and added cholesterol), seeded with 150 μl of OP50 bacteria that had been washed three times with M9 buffer and concentrated 20:1 from the initial culture volume (Yochem et al., 1999). For RNAi experiments, percent dauer formation was calculated as the average of at least three different sets of double-stranded (ds) RNA injections, with a minimum of ten hermaphrodites per injection.

Animals homozygous for either *lin-42(n1089)* or *lin-42(ve11)* in combination with the *daf-12* LBD mutations (*rh193*, *rh284*, *rh285* and *rh61*) are sterile. To extrapolate the SynDaf phenotype of double-homozygous strains, the progeny of *lin-42; daf-12/+* hermaphrodites were scored for dauer formation. The background rate for each *daf-12* strain was subtracted from the rate of dauer formation for each heterozygous brood

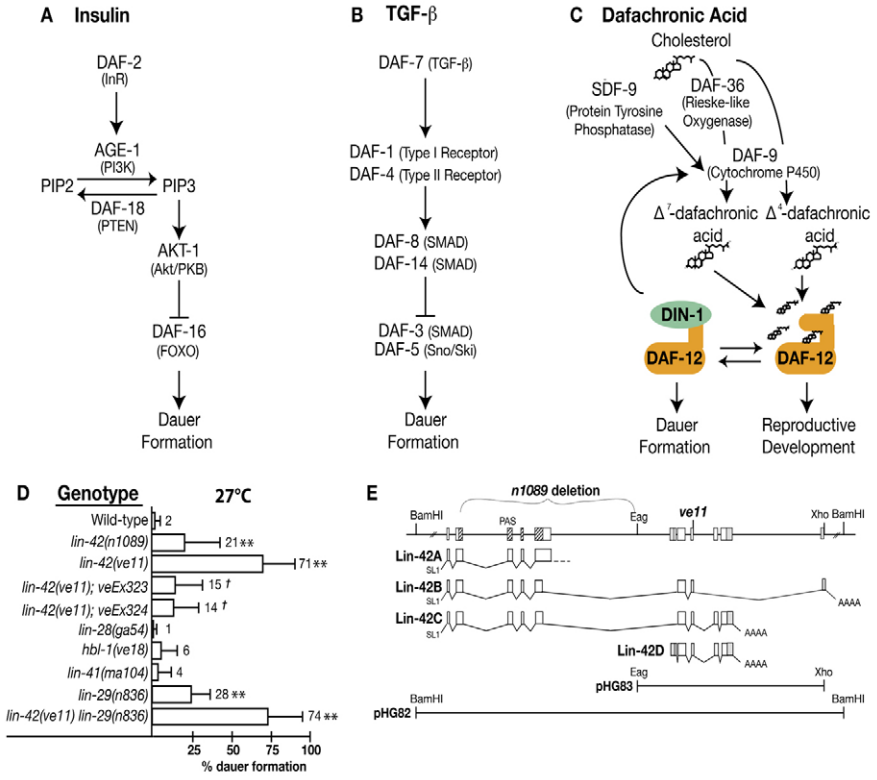

Fig. 1. Dauer regulation by insulin, TGFβ, dafachronic acid signaling pathways and *lin-42*. (**A-C**) Simplified pathways illustrating inputs into dauer formation. (**D**) *lin-42* mutants are Daf-c at 27°C. Horizontal bars and the numbers to the right indicate the average percentage dauer formation. Here, and in subsequent figures, error bars represent s.d. and *P*-values were calculated using two-tailed Student's *t*-tests with unequal variance. **, $P \leq 0.005$ compared with the wild type. $n \geq 200$ worms for all strains. The *lin-42(ve11)* high-temperature-induced dauer (Hid) phenotype is rescued by the *lin-42*D-containing transgene arrays *veEx323* or *veEx324* (pHG83 plus *str-1::gfp*) (Tennessen et al., 2006). † indicates values for GFP(+) animals; they formed significantly fewer dauers than did their nontransgenic siblings ($P < 5 \times 10^{-8}$; $n > 200$). Four *str-1::gfp* control lines formed dauers at a rate indistinguishable from *ve11* (74%; $n = 372$), and the dauers observed were not biased for animals that had lost the array. (**E**) The *lin-42* locus, alleles and genomic fragments used. *lin-42* encodes four transcription units, two of which are non-overlapping (Lin-42A and Lin-42D) (Tennessen et al., 2006). Exons in Lin-42A encode the hallmark PAS domain, whereas the 3′ exons in Lin-42D encode smaller domains that are conserved in Period proteins. Expression of the 3′ exons (whether from Lin-42B, C or D) appears to be key to heterochronic function, as transgenic expression of this region (pHG83) rescues heterochronic defects produced by all *lin-42* mutations, whereas expression of the upstream region alone does not. On WormBase (www.wormbase.org) LIN-42A=F47F6.1c, LIN-42C=F47F6.1b, LIN-42D=F47F6.1a, and LIN-42B is not defined.

and multiplied by four. A SynDaf phenotype was not observed when *daf-12(rh193)* was assayed in combination with *lin-28(ga54)*, *lin-28(RNAi)*, *hbl-1(ve18)* or *lin-41(RNAi)* (0% in each case; $n \geq 360$).

RNAi

dsRNA synthesis and microinjections were conducted as described previously (Kamath et al., 2001) using pPD129.36. *lin-42* dsRNA was synthesized from pJT27, which contains cDNA spanning exons 6-9 (Tennessen et al., 2006). *lin-41* dsRNA was synthesized as described previously (Lin et al., 2003), and *lin-28* dsRNA was made from an *Age*I/*Sac*I fragment (pJT101). For each strain analyzed, the progeny of ten injected hermaphrodites were scored for dauer formation, omitting from analysis embryos laid in the first 24 hours post-injection. For statistical purposes, each injection set was scored as one value and at least three sets were scored for each strain.

Yeast two-hybrid assays

daf-12 and *lin-42* cDNAs were cloned into the yeast two-hybrid vectors pGBKT7 and pGADT7, and assays were performed using the Clontech Matchmaker GAL4 Two Hybrid System 3 (Clontech). To test for interactions, constructs were transformed pairwise into yeast strain AH109. Transformants were grown overnight in minimal media lacking leucine and tryptophan (SD-L-T), and interactions were tested for by growth on SD-L-T-histidine-adenine+X-α-gal plates, assessing activation of three reporters: ADE2, HIS3 and MEL1. Murine p53 and SV40 large T-antigen were used as a positive control, and T-antigen and Lamin C were used as negative controls. None of the test clones caused appreciable activation of the reporters when tested alone or versus empty vectors. Western analyses confirmed accumulation of fusion proteins in all strains.

RESULTS

lin-42 regulates dauer formation

lin-42 and *daf-12* act antagonistically during continuous reproductive development (Tennessen et al., 2006). If this relationship were to hold true for the regulation of dauer formation, *lin-42* mutants should have a phenotype opposite to the dauer defective (Daf-d) phenotype of *daf-12(lf)* mutants and should be hypersensitive to forming dauers. Although *lin-42* mutants do not form dauers inappropriately under normal growth conditions, they are sensitized toward dauer formation. When grown in the presence of food and at low population density, but under conditions of temperature stress (27°C), 71% of *lin-42(ve11)* and 21% of *lin-42(n1089)* animals became dauer larvae compared with 2% of wild-type larvae (Fig. 1D). This characteristic of revealing a dauer phenotype when exposed to temperature stress is shared by

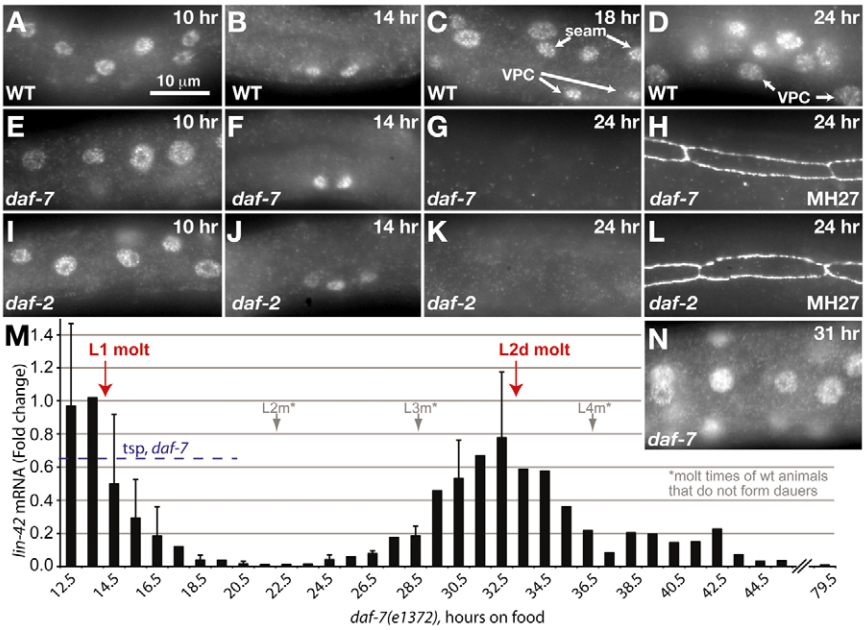

Fig. 2. LIN-42 is not detected during the L2d intermolt. Micrographs (**A-L**,**N**) of animals stained with anti-LIN-42 sera (Tennessen et al., 2006) unless noted otherwise. Hours indicate time of larval development. In wild-type larvae (A-D), LIN-42 accumulates in the nuclei of most somatic cells during mid-L1, including hypodermal cells (A), and is undetectable except in the somatic gonad during late L1 (B). During mid-L2 (C) and mid-L3 (D), LIN-42 accumulates in the hypodermal seam and most other somatic cells. Arrows indicate hypodermal seam and vulval precursor cells (VPCs). Mid- and late L1 stage *daf-7(e1372)* (E,F) and *daf-2(e1370)* (I,J) look similar to wild-type animals. However, LIN-42 is not detected during the L2d intermolt (G,K) even though these animals stain with the control MH27 antibody (H,L). *daf-7* and *daf-2* mutants were stained at 2-hour intervals, from 18 (mid-L2) to 26 hours without detection of LIN-42. Shortly after 26 hours, these populations began to enter the L2d molt. By 26 hours, wild-type animals were at the L3 stage and showed the corresponding LIN-42 pattern. (N) LIN-42 accumulation at 31 hours in *daf-7(e1372)*. Scale bar in A also applies to B-L and N. (**M**) Fold change in *lin-42* mRNA levels in *daf-7(e1372)* mutants grown at 25°C relative to mid-L1 (10.5 hours) levels from wild-type animals assayed in parallel. The 10.5 hour time point in wild-type animals corresponds to ~12.5 hours of *daf-7* development. The bar-chart shows the mean values of four time courses (shown individually in Fig. S1 in the supplementary material). Error bars (s.d.) are shown for time points represented by at least three independent time courses. By 32.5 hours, most animals had stopped pumping, indicating entry into the L2d molt. Red arrows indicate the approximate times of the L1 and L2d molts for *daf-7* animals grown at 25°C. Grey arrows indicate the approximate times of each molt for wild-type animals developing continuously at 25°C. The cyclical pattern of *lin-42* mRNA accumulation in the wild type was reported previously for continuous development; levels peak in the intermolt and are low during the molts (Jeon et al., 1999; Gissendanner et al., 2004). The temperature-sensitive period of *daf-7(e1372)* (Golden and Riddle, 1984) is indicated with a dashed line.

mutations in several genes that alter the outputs of dauer signaling pathways (Ailion and Thomas, 2000; Ailion and Thomas, 2003; Rottiers et al., 2006).

The observation that *ve11* exhibits a more penetrant phenotype than does *n1089* parallels previous studies of their heterochronic phenotypes (Tennessen et al., 2006) and suggests that the more 3′ exons of *lin-42* are important in the control of dauer formation. Therefore, studies reported here focus on the *lin-42(ve11)* allele and elimination of *lin-42* activity via RNAi. Transgenic expression of *lin-42D*, the downstream isoform that rescues *lin-42(lf)* heterochronic defects (Tennessen et al., 2006), also rescued the *lin-42(ve11)* dauer phenotype (Fig. 1D,E), demonstrating that one function of wild-type LIN-42 is to inhibit dauer formation.

The *lin-42(lf)* high-temperature-induced dauer (Hid) phenotype could be a non-specific consequence of the precocious phenotype exhibited by these mutants. For example, if an inhibitor of dauer formation is expressed in a stage-specific manner, the temporal shift in tissue identities associated with *lin-42(lf)* might indirectly produce a Hid phenotype by changing the way dauer development is regulated. In this case, other precocious mutants should exhibit a similar Hid phenotype. However, when the precocious heterochronic mutants *hbl-1(ve18)*, *lin-28(ga54)* and *lin-41(ma104)* (Moss et al., 1997; Slack et al., 2000; Abrahante et al., 2003; Lin et al., 2003) were raised at 27°C and scored for dauer formation, the animals did not form dauers at a significantly higher frequency than did the wild type (Fig. 1D). Furthermore, all of the *lin-42* heterochronic phenotypes are dependent on the zinc-finger transcription factor LIN-29 (Rougvie and Ambros, 1995; Tennessen et al., 2006), but the *lin-42(lf)* Hid phenotype is not *lin-29* dependent. The majority of *lin-42(ve11) lin-29(n836)* double mutants raised at 27°C formed dauers, demonstrating that suppression of the *lin-42(lf)* heterochronic phenotypes has no effect on the dauer phenotype (Fig. 1D). *lin-29* single mutants also exhibited a mild Hid phenotype, an observation consistent with previous studies implicating *lin-29* in inhibition of dauer formation (Liu and Ambros, 1989; Liu et al., 2004).

LIN-42 accumulation is altered significantly in animals that will form dauers

Because LIN-42 acts to inhibit dauer formation, the LIN-42 accumulation pattern might differ between L2 stage animals undergoing continuous development and those in the pre-dauer state (L2d) that are destined to form dauers. To test this hypothesis, LIN-42 accumulation was analyzed in *daf-7(e1372)* and *daf-2(e1370)* temperature-sensitive mutants, which are dauer constitutive (Daf-c) when raised at 25°C (Riddle et al., 1981).

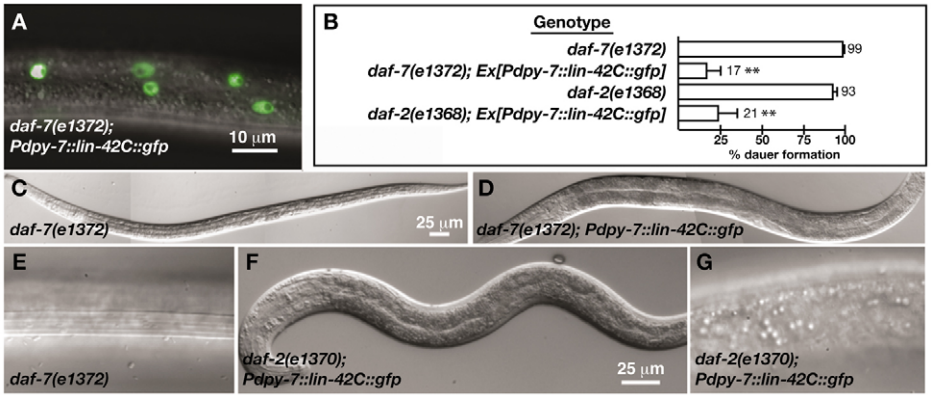

Fig. 3. LIN-42 inhibits dauer formation. (**A**) Transgenic *daf-7(e1372)* animals accumulate LIN-42C::GFP in hypodermal nuclei during the L2 stage; a similar pattern was observed in *daf-2* mutants. (**B**) The temperature-dependent Daf-c phenotype of *daf-7(lf)* and *daf-2(lf)* mutants is suppressed by ectopic *lin-42* expression. **, $P \leq 0.005$ relative to non-transgenic siblings; $n \leq 500$ worms for all strains. Control arrays containing $P_{dpy\text{-}7}$*::gfp* plus *str-1::gfp* did not alter dauer formation phenotypes. For *daf-7* controls, 94±4% of transgenic animals and 95±2% of their nontransgenic siblings formed dauers ($n>500$). Similarly, for *daf-2* controls, nearly all transgenic animals and their nontransgenic siblings formed dauers (99±1% each; $n>300$). (**C-E**) *daf-7(e1372)* mutants arrest as dauers (C) with hallmark dauer alae (E), whereas transgenic siblings expressing LIN-42::GFP do not (D). (**F**,**G**) Transgenic *daf-2(e1370)* mutants also do not form dauers but instead arrest as L3-like animals (F) lacking dauer alae (G). Scale bar in A also applies to E and G; scale bar in C also applies to D. All assays were carried out at 25°C.

During continuous development in wild-type animals, LIN-42 levels cycle during each larval stage, peaking in the intermolt and becoming undetectable in the molt (Tennessen et al., 2006). This pattern was observed during the L1 stage in wild-type, *daf-2* and *daf-7* mutants; LIN-42 accumulation was spatially and temporally indistinguishable among these strains (Fig. 2; data not shown). However, the pattern differed significantly between wild-type and Daf-c mutants after the L1 molt. LIN-42 was detected in wild-type L2 stage animals soon after the first molt and remained detectable (Fig. 2C) until the L2 molt, after which it disappeared, returning following the molt in the L3 stage (Fig. 2D). By contrast, LIN-42 was absent from *daf-7* and *daf-2* mutants in the early L2 stage and remained undetectable in the intermolt through 26 hours of larval development (Fig. 2G,K; data not shown), ~7-8 hours after the temperature-sensitive period for dauer formation ends in each of these mutants (Swanson and Riddle, 1981). This suggests that the presence of LIN-42 could be inhibitory to dauer formation. A pulse of LIN-42 protein accumulation was observed during the L2d molt in Daf-c mutants, centered around 32 hours (Fig. 2N; data not shown). At this time, *daf-7* mutants are molting into the dauer stage and undergoing dauer remodeling, which includes constriction of the pharynx and body and synthesis of the dauer cuticle (Swanson and Riddle, 1981) (K.J.O. and A.E.R., unpublished), suggesting that LIN-42 might contribute to these processes.

The response of *lin-42* expression to dauer entry is manifest at the RNA level. We performed a series of qRT-PCR experiments to examine *lin-42* mRNA levels in *daf-7* mutants grown at 25°C; a summary time course is shown in Fig. 2M and additional information is shown in Fig. S1 in the supplementary material. qRT-PCR confirmed the previously reported cyclical pattern of *lin-42* mRNA accumulation in wild-type animals (Jeon et al., 1999), with levels peaking during each intermolt and declining during the molts. A similar pattern was observed during the L1 stage in *daf-7* mutants grown at 25°C (Fig. 2M). However, in contrast to the wild type, *lin-42* mRNA levels did not rise again in the early L2; they remained low or absent in *daf-7* mutants until the L2d molt, an additional 12-15 hours. By this time, wild-type animals had completed the L2 and L3 stages.

Misexpression of *lin-42* during the L2d inhibits dauer formation

Many genes are downregulated as animals initiate dauer development and transit through the dauer stage (Wang and Kim, 2003; Liu et al., 2004). Thus, an important question is whether the temporal alteration of LIN-42 accumulation during the L2d intermolt is functionally significant for dauer regulation or is instead a passive consequence of the physiological changes associated with preparing for dauer entry. To address this question, dauer formation was examined in *daf-7* and *daf-2* mutant animals engineered to express *lin-42C::gfp* from a *dpy-7* promoter (Fig. 3; see Table S1 in the supplementary material; data not shown). In contrast to the absence of endogenous LIN-42 during the early second larval stage of *daf-7* mutants, LIN-42C::GFP was detected in the L2 stage hypodermis of *daf-7(e1372); veEx[Pdpy-7::lin-42C::gfp]* animals raised at 25°C (Fig. 3A). Although these animals appeared to develop through an L2d-like stage, as did *daf-7* mutants raised at 15°C (as judged by their dark intestine and the extended length of the larval stage), 83% of these animals developed directly into L3 larvae rather than forming dauers. By contrast, their nontransgenic siblings instead arrested as dauer larvae, as did control transgenic animals expressing *gfp* alone from the *dpy-7* promoter (Fig. 3B-E). Similarly, 79% of *daf-2(e1368); veEx[Pdpy-7::lin-42C::gfp]* animals raised at 25°C did not form dauers (Fig. 3B). These results indicate that forced expression of LIN-42 during L2d can interfere with dauer formation. Interestingly, when a stronger *daf-2* allele, *e1370*, was used, 34% of the transgenic animals bypassed dauer formation, but they failed to complete development and instead arrested as L3 stage larvae (Fig. 3F,G), a phenotype reminiscent of *daf-2(lf); daf-12(lf)* double mutants or *daf-2(lf)* mutants treated with exogenous DAF-12 ligand (Vowels and Thomas, 1992; Motola et al., 2006).

lin-42 interacts genetically with the dafachronic acid signaling pathway

Dauer formation is controlled by multiple inputs, including signaling through conserved insulin/IGF and TGFβ pathways that converge upon the NR DAF-12. In order to position *lin-42* with

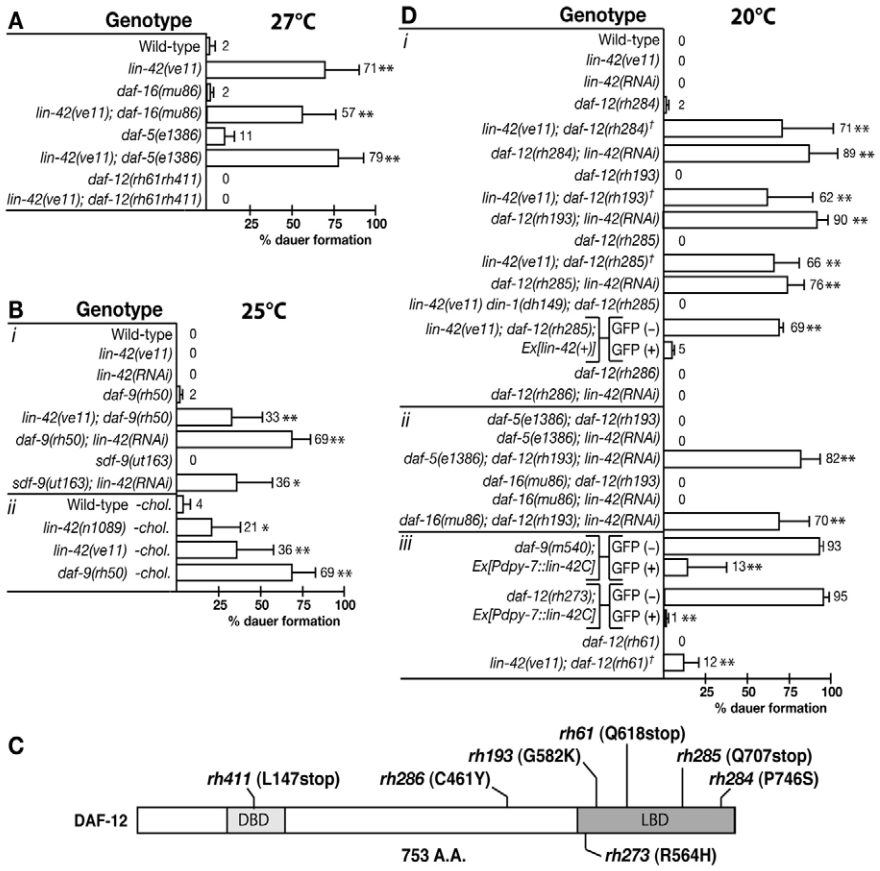

Fig. 4. LIN-42 modulates ligand-free DAF-12 signaling. Horizontal bars and the numbers to the right indicate the average percentage of dauers formed. *, $P \leq 0.05$; **, $P \leq 0.005$. $n \geq 380$ worms were scored for all strains. (**A**) At 27°C, the *lin-42* Hid phenotype is not significantly altered by the Daf-d mutations *daf-16(mu86)* or *daf-5(e1386)*, but is completely suppressed by *daf-12(rh61rh411)*, a null allele. (**B**) At 25°C, *lin-42(lf)* is SynDaf in combination with *daf-9* or *sdf-9* mutations that decrease dafachronic acid synthesis, and Daf-c when raised on cholesterol-depleted media. (**C**) DAF-12 mutations used (Antebi et al., 2000). DBD, DNA-binding domain; LBD, ligand-binding domain. (**D**) At 20°C, *lin-42(lf)* is SynDaf with *daf-12* LBD mutations. The phenotype is dependent on *din-1* and can be rescued by an array containing the *lin-42* locus, *Ex[lin-42(+)]* (pHG82; see Fig. 1E), and *sur-5::gfp* as a marker. Attempts to rescue the SynDaf phenotype with the smaller fragment, pHG83, were hampered by its inability to restore fertility to *daf-12(LBD); lin-42(lf)* animals. Neither *daf-5(lf)* nor *daf-16(lf)* can suppress dauer formation in *lin-42(ve11); daf-12(rh193)* mutants. Ectopic LIN-42 can suppress the *daf-9(lf)* Daf-c phenotype. † indicates a genotype that was sterile and the values were calculated from the progeny of *lin-42(ve11); daf-12/+* hermaphrodites (see Materials and methods). For strains bearing extrachromosomal arrays, GFP(+) indicates transgenic animals whereas GFP(–) refers to their nontransgenic siblings. Values are averaged from at least three independent strains (see Table S1 in the supplementary material). The SynDaf phenotypes appear to be dependent upon disruption of 3′ *lin-42* exons. *ve11* does not disrupt the Lin-42A transcription unit, and yet produces a SynDaf phenotype. By contrast, *lin-42(n1089)*, which deletes most of the upstream region but leaves the D isoform intact, exhibits a weak Hid phenotype and is not SynDaf with *daf-12(rh284)* or *daf-12(rh285)* at 20°C, or with *daf-9(rh50)* at 25°C (<1% dauer formation for all strains; $n>1000$). *lin-42(mg152)* contains a premature stop in exon 2 and also fails to produce a SynDaf phenotype in these backgrounds. Thus, the 3′ *lin-42* exons appear to be important to LIN-42 function in regulating dauer development.

respect to these well-established dauer formation pathways, the *lin-42(lf)* Hid phenotype was examined in combination with dauer defective (Daf-d) mutations in *daf-16* and *daf-5*, which encode the most downstream components of the insulin and TGFβ signaling pathways, respectively (Fig. 1A,B) (Ogg et al., 1997; da Graca et al., 2004). *daf-16(lf)* and *daf-5(lf)* mutations failed to suppress the *lin-42(lf)* Hid phenotype (Fig. 4A), placing *lin-42* downstream of, or in parallel to, these pathways. By contrast, *lin-42(ve11); daf-12(rh61rh411)* double mutants failed to form dauers when raised at 27°C (Fig. 4A), indicating that the Hid phenotype requires *daf-12* activity and that *lin-42* acts through (or in parallel to) *daf-12*.

Previous studies have shown that Daf-c mutations in components of the DAF-12-based steroid hormone signaling pathway result in the inappropriate formation of partial dauers with normal dauer alae, but they fail to radially constrict their pharynxes (Antebi et al., 2000; Gerisch et al., 2001; Jia et al., 2002; Ohkura et al., 2003; Li et al., 2004; Rottiers et al., 2006). *lin-42* mutants raised at 27°C similarly formed partial dauers with robust dauer alae and unconstricted pharynxes (Fig. 5A-D), suggesting that LIN-42 and DAF-12 regulate dauer development through a common mechanism.

lin-42(lf) causes dauer formation when dafachronic acid signaling is compromised

To further examine the relationship between *lin-42* and DAF-12-based hormone signaling, *lin-42* activity was depleted in animals weakly compromised for function of the P450 DAF-9, which is involved in ligand synthesis (Fig. 1C) (Gerisch et al., 2001; Jia et al., 2002; Motola et al., 2006). Animals bearing *lin-42(lf)* mutations or the weak *daf-9(lf)* allele *rh50* rarely formed dauers when raised

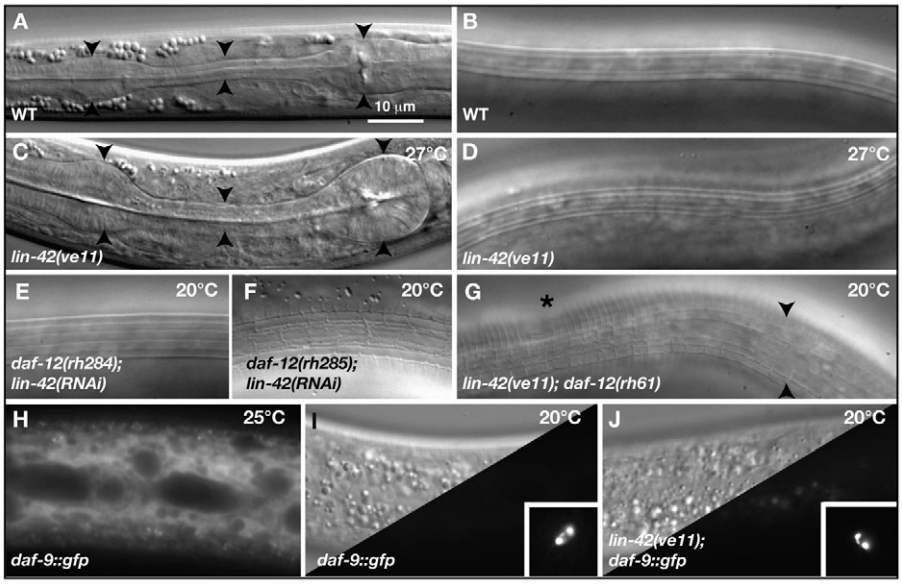

Fig. 5. *lin-42(lf)* Hid and SynDaf phenotypes. (**A**,**B**) Wild-type dauers have (A) constricted pharynxes (arrowheads) and (B) dauer alae. (**C**,**D**) *lin-42(ve11)* animals form dauer-like larvae at 27°C. They lack constricted pharynxes (C; arrowheads) but have dauer alae (D). (**E**) Dauer alae in a *daf-12(rh284); lin-42(RNAi)* animal raised at 20°C. (**F**) *daf-12(rh285); lin-42(RNAi)* dauers have poorly formed alae. (**G**) *lin-42(ve11); daf-12(rh61)* mutants often form incomplete dauers. Asterisk denotes the boundary between the radially constricted (dauer-like) anterior half (left) and the unconstricted, L3-like posterior half. Arrowheads indicate where the dauer alae begin to dissipate. (**H-J**) *daf-9::gfp* is expressed in wild-type L2s raised at 25°C (H), but is undetectable in the hypodermis of the wild type (I) or *lin-42(ve11)* (J) L2s raised at 20°C. Insets show similar expression of *daf-9::gfp* expression in XXX neurons in wild-type (I) and *lin-42(ve11)* (J) mutants.

at 25°C. By contrast, 33% of *lin-42(ve11); daf-9(rh50)* and 69% of *lin-42(RNAi); daf-9(rh50)* animals produced a synthetic constitutive dauer formation phenotype (SynDaf) (Fig. 4Bi). Similarly, loss of function of SDF-9, a phosphatase that appears to act upstream of DAF-9 (Ohkura et al., 2003), did not induce dauer formation in animals raised at 25°C, but 36% of *sdf-9(ut163); lin-42(RNAi)* animals grown in parallel were SynDaf (Fig. 4Bi). The *lin-42* SynDaf phenotype appears to be specific to components of the dafachronic acid pathway, as *lin-42(ve11)* failed to enhance the dauer formation phenotypes of either *daf-2(e1370)* or *daf-7(e1372)* mutants when raised at 20°C, and *lin-42(RNAi)* did not produce a dauer phenotype in combination with other mutations tested for which SynDaf phenotypes have been described [*akt-1(ok525)*, *unc-3(e151)* or *unc-31(e169)* mutants raised at 25°C (see Table S2 in the supplementary material)] (Ailion and Thomas, 2000; Ailion and Thomas, 2003). The observed genetic interactions are consistent with the idea that loss of *lin-42* activity makes animals hypersensitive to changes in DAF-12-based hormonal signaling.

Mutations in genes such as *daf-9* and *sdf-9* that lower dafachronic acid signaling cause animals to form dauers when raised on plates containing food but lacking cholesterol (Gerisch et al., 2001; Jia et al., 2002; Ohkura et al., 2003; Rottiers et al., 2006). *lin-42* mutants behaved in a similar fashion; they formed dauers when grown on cholesterol-depleted media (Fig. 4Bii), again linking *lin-42* function with dafachronic acid signaling.

If *lin-42(lf)* makes animals hypersensitive to changes in DAF-12 ligand production, then mutations that weaken the interaction between DAF-12 and its ligand might also result in SynDaf phenotypes in combination with loss of *lin-42* function. Indeed, the *daf-12* LBD mutations *rh193*, *rh284* and *rh285* (Fig. 4C) (Antebi et al., 2000), which alone are non-Daf, produced a strong SynDaf interaction when *lin-42* activity was also disrupted via RNAi or *ve11* (Fig. 4Di, Fig. 5E,F), and this phenotype was rescued by transgenic expression of wild-type *lin-42* (Fig. 4Di; see Table S1 in the supplementary material). The SynDaf phenotype is dependent on increased amounts of ligand-free DAF-12, rather than a general disruption of *daf-12* activity, because *lin-42(RNAi)* performed in the presence of *daf-12(rh286)*, a mis-sense allele that does not disrupt the LBD (Antebi et al., 2000), was not SynDaf at 20°C or 25°C (Fig. 4C,Di; data not shown).

To determine whether the SynDaf phenotype of *lin-42; daf-12(LBD)* mutants requires insulin or TGFβ signaling, *lin-42* RNAi was conducted in *daf-16(lf); daf-12(rh193)* and *daf-5(lf); daf-12(rh193)* double mutants. Similar to the *lin-42(lf)* Hid phenotype, dauer formation in *daf-12(rh193); lin-42(RNAi)* double mutants was not suppressed by the absence of either the FoxO gene *daf-16* or the Sno gene *daf-5* (Fig. 4Dii) (Ogg et al., 1997; da Graca et al., 2004), indicating that LIN-42 does not act in these two pathways.

Finally, the SynDaf phenotype is not simply a general interaction between *daf-12(LBD)* alleles and precocious heterochronic mutants, because depletion of *lin-28*, *lin-41* or *hbl-1* in a *daf-12(rh193)* background failed to elicit this response (see Materials and methods). Rather, the SynDaf interaction between *lin-42* and *daf-12* is unique among the known heterochronic genes.

LIN-42 antagonizes the dauer-inducing signal from ligand-free DAF-12

LIN-42 function in dauer formation is also independent of dafachronic acid signaling. *daf-9(m540)* and *daf-12(rh273)* mutants have strong Daf-c phenotypes, albeit for different reasons: *daf-9(m540)* mutants form dauers because they do not produce dafachronic acid (Jia et al., 2002), whereas the *daf-12(rh273)* mutation renders the DAF-12 LBD unable to bind dafachronic acid (Antebi et al., 2000; Motola et al., 2006). Expression of LIN-42 from the P*dpy-7::lin-42C::gfp* transgene suppresses the Daf-c phenotypes of these mutants, indicating that LIN-42 can block dauer formation in the absence of dafachronic acid signaling (Fig. 4Diii; see Table S1 in the supplementary material).

These genetic experiments suggest that LIN-42 antagonizes the activity of ligand-free DAF-12 downstream of the known dauer signaling pathways. The only other factor known to act at this point in dauer signaling is DIN-1, the SHARP co-repressor that binds ligand-free DAF-12, forming a dauer-promoting complex (Ludewig et al., 2004). *din-1(lf)* mutations cause a Daf-d phenotype and, previous to this study, were the only reported suppressors of the *daf-12(LBD)* Daf-c phenotype. We found that the SynDaf phenotype of *lin-42(lf); daf-12(lbd)* mutants is dependent on formation of a DAF-12–DIN-1 complex. A *lin-42(ve11); din-1(dh149); daf-12(rh285)* triple mutant did not form dauers (Fig. 4Di), consistent with a model in which LIN-42 antagonizes the

DAF-12–DIN-1 complex. As an additional means of testing this model, we examined the interaction between *lin-42(ve11)* and *rh61*, an unusual allele of *daf-12*. The *daf-12(rh61)* lesion results in a premature stop early in the LBD that renders the DAF-12 protein insensitive to its ligand but also decreases the binding affinity between the DAF-12 LBD and DIN-1 (Fig. 4C) (Antebi et al., 2000; Motola et al., 2006). As a result of decreased DIN-1 binding, *rh61* mutants are Daf-d even though they are unable to bind dafachronic acid. If LIN-42 inhibits dauer formation by antagonizing the DAF-12–DIN-1 complex, removal of LIN-42 in a *rh61* background might allow this mutant to form dauers even with decreased complex formation. Consistent with this prediction, 12% of *lin-42(ve11); daf-12(rh61)* animals formed partial dauers (Fig. 4Diii). Interestingly, many of these animals formed dauer alae only over the anterior seam cells, whereas the posterior half appeared similar to that of L3 larvae (Fig. 5G), suggesting that positional information influences the dauer decision.

Finally, we monitored expression of a *daf-9::gfp* reporter (Gerisch and Antebi, 2004; Mak and Ruvkun, 2004) in *lin-42(ve11)* mutants. As previously reported, *daf-9::gfp* expression is upregulated under conditions with abnormally low levels of the ligand-bound DAF-12, such as in animals raised on cholesterol-deficient media or at high temperature (Fig. 5H,I) or in those carrying the LBD mutation *daf-12(rh273)* (Gerisch and Antebi, 2004; Mak and Ruvkun, 2004). Hypodermal DAF-9::GFP levels appeared to be unchanged in *lin-42(ve11)* animals raised at 20°C (Fig. 5I,J), indicating that *lin-42(lf)* neither appreciably decreases the production of the DAF-12 ligand nor alters the ability of DAF-12 to bind its ligand.

LIN-42 interacts with DAF-12 in yeast

The genetic experiments described above indicate that LIN-42 and DAF-12 might interact in a transcriptional complex that regulates dauer formation. To test this hypothesis, LIN-42 isoforms were assessed for their ability to interact with DAF-12 peptides in a yeast two-hybrid system. In pairwise tests, LIN-42B interacted with DAF-12 in both bait and prey conformations, whereas LIN-42C interacted with DAF-12 only when expressed as bait (Fig. 6). These LIN-42 isoforms also interacted with DAF-12 peptides that represent the *rh61* and *rh285* truncations, and the interactions were noticeably stronger than those between LIN-42B/C and the longer DAF-12 peptide containing an intact LBD (Fig. 6). These results indicate that the C-terminus of DAF-12 can influence its ability to interact with LIN-42, perhaps reflecting a role for the AF2 homology domain, which regulates NR transcriptional activity via ligand-dependent conformational changes (reviewed by Wärnmark et al., 2003). Results from the smallest DAF-12 peptide tested, which contains the LBD but lacks the hinge domain and C-terminus, indicated that sequences within the LBD contribute to the interaction detected. The two shorter LIN-42 isoforms revealed little, if any, interaction with DAF-12 (data not shown), suggesting that the role of LIN-42D in dauer formation involves a weaker interaction with DAF-12 or that it regulates dauer formation through additional binding partners.

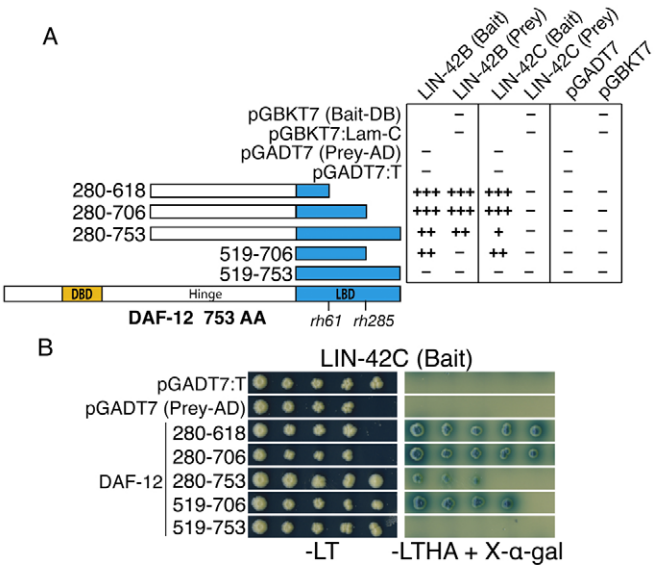

Fig. 6. LIN-42 interacts with DAF-12 in yeast. (**A**) Summary of three independent experiments for each pair represented qualitatively by the strength of interaction: strong (+++), intermediate (++), weak (+) and no interaction (–). The DAF-12 diagram notes the positions of the DNA-binding domain (DBD), hinge, ligand-binding domain (LBD) and *daf-12* mutations. Empty vectors and constructs expressing Lamin C or SV40 T antigen (T) were used as negative controls. Diagrams of the five DAF-12 peptides used are shown with the corresponding amino acids listed to the left. (**B**) A representative experiment in which the LIN-42C bait construct was co-transformed with the various prey constructs. Each co-transformation was grown overnight and four 5-fold serial dilutions were made and pin-replicated from left to right on media lacking leucine and tryptophan (–LT) or also lacking histidine and adenine and containing X-α-gal (–LTHA+X). Positive interactions produced blue colonies on –LTHA+X plates.

DISCUSSION

lin-42 controls developmental timing and dauer formation

We report the identification of the developmental timing protein LIN-42 as a stress-responsive negative regulator of dauer formation. Our work positions LIN-42, a Period (Per) family member, at the nodal point where nutrient sensing interfaces with DAF-12 to mediate the choice between continuous reproductive development and dauer formation. Several lines of evidence converge to place LIN-42 function at the level of DAF-12: (1) when *lin-42* mutants are stressed and inappropriately enter the dauer pathway, they form partial dauers similar to those produced by mutants with defects in dafachronic acid signaling, suggesting misregulation of DAF-12 activity; (2) ectopic LIN-42 expression can suppress the Daf-c phenotype of *daf-9(m540)* and *daf-12(rh273)*, indicating that LIN-42 can act independently of dafachronic acid signaling; and (3) overexpression of *lin-42* in a strong *daf-2* loss-of-function mutant background causes animals to arrest as L3 larvae, a synthetic phenotype described previously for *daf-2(lf); daf-12(lf)* double mutants (Vowels and Thomas, 1992), or *daf-2(lf)* mutants treated with dafachronic acid (Motola et al., 2006), suggesting that *lin-42* and *daf-12* act at a similar position. Furthermore, consistent with a model of interplay between LIN-42 and DAF-12, the precocious defects associated with *lin-42(lf)* during continuous development are partially suppressed in *lin-42(lf); daf-12(LBD)/+* animals (see Table S3 in the supplementary material), even though *daf-12(LBD)/+* animals do not have retarded defects (Antebi et al., 1998). This finding indicates that even a modest increase in ligand-free DAF-12 can compensate for a lack of LIN-42.

Our results indicate that *lin-42* acts antagonistically to *daf-12* to regulate developmental timing and dauer formation, and together these two genes ensure proper development in a changing environment. As depicted in the model in Fig. 7 and as proposed

by Antebi and colleagues (for a review, see Magner and Antebi, 2008), the balance between ligand-free and ligand-bound DAF-12 gates entry to dauer formation. Under favorable growth conditions of mild temperature, low population density and abundant food, dafachronic acids are synthesized and shift the balance of power to ligand-bound DAF-12, thereby disrupting the L3-inhibitory and dauer formation signals of ligand-free DAF-12, and continuous development ensues (Fig. 7A). Under these conditions, the presence of LIN-42 protein is required to prevent inappropriately early initiation of L3 fates (Jeon et al., 1999). Based on the findings reported here, we propose that when animals are stressed and dafachronic acid signaling is lowered, the presence or absence of LIN-42 is a crucial factor in the decision of whether to become a dauer. If animals encounter mild stress that does not necessitate dauer development, dafachronic acid signaling decreases, but the presence of LIN-42 prevents ligand-free DAF-12 from initiating dauer formation (Fig. 7B). By contrast, when harsh environmental growth conditions trigger dauer formation, both LIN-42 and ligand levels drop. The decreased concentrations of these components shift the equilibrium to the ligand-free DAF-12–DIN-1 complex, which, in the absence of LIN-42, now promotes dauer entry through the L2d predauer stage and inhibits L3 development (Fig. 7C). Thus, the interplay of LIN-42 and DAF-12 activities provides the worm with an additional level of modulation of the response to environmental conditions, increasing the robustness of the system and allowing reproductive development in cases of mild stress.

Important challenges for the future are to determine how environmental conditions and/or nutritional status regulate LIN-42 accumulation, and how changes in LIN-42 levels in turn modulate hormonal signaling via DAF-12. An interesting possibility suggested by our yeast two-hybrid assays is that LIN-42 regulates the activity of a DAF-12 transcriptional complex, perhaps with relevance to the recently described role for DAF-12 in modulating levels of heterochronic miRNAs (Bethke et al., 2009; Hammell et al., 2009). LIN-42, however, appears to be properly localized in a *daf-12(0)* mutant, as judged by anti-LIN-42 staining (data not shown), and thus is not an obligate DAF-12 partner for either stability or localization.

Links between heterochronic genes and dauer formation have long been known. For example, *lin-14* activity prevents precocious dauer formation at the L1 molt (Liu and Ambros, 1989), and the phenotypes of most heterochronic mutants are suppressed by development through the dauer pathway (Liu and Ambros, 1989; Euling and Ambros, 1996). However, none of these other heterochronic genes appears to participate in the relationship between *lin-42* and *daf-12*; the role of *lin-42* in modulating the stress response is unique. The LIN-42 PAS domain could be a key component of this aspect of LIN-42 function by contributing to the sensing of growth conditions, as PAS domains are deployed as important environmental sensors in organisms ranging from archaea to humans (Taylor and Zhulin, 1999). Conceivably, PAS domain-containing LIN-42 isoforms could be regulated by metabolic cues. However, transgenic expression of LIN-42D, which lacks the PAS domain, is capable of rescuing the dauer phenotypes, so the PAS domain is not an obligate component of LIN-42-mediated inhibition of dauer entry.

A conserved role for Per family members in responding to environmental stress

This study demonstrates a role for a Per family protein in the mediation of a response to deteriorating environmental conditions and suggests an ancient role for Per proteins in the regulation of stress responses. Similar to the role of *lin-42* in regulating dauer formation, the expression of both mouse and fly Per genes is sensitive to changes in external and intrinsic stressors. *Per1* is rapidly upregulated when mice are exposed to physical stress or hormone fluctuations, suggesting that there is an increased demand for PER1 under stressful conditions (Balsalobre et al., 2000; Takahashi et al., 2001). Similarly, expression of *Drosophila period* is sensitive to a combination of insulin signaling and oxidative stress (Zheng et al., 2007). The dynamic role for LIN-42 in the control of dauer formation demonstrates a stress-responsive role for a Per family member in the nematode, and suggests that stress-induced changes in Per expression might prove to be functionally significant in other organisms.

LIN-42 modulation of ligand-free DAF-12 signaling provides an important insight into the relationship between Per proteins and NR signaling. Using a mechanism that is reminiscent of the genetic hierarchy between *daf-12* and *lin-42*, circadian rhythms in mice are controlled by an elegant feedback loop between Per family

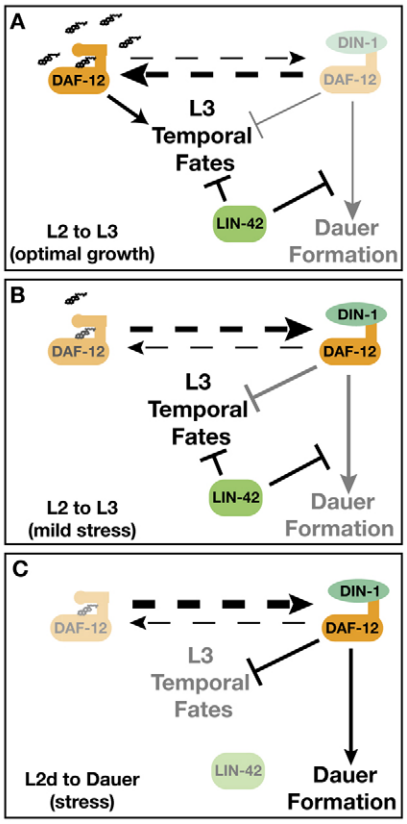

Fig. 7. A model for LIN-42 and DAF-12 genetic interactions. Solid arrows indicate genetic relationships. Dashed arrows represent the equilibrium between ligand-bound and ligand-free forms of DAF-12. Faded colors indicate reduced activity (DAF-12, DIN-1) or absence (LIN-42) of the protein. (**A**) When animals are raised under normal growth conditions, dafachronic acid is present and binds DAF-12, preventing DAF-12–DIN-1 interaction, thereby promoting L2 and L3 development. Under these conditions, LIN-42 prevents inappropriate initiation of L3 temporal fates during the L2 stage. (**B**) Although dafachronic acid signaling is decreased in animals that encounter mild stress, LIN-42 inhibits dauer formation by antagonizing the DAF-12–DIN-1 complex. (**C**) When animals encounter environmental conditions that necessitate dauer formation, the absence of LIN-42 during the L2d allows the DAF-12–DIN-1 complex to promote dauer entry.

members and the NRs REV-ERBα (Nr1d1 – Mouse Genome Informatics) and RORα (Preitner et al., 2002; Sato et al., 2004). As both REV-ERBα and RORα respond to metabolic changes, the interaction between Per proteins and these NRs could potentially coordinate physiology with circadian rhythms (for a review, see Ramakrishnan and Muscat, 2006). Indeed, PER2 was recently found to directly interact with a number of NRs including REV-ERBα, and this interaction with REV-ERBα appears to play a role in the regulation of glycogen storage (Schmutz et al., 2010).

Per proteins are also indirectly associated with other steroid hormone signaling pathways. For example, the levels of cortisol in humans and other mammals fluctuate with a 24-hour circadian cycle (Hellman et al., 1970), and the release of ecdysone in insects is often coordinated with circadian rhythms (Nijhout, 1994). Together with our work, these studies suggest that the interrelationship between Per proteins and environmentally sensitive NR signaling represents an ancient genetic framework by which biological clocks can adapt to changes in external growth conditions. Future analysis of *lin-42* should allow a mechanistic dissection of how a per family member coordinates environmental conditions with intrinsic timing mechanisms.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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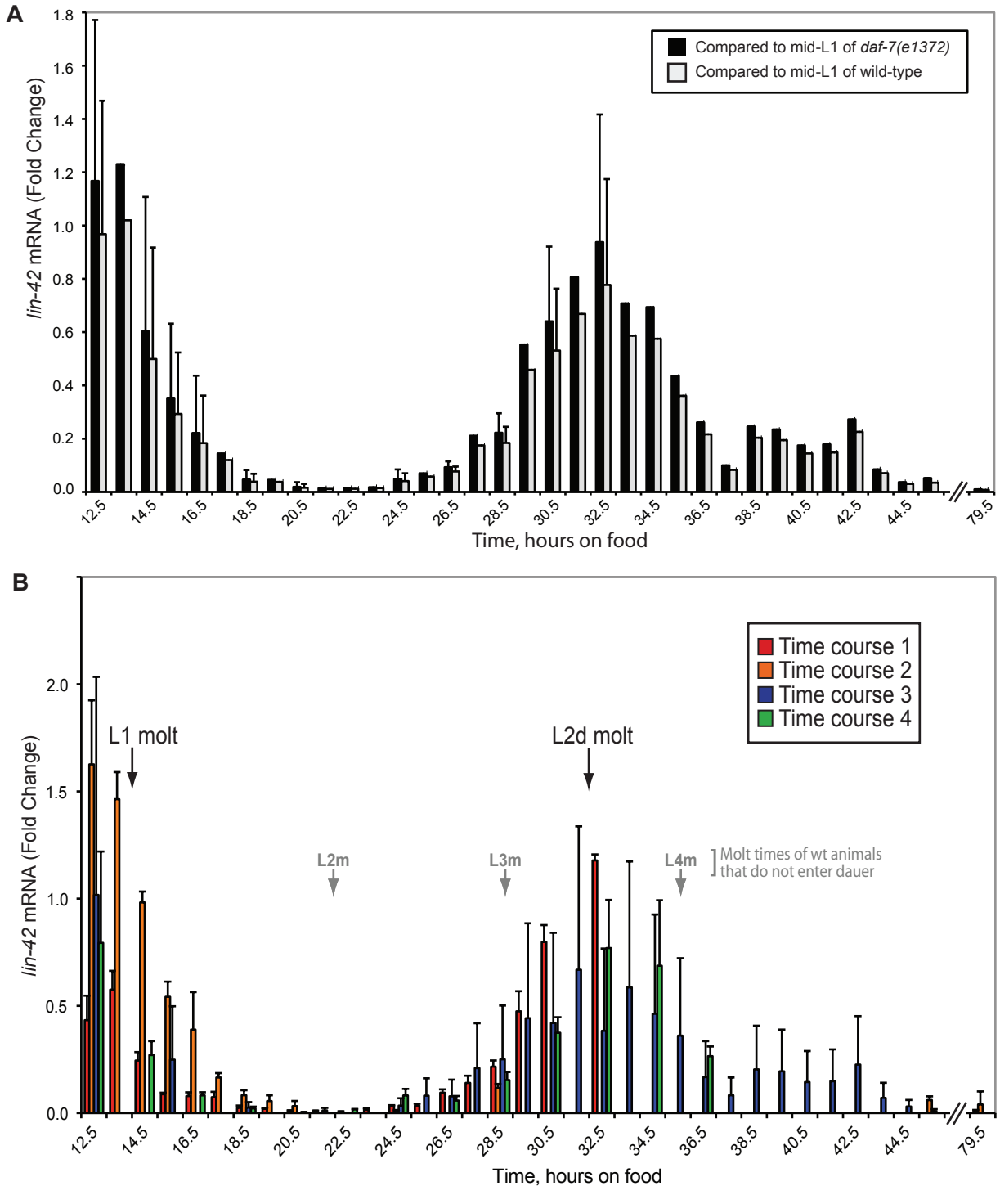
A
lin-42 mRNA (Fold Change)
Compared to mid-L1 of daf-7(e1372)
Compared to mid-L1 of wild-type
Time, hours on food
B
lin-42 mRNA (Fold Change)
Time course 1
Time course 2
Time course 3
Time course 4
L1 molt
L2d molt
L2m
L3m
L4m
Molt times of wt animals that do not enter dauer
Time, hours on food

Table S1. Dauer formation in transgenic lines expressing *lin-42*

Genotype*	Array #	GFP (+)†		GFP (–)‡		Broods scored	Animals scored§
		Dauer formation (%)	s.d.	Dauer formation (%)	s.d.		
lin-42(ve11); daf-12(rh285); Ex[pHG82]	*veEx426*	3	1	67	1	3	301
	veEx582	6		71		1	48
	veEx427	8		69		1	50
daf-7(e1372); Ex[Pdpy-7::lin-42C]	*veEx478*	6	6	97	4	14	817
	veEx479	15	11	99	1	12	1221
	veEx477	26	18	99	1	12	1112
daf-2(e1368); Ex[Pdpy-7::lin-42C]	veEx528	25	19	89	16	22	1552
	veEx529	21	16	96	8	20	1019
	veEx530	14	17	96	9	18	498
daf-2(e1370); Ex[Pdpy-7::lin-42C]	*veEx482*	72	19	99	1	16	291
	veEx480	52	24	100	0	20	448
	veEx481	58	32	99	2	17	427
	veEx531	13	15	98	2	13	676
	veEx532	48	14	96	5	6	551
daf-9(m540); Ex[Pdpy-7::lin-42C]	*veEx583*	48	25	97	3	7	828
	veEx473	11	9	93	8	9	679
	veEx474	8	12	88	25	9	338
	veEx475	2	4	83	32	10	251
daf-12(rh273); Ex[Pdpy-7::lin-42C]	*veEx520*	1	1	93	8	9	687
	veEx521	0	0	91	3	11	649
	veEx522	1	3	98	3	6	292
	veEx523	0	0	91	11	8	285
	veEx524	0	0	100	0	4	141

Strains were grown at 20°C except for the *daf-7* and *daf-2* lines, which were analyzed at 25°C. s.d. could not be calculated for two control strains where a single brood was assayed. The dauer formation data averaged from different strains of the same genotype are presented in Figs 1, 3 and 4.

*Constructs were co-injected with *str-1::gfp* (100 ng/μl) which is expressed in the two AWB neurons (Troemel et al., 1997), which had no effect on dauer formation when assayed alone (see Fig. 1 legend).

†Animals carried the extrachromosomal array as determined by *str-1::gfp* expression.

‡Animals lacked the extrachromosomal array as determined by absence of *str-1::gfp* expression.

§Total number of animals scored for each transgenic line.

Table S2. *lin-42(lf)* does not cause a SynDaf phenotype with *daf-7*, *daf-2* or other mutations for which SynDaf phenotypes have been described

Genotype	Temperature	Dauer formation (%) ± s.d.
daf-7(e1372)	20°C	11±8
daf-7(e1372); lin-42(ve11)	20°C	11±10
daf-7(e1372); lin-42(RNAi)	20°C	17±9
daf-2(e1370)	20°C	0
daf-2(e1370); lin-42(ve11)	20°C	2±3
daf-2(e1370); lin-42(RNAi)	20°C	0
akt-1(ok525)	25°C	0
akt-1(ok525); lin-42(RNAi)	25°C	0
unc-3(e131)	25°C	0
unc-3(e131); lin-42(RNAi)	25°C	0
unc-31(e169)	25°C	0
unc-31(e169); lin-42(RNAi)	25°C	0

Individual hermaphrodites were allowed to lay eggs for 12-16 hours at the indicated temperature on plates seeded with *E. coli* OP50. Two days later each brood was scored for dauer formation. $n>300$ for all genotypes. All RNAi experiments were repeated twice.

Table S3. Semidominant suppression of *lin-42(lf)* by *daf-12(lbd)* mutations

Genotype of parent	Animals with L3 molt alae (%)			*n*
	Complete	Partial	None	
lin-42(mg152)	55	45	0	20
lin-42(n1089)	73	28	0	80
lin-42(ve11)	68	31	0	60
lin-42(ve11); daf-12(rh193)/+	21	43	36	28
lin-42(n1089); daf-12(rh285)/+	10	65	25	20
lin-42(ve11); daf-12(rh285)/+	21	41	38	34
lin-42(mg152); daf-12(rh61)/+	25	40	35	20
lin-42(n1089); daf-12(rh61)/+	18	50	32	22

Only non-Mig, non-SynDaf animals were scored for precocious alae formation at the L3 molt (20°C). The majority of *lin-42; daf-12* homozygotes developed into dauers and those that did not were eliminated from the analysis by their Mig phenotype. Thus, 2/3 of the scored progeny of *lin-42; daf-12*/+ animals should be heterozygous for *daf-12*.