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Specific insulin-like peptides encode sensory information to regulate distinct developmental processes

Astrid Cornils[1], Mario Gloeck[1], Zhunan Chen[2], Yun Zhang[2] and Joy Alcedo[1,*]

SUMMARY

An insulin-like signaling pathway mediates the environmental influence on the switch between the *C. elegans* developmental programs of reproductive growth versus dauer arrest. However, the specific role of endogenous insulin-like peptide (ILP) ligands in mediating the switch between these programs remains unknown. *C. elegans* has 40 putative insulin-like genes, many of which are expressed in sensory neurons and interneurons, raising the intriguing possibility that ILPs encode different environmental information to regulate the entry into, and exit from, dauer arrest. These two developmental switches can have different regulatory requirements: here we show that the relative importance of three different ILPs varies between dauer entry and exit. Not only do we find that one ILP, *ins-1*, ensures dauer arrest under harsh environments and that two other ILPs, *daf-28* and *ins-6*, ensure reproductive growth under good conditions, we also show that *daf-28* and *ins-6* have non-redundant functions in regulating these developmental switches. Notably, *daf-28* plays a more primary role in inhibiting dauer entry, whereas *ins-6* has a more significant role in promoting dauer exit. Moreover, the switch into dauer arrest surprisingly shifts *ins-6* transcriptional expression from a set of dauer-inhibiting sensory neurons to a different set of neurons, where it promotes dauer exit. Together, our data suggest that specific ILPs generate precise responses to dauer-inducing cues, such as pheromones and low food levels, to control development through stimulus-regulated expression in different neurons.

KEY WORDS: Developmental plasticity, Insulin-like peptides, ILP code, Sensory neurons, *Caenorhabditis elegans*

INTRODUCTION

The environment has long been known to influence physiology. In *C. elegans*, the nature of its environment determines its developmental program (Golden and Riddle, 1984). Under conditions of abundant food supply, low population density and optimal temperatures, *C. elegans* develops through four larval stages (L1-L4) to become a reproductive adult (Golden and Riddle, 1982; Golden and Riddle, 1984). However, high population density, food scarcity and/or high temperatures can induce first-stage larvae (L1) to enter a different program, known as dauer arrest (Golden and Riddle, 1982; Golden and Riddle, 1984). Dauers, which are alternative third-stage larvae (L3) and anatomically distinct from L3s grown under optimal conditions, are highly stress-resistant and equipped for long-term survival (Cassada and Russell, 1975; Golden and Riddle, 1982; Golden and Riddle, 1984; Riddle et al., 1981).

The entry into the dauer state depends on a high ratio of a glycosidic pheromone mixture (Butcher et al., 2007; Jeong et al., 2005) to food cues (Golden and Riddle, 1982; Golden and Riddle, 1984). This dauer-inducing pheromone mixture, which is secreted by each animal throughout its life and thus reflects population density, is sensed, together with the food cues, by specific neurons that regulate dauer entry (Bargmann and Horvitz, 1991; Kim et al., 2009; Schackwitz et al., 1996). Conversely, the exit from the dauer state into the last larval stage (L4), prior to becoming fertile adults, is promoted by a subsequent improvement in the environment (Golden and Riddle, 1984), such as an increase in food levels, which is sensed by a different set of neurons (Bargmann and Horvitz, 1991; Ouellet et al., 2008).

An important pathway that mediates the sensory influence on *C. elegans* development is the insulin DAF-2 pathway (Kimura et al., 1997; Riddle et al., 1981; Vowels and Thomas, 1992). Mutations that downregulate the insulin receptor ortholog DAF-2 (Kimura et al., 1997) lead to dauer arrest, which requires the activity of the FOXO transcription factor DAF-16 (Gottlieb and Ruvkun, 1994; Lin et al., 1997; Ogg et al., 1997; Riddle et al., 1981; Vowels and Thomas, 1992). Moreover, whereas a strong downregulation in DAF-2 signaling induces dauers that arrest constitutively, a weaker reduction of DAF-2 activity leads to transient dauer formation (Gems et al., 1998). This suggests that the DAF-2 pathway determines not only when the animals should enter dauer arrest but also when they should exit from it.

Although there is only one known *C. elegans* insulin receptor ortholog, DAF-2 (Kimura et al., 1997), there are 40 insulin-like genes that have been predicted to encode insulin-like peptides (ILPs) (Li et al., 2003; Pierce et al., 2001) (see www.wormbase.org). Many of the ILPs are expressed in overlapping subsets of sensory neurons and/or interneurons, including the sensory neurons (Kodama et al., 2006; Li et al., 2003; Pierce et al., 2001) that regulate dauer entry or exit (Bargmann and Horvitz, 1991; Kim et al., 2009; Ouellet et al., 2008; Schackwitz et al., 1996). Interestingly, the mechanism that regulates entry into dauer arrest differs from the mechanism that regulates exit from this state, not only at the neuronal but also at the molecular level (Bargmann and Horvitz, 1991; Kim et al., 2009; Ouellet et al., 2008; Schackwitz et al., 1996; Tissenbaum et al., 2000). Since the endogenous roles of individual ILPs in these developmental switches are unknown, the different ILP gene expression patterns have led us to consider the hypothesis that they encode discrete sets of sensory information to regulate dauer entry versus exit.

[1]Friedrich Miescher Institute for Biomedical Research, Maulbeerstrasse 66, CH-4058 Basel, Switzerland. [2]Department of Organismic and Evolutionary Biology, Center for Brain Science, Harvard University, Cambridge, MA 02138, USA.

*Author for correspondence (joy.alcedo@fmi.ch)

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Here, we have tested our hypothesis by analyzing the functions of the ILPs *daf-28*, *ins-6* and *ins-1* in regulating these two developmental switches. We find that the ILP requirements for dauer entry versus exit are dissimilar. Surprisingly, we observe that the relative importance of *daf-28* versus *ins-6* in dauer entry is reversed in dauer exit. In addition, we show that environmental information is encoded by these ILPs through cue-driven expression in distinct sensory neurons, which in turn could elicit precise physiological responses by modulating the activities of the affected sensory circuits and/or their target tissues.

MATERIALS AND METHODS

Worm strains and culture

All mutants used in this study were backcrossed six times to the wild-type (N2) strain before any phenotypic analysis was performed. To directly study the relative contributions of the ILPs that have been previously implicated through indirect approaches in regulating the dauer program, we used deletion alleles, with each serving as a molecular null specific for its corresponding ILP (Pierce et al., 2001) (see www.wormbase.org). Worms were continuously fed *E. coli* OP50 for at least two generations before each assay.

Transgenic worms

Independent lines were generated using standard methods and the *ofm-1p::gfp* (Miyabayashi et al., 1999) co-injection marker (injected at 25 ng/μl). For controls, we generated wild-type and mutant worms that carry the *ofm-1p::gfp* co-injection marker alone.

ins-6 rescue lines

To generate the full rescue construct for *ins-6* (pQZ11), we used a 4.2 kb fragment of the *ins-6* genomic locus that includes the 1.7 kb sequence upstream of its start codon and the 2.1 kb sequence downstream of its stop codon, which was inserted into the pCR-BluntII-TOPO vector (Invitrogen, UK). We injected this construct at two different doses (2 and 25 ng/μl) into wild type or *ins-6(tm2416)* mutants. We then crossed the resulting extrachromosomal arrays into (1) the *ins-6(tm2416)*; *daf-28(tm2308)* mutants to assay for rescue of the *ins-6*-dependent dauer entry phenotype or (2) the *ins-6(tm2416)*; *daf-2(e1368)* mutants to test for rescue of the *ins-6*-dependent dauer exit phenotype.

daf-28 rescue lines

To generate the full rescue for *daf-28* (pQZ43), we inserted into the pCR-BluntII-TOPO vector a 4.09 kb fragment of the *daf-28* genomic locus that spans the 3.3 kb sequence upstream of the start codon to the 70 bp region downstream of the stop codon. This construct was introduced as extrachromosomal arrays at two different doses (2ng/μl and 25 ng/μl) into *ins-6(tm2416)*; *daf-28(tm2308)* or *daf-2(e1368)*; *daf-28(tm2308)* mutants.

ILP expression lines

To determine the *ins-1* expression pattern, we generated a transcriptional *ins-1p::cfp* reporter construct (pQZ6) using the Gateway Technology vectors (Invitrogen). In pQZ6, *cfp* is flanked by the 4.3 kb sequence upstream of the *ins-1* start codon and by the 1.1 kb sequence downstream of the *ins-1* stop codon. In addition, the 0.8 kb sequence of the largest intron, which might contain regulatory elements required for expression, is fused downstream of the 3′ cis sequences. pQZ6 was injected into wild-type worms at 100 ng/μl. Three independent lines were recovered, which show identical patterns of *cfp* expression.

To determine the *ins-6* expression pattern, we constructed a transcriptional *ins-6p::mCherry* reporter (pQZ10) using the Gateway system. The *mCherry* is flanked by the 1.7 kb sequence upstream of the *ins-6* start codon and by the 2.0 kb sequence downstream of the *ins-6* stop codon. pQZ10 was injected into wild-type worms at 100 ng/μl. Three lines were recovered, which have the same *mCherry* expression pattern.

Generation of worms genetically ablated for ASJ neurons

To genetically ablate the ASJ neurons, we drove human caspase 1 (*ICE*, or *CASP1*) [a gift of V. Maricq (Zheng et al., 1999)] transcription from the 1 kb thioredoxin (*trx-1*) promoter [gift of P. Swoboda (Miranda-Vizuete et al., 2006)] as *trx-1p::ICE* in the pPD95.77 vector (pQZ37). *trx-1* is specifically expressed in the ASJ neurons (Miranda-Vizuete et al., 2006). We introduced pQZ37 at 100 ng/μl into either *ins-6(tm2416)*; *daf-2(e1368)* mutants or *daf-2(e1368)* mutants.

Dauer entry assays

The worms were grown at 25°C and allowed to lay eggs at this temperature for 3-7 hours. The eggs were then allowed to develop either at 25°C or 27°C and scored ~24 hours after egg-laying for L1 arrest phenotypes. Like wild type, we observed no L1 arrest phenotypes for any worms carrying deletions in *ins-1*, *ins-6* and/or *daf-28* at these temperatures. Forty-eight hours after the egg-laying midpoint, the fraction of dauers and L4s/adults was counted. Animals carrying transgenes or the *daf-2* or *daf-16* mutation were grown at 20°C and allowed to lay eggs for 3-7 hours, which were then allowed to develop either at 20°C or shifted to 22.5°C, 25°C or 27°C. At 48 hours after the egg-laying midpoint, the fraction of dauers and L4s/adults was counted. To compare the different genotypes, we used the Wilcoxon Mann-Whitney rank sum test (Hothorn et al., 2008; R Development Core Team, 2009).

Dauer exit assays

The eggs that were laid for 3-7 hours at 20°C were shifted to 25°C. All dauers that were formed 48 hours after the egg-laying midpoint were collected and scored daily for dauer exit. We used JMP 5.1 (SAS) software to determine Kaplan-Meier probability estimates of dauer exit events and for all statistical comparisons. *P*-values were determined by the log-rank test.

To measure dauer exit in the ASJ-ablated and control animals, eggs that were laid for 4 hours at 20°C were transferred to 25°C on 3.5-cm plates containing 5 ml nematode-growth (NG) agar (Brenner, 1974), 150 μl of a crude dauer pheromone mixture [prepared according to Thomas et al. (Thomas et al., 1993)] and 50 μl *E. coli* OP50. After 48 hours, all dauers were collected and transferred onto plates lacking the exogenously added pheromone. These dauers were again scored daily at 25°C for exit into the L4 stage.

Analyses of ILP gene expression in response to environmental cues

Dauer pheromone

To assess the effect of dauer pheromone on ILP gene expression, we placed ~100 embryos on 3.5-cm NG agar plates, to which 100-200 μl dauer pheromone and 50 μl *E. coli* OP50 were added. The concentration of the crude dauer pheromone mixture used in these assays caused ~45-55% of wild-type L1s to form dauers at 25°C. We monitored the ILP gene expression of the different larvae and adults that subsequently developed on these plates.

Starvation

The effect of low food availability on ILP gene expression was tested by several methods. First, we compared the ILP gene expression of well-fed larvae and young adults with those of age-matched larvae (L1 and L4) and young adults from starved plates. Second, we bleached gravid adults to collect a number of eggs that either (1) were placed directly on NG agar plates in the absence of food, which caused the animals to undergo L1 arrest or (2) were permitted to develop to L1 or L2 on plates with food, before being harvested and washed at least twice with M9 buffer (Lewis and Fleming, 1995) to remove the bacteria. These harvested L1 and L2 animals were then placed on plates without food, which caused the animals to arrest as L2 or L3, respectively. The starved animals were all scored for ILP gene expression within a day, and sometimes for several days afterwards.

Temperature

To determine the influence of temperature, the ILP gene expression of animals that developed at 20°C was compared with that of age-matched animals that developed at 27°C from eggs laid at 20°C.

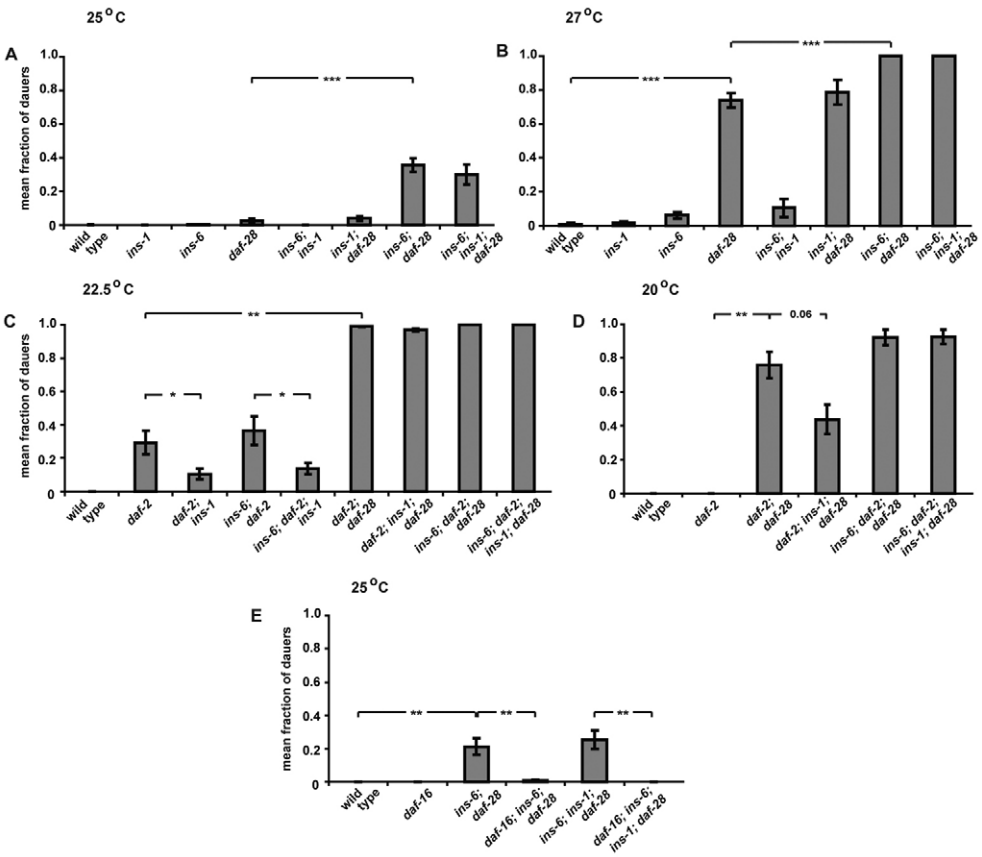

Fig. 1. *daf-28* acts with *ins-6* to inhibit dauer entry, whereas *ins-1* promotes it. (**A**,**B**) The mean fractions of wild type and insulin-deletion *C. elegans* mutants that form dauers at 25°C (A) or 27°C (B). Each mean ± s.e.m. includes at least three independent trials of ~100 worms per trial. The detailed statistical comparisons between the dauer entry phenotypes of different genotypes under different conditions in this and subsequent figures can be found in Table S1 in the supplementary material. (**C**,**D**) The effect of different insulin deletions on the dauer entry of *daf-2(e1368)* mutants at 22.5°C (C) and 20°C (D). (**E**) The dauer entry of *ins-6*; *daf-28* deletion mutants is suppressed by *daf-16(mu86)* at 25°C. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$.

RESULTS

daf-28 has a more prominent role than *ins-6* in inhibiting dauer entry

Previous gain-of-function experiments suggested that the ILPs *daf-28*, *ins-6* and *ins-1* play a role in dauer entry (Li et al., 2003; Malone et al., 1996; Pierce et al., 2001). However, these earlier studies involved indirect manipulations of ILP function, which do not allow the direct comparison of the endogenous contributions of specific ILPs in regulating not only dauer entry but also dauer exit. Thus, to directly test the role of each ILP in these two processes, we studied deletion mutants in which gene function is completely and specifically eliminated. We first examined the dauer entry phenotypes of the worms carrying the following single or combined deletions: *daf-28(tm2308)*, *ins-6(tm2416)* and *ins-1(nr2091)*. Like wild type, the *ins-6* and *ins-1* deletion mutants formed no dauers at 25°C and very few dauers at 27°C, a temperature known to stimulate dauer entry (Fig. 1A,B and see Table S1 in the supplementary material). By contrast, the *daf-28* deletion mutants formed few dauers at 25°C and a much larger fraction of dauers at 27°C (Fig. 1A,B and see Table S1 in the supplementary material). In addition, loss of *ins-6* enhanced the dauer entry phenotype of *daf-28* deletion mutants at both temperatures, whereas removing *ins-1* did not (Fig. 1A,B and see Table S1 in the supplementary material). Together, these data indicate that *daf-28* acts with *ins-6* to inhibit dauer entry, which is consistent with the reported rescue of the dauer formation phenotype of the gain-of-function *daf-28(sa191)* mutant upon overexpression of wild-type *daf-28* or *ins-6* (Li et al., 2003). At the same time, by comparing null mutants, we unexpectedly identified a stronger role for *daf-28* than *ins-6* in inhibiting this process.

Because *daf-28* and *ins-6* might encode ligands for the DAF-2 receptor, we tested the effect of these two ILPs on the temperature-sensitive dauer entry phenotype of the reduction-of-function *daf-2(e1368)* mutants. At temperatures that induce few or no *daf-2(e1368)* dauers, loss of *daf-28* strongly enhanced dauer entry in these *daf-2* mutants, whereas the *ins-6* deletion had little or no effect (Fig. 1C,D and see Table S1 in the supplementary material). Since *daf-2* requires the activity of *daf-16* to regulate dauer formation (Riddle et al., 1981), we next tested whether the same is true for *ins-6*; *daf-28* double mutants. We found that the dauer entry of *ins-6*; *daf-28* mutants was suppressed by loss of *daf-16* (Fig. 1E and see Table S1 in the supplementary material), which suggests that DAF-28 and INS-6 activate the DAF-2 receptor to inhibit dauer entry via inhibition of DAF-16.

ins-1 promotes dauer entry

Next, we analyzed how *ins-1* interacts with *daf-28* and *ins-6* in the presence of wild-type or downregulated *daf-2* activity. Unlike *daf-28* and *ins-6*, deletion of *ins-1* suppressed dauer entry in *daf-2(e1368)* at 22.5°C (Fig. 1C and see Table S1 in the supplementary material). Likewise, loss of *ins-1* decreased the number of dauers formed by *ins-6*; *daf-2(e1368)* mutants (Fig. 1C and see Table S1 in the supplementary material). By contrast, an *ins-1* deletion only suppressed the dauer entry of the *daf-2*; *daf-28* double mutants at a lower temperature, 20°C, which is a weaker dauer-inducing condition, and not at 22.5°C, a stronger dauer-inducing condition (Fig. 1C,D and see Table S1 in the supplementary material). Consistent with these observations, loss of *ins-1* also had no effect on dauer entry in *ins-6*; *daf-2(e1368)*; *daf-28* triple mutants, which all formed dauers at both temperatures (Fig. 1C,D and see Table S1 in the supplementary

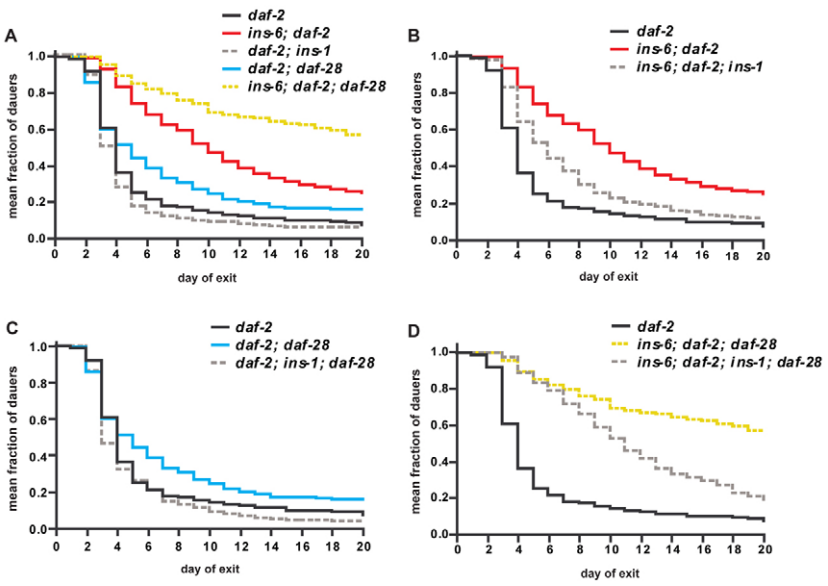

Fig. 2. *ins-6* acts with *daf-28* to promote dauer exit, whereas *ins-1* inhibits it. (**A-D**) The rates of dauer exit at 25°C of animals carrying different combinations of insulin deletions in a *daf-2(e1368)* mutant background. Each curve represents cumulative data from six independent trials. All curves are significantly different from that of the *daf-2(e1368)* control ($P \leq 0.001$, log-rank test). The complete statistical comparisons between the dauer exit phenotypes of the different groups of animals in this and subsequent figures are shown in Table S2 in the supplementary material.

material). Thus, these findings suggest that *ins-1* functions to promote dauer entry, which is in agreement with the increased dauer formation previously observed in weak reduction-of-function *daf-2* mutants that overexpress wild-type *ins-1* (Pierce et al., 2001). Yet, these present studies also suggest that *ins-1* only weakly antagonizes the activity of the DAF-2 pathway in regulating this switch between the developmental programs.

ins-6 has a more prominent role than *daf-28* in promoting dauer exit

DAF-2 signaling also regulates exit from dauer arrest (Gems et al., 1998; Kao et al., 2007). For example, through a loss-of-function mutation, *daf-28* has been shown to affect exit from the dauer state (Kao et al., 2007). To determine whether *ins-6* and *ins-1* also regulate dauer exit, we analyzed the effects of loss of these two ILPs, either singly or in combination and with or without *daf-28*, on the dauer exit phenotype of *daf-2(e1368)* mutants, which are known to form dauers at 25°C that exit after a few days (Gems et al., 1998). We found that deletion of *ins-6* in *daf-2(e1368)* mutants strongly inhibited dauer exit, but removal of *daf-28* only slightly delayed it (Fig. 2A and see Table S2 in the supplementary material). Moreover, removal of both *ins-6* and *daf-28* in *daf-2* mutants caused the greatest delay in dauer exit (Fig. 2A and see Table S2 in the supplementary material). Thus, unpredicted from previous studies on dauer entry (*daf-28* and *ins-6*) and dauer exit (*daf-28*) (Kao et al., 2007; Li et al., 2003; Malone et al., 1996), these data indicate that although both ILPs act together to promote dauer exit, *ins-6* has a more significant role than *daf-28* in this process.

This difference in the relative importance of *ins-6* in dauer entry versus exit was also reflected by the *ins-6* levels required to rescue dauer entry versus those needed to rescue dauer exit. A low level of *ins-6* was sufficient to rescue the dauer entry of *ins-6*; *daf-28* double mutants back to that of *daf-28* single mutants (Fig. 3A,B and see Table S1 in the supplementary material). Conversely, the dauer exit phenotype of *ins-6*; *daf-2* double mutants could only be fully rescued to the exit phenotype of *daf-2* single mutants with high levels of *ins-6* (Fig. 4A,B and see Table S2 in the supplementary material). It should be noted that high *ins-6* levels did not completely rescue the dauer entry of *ins-6*; *daf-28* double mutants back to wild type (Fig. 3B and see Table S1 in the supplementary material), which again suggests that these ILPs do not act completely redundantly with each other.

Similarly, we showed that the *daf-28* levels that were necessary to rescue the two phenotypes also reflected the greater requirement for *daf-28* in dauer entry versus exit. A higher level of *daf-28* was needed to rescue the dauer entry of *ins-6*; *daf-28* mutants (Fig. 3C,D and see Table S1 in the supplementary material), whereas a lower level of *daf-28* was sufficient to rescue the dauer exit phenotype of *daf-2*; *daf-28* double mutants back to that of *daf-2* single mutants (Fig. 4C,D and see Table S2 in the supplementary material). Together, our data indicate that the ILP regulation of dauer entry is different from that of dauer exit: higher *daf-28* levels are required to inhibit entry than to promote exit, whereas the reverse is true for *ins-6* (see Fig. 6B).

ins-1 inhibits dauer exit

Overexpression of *ins-1* has been shown to increase dauer formation (Pierce et al., 2001). However, it is unclear from that study whether *ins-1* regulates dauer entry or exit or both. Since we already showed that *ins-1* promotes dauer entry (Fig. 1C,D and see Table S1 in the supplementary material), we next analyzed *ins-1* for a role in dauer exit. We observed that loss of *ins-1*, which had little effect on dauer exit in *daf-2* single mutants (Fig. 2A and see Table S2 in the supplementary material), enhanced dauer exit in all other *daf-2* mutants that lack *ins-6* and/or *daf-28* (Fig. 2B-D and see Table S2 in the supplementary material). This suggests that *ins-1* not only plays a role in dauer entry but also in dauer exit and that the wild-type *ins-1* function is to ensure dauer arrest under harsh environments.

ins-6 expression switches between two sensory neurons to control dauer entry versus exit

The switch between reproductive growth and dauer arrest is regulated by specific sensory neurons (Bargmann and Horvitz, 1991; Schackwitz et al., 1996) that express some ILP genes (Li et al., 2003; Pierce et al., 2001). Dauer entry is inhibited by the sensory neurons ADF, ASI and ASG (Bargmann and Horvitz, 1991) and is promoted by the sensory neurons ASJ and ASK (Kim et al., 2009; Schackwitz et al., 1996). However, dauer exit is

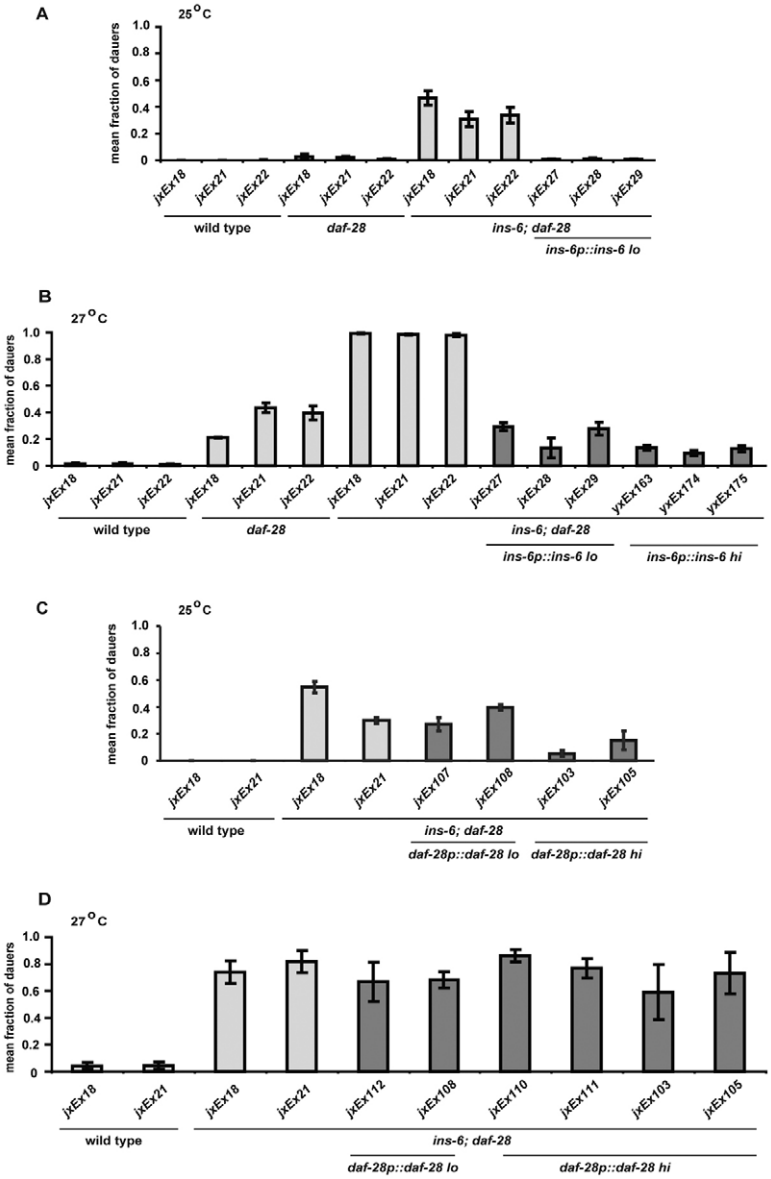

Fig. 3. Inhibition of dauer entry requires low levels of *ins-6* and high levels of *daf-28*. (**A**,**B**) The mean fractions of dauers at 25°C (A) or 27°C (B) in *ins-6*; *daf-28* mutants rescued with low (2 ng/μl, lo) or high (25 ng/μl, hi) levels of *ins-6* are compared with those of wild-type *C. elegans* or with insulin-deletion mutants that carry the *ofm-1p::gfp* co-injection marker alone (*jxEx18*, *jxEx21* or *jxEx22*). (**C**,**D**) The mean fractions of dauers at 25°C (C) or 27°C (D) in *ins-6*; *daf-28* mutants rescued with low (2 ng/μl) or high (25 ng/μl) levels of *daf-28* are compared with those of wild type carrying the co-injection marker alone. The *daf-28* levels needed to fully rescue the dauer entry phenotype of the *ins-6*; *daf-28* mutants are higher than 25 ng/μl. Error bars represent s.e.m.

inhibited by the sensory neurons IL2 and promoted by the sensory neurons ASJ and AWC (Bargmann and Horvitz, 1991; Ouellet et al., 2008).

Although *daf-28* is expressed in ASI and ASJ of well-fed animals and is downregulated in both neurons by low food availability, a dauer pheromone mixture or entry into dauer arrest (Li et al., 2003), the cells from which *ins-1* or *ins-6* might act to regulate the developmental switches are unknown. *ins-1* is expressed in many neurons, including those that regulate entry into and exit from the dauer program (Kodama et al., 2006; Tomioka et al., 2006). Unlike *daf-28*, the switch in developmental programs had little or no effect on *ins-1* expression in ASI and ASJ (Table 1), as determined from a *cfp* transcriptional reporter fused to the *ins-1* 5′ and 3′ cis regulatory sequences.

By contrast, we observed that *ins-6* expression, which was based on an *mCherry* transcriptional reporter fused to the upstream and downstream regulatory regions of *ins-6* (*ins-6p::mCherry*), is rare in non-neuronal tissues and restricted to the ASI neurons of well-fed larvae and adults (Fig. 5). This is different from the previously described expression of *ins-6* in many neurons (Pierce et al., 2001), including ASI (A.C. and J.A., data not shown), which was determined with a *gfp* or an *mCherry* transcriptional reporter fused only to the *ins-6* upstream sequences. This suggests that sequences downstream of *ins-6* contain element(s) that repress its expression in other cells.

Interestingly, the switch into dauer arrest shifted the transcription of *ins-6p::mCherry* from ASI to ASJ (Fig. 5B,C, Table 1). In addition, as the animals started to exit from dauer, ASJ expression of *ins-6p::mCherry* appeared to become stronger (A.C. and J.A., data not shown) in response to the improved environment. We also saw this activation of *ins-6* in the ASJ of worms carrying a transcriptional reporter fused only to the *ins-6* upstream sequences (A.C. and J.A., data not shown). Together, our data suggest that *ins-6* functions in ASI to inhibit dauer entry, and that it also functions in ASJ to promote dauer exit.

Since overexpression of *ins-6* from either ASI or ASJ was able to rescue both the dauer entry phenotype of *ins-6*; *daf-28* mutants and the dauer exit phenotype of *ins-6*; *daf-2* mutants (see Figs S1 and S2 and Tables S1 and S2 in the supplementary material), we asked whether endogenous *ins-6* acts primarily in ASJ to promote dauer exit. We analyzed worms in which the ASJ neurons were genetically

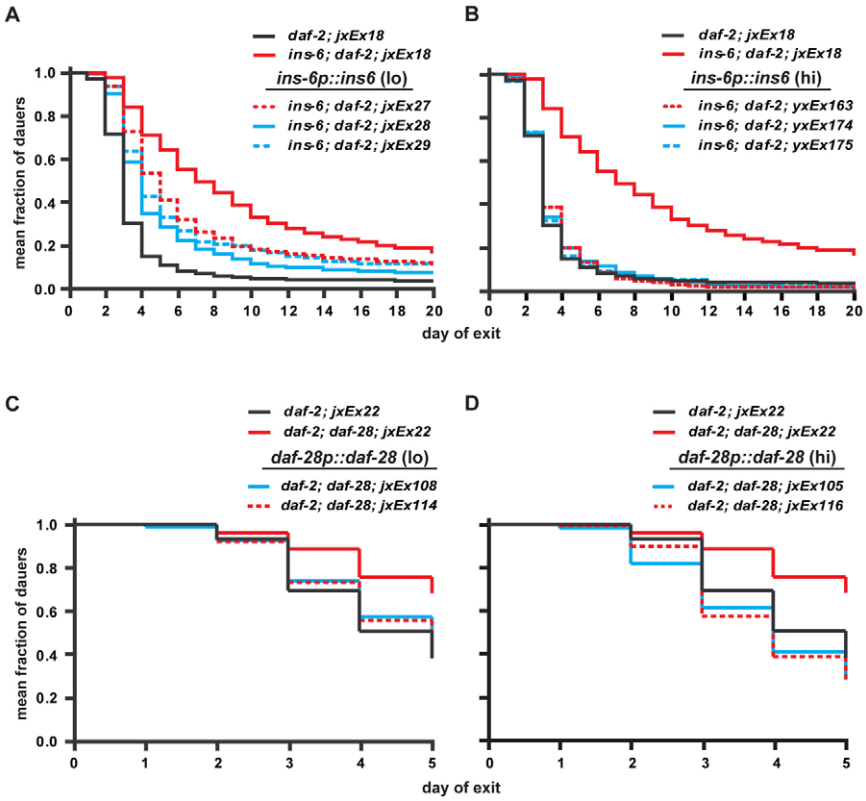

Fig. 4. Unlike dauer entry, higher *ins-6* levels and lower *daf-28* levels are required to promote dauer exit. (**A**,**B**) The rates of dauer exit of *ins-6*; *daf-2(e1368)* mutants that were rescued with low (A) or high (B) *ins-6* levels are compared with those of *daf-2(e1368)* or *ins-6*; *daf-2(e1368)* mutants that carry the *ofm-1p::gfp* co-injection marker alone (*jxEx18*). Each curve represents the cumulative data from at least seven trials. The low-expressing *ins-6* rescue lines are significantly different from the *daf-2* control ($P<0.0001$), whereas the high-expressing *ins-6* rescue lines behave the same as the *daf-2* control. (**C**,**D**) The rates of dauer exit of *daf-2(e1368)*; *daf-28* mutants that were rescued with low (C) or high (D) *daf-28* levels are compared with those of *daf-2(e1368)* or *daf-2(e1368)*; *daf-28* mutants that carry the *jxEx22* co-injection marker. Each curve represents the cumulative data from three trials. See Table S2 in the supplementary material for a comparison of the rescue lines with other, additional control lines.

ablated through the ASJ-specific expression of human caspase 1 (Zheng et al., 1999). As expected, ASJ ablation delayed the exit of *daf-2* single-mutant dauers (Fig. 6A and see Table S2 in the supplementary material). Nonetheless, killing ASJ also largely abolished the difference between the dauer exit phenotypes of *daf-2* single and *ins-6*; *daf-2* double mutants (Fig. 6A and see Table S2 in the supplementary material). These findings support the hypothesis that *ins-6* expression shifts to ASJ to induce the switch from dauer arrest to reproductive growth.

The dauer pheromone and the dauer state have distinct effects on *ins-6* expression

We next asked which cues would downregulate *ins-6p::mCherry* in ASI and which cues would induce it in ASJ. We observed that high concentrations of the dauer-inducing pheromone downregulated *ins-6p::mCherry* in the ASI of pre-dauer (L2d) or L4 larvae or adults (Table 1). However, the dauer pheromone mixture by itself induced little or no *ins-6p::mCherry* expression in the ASJ of different stages of well-fed larvae and adults (Table 1). Surprisingly, induction of dauer arrest by shifting *daf-2(e1368)* mutants to 25°C, but under low pheromone levels, was also insufficient to fully activate *ins-6p::mCherry* in ASJ (Table 1). Indeed, the switch in *ins-6p::mCherry* from ASI to ASJ was only completely executed in dauers that were induced by high levels of dauer pheromone, either by the direct addition of the pheromone mixture or by high population density (Table 1). Since we also never observed *ins-6p::mCherry* expression in the ASJ of partial dauers, our data suggest that the combined activities of the dauer state and the dauer pheromone, which presumably induces a stronger arrest, are required for this expression shift (Table 1).

In addition, we found that starvation alone or high temperature (27°C) had little or no effect on *ins-6p::mCherry* expression, as compared with well-fed worms at 20°C (Fig. 5F,G, Table 1). Moreover, we detected no significant effect on *ins-6p::mCherry* expression in continuously well-fed animals lacking *daf-16* or having reduced *daf-2* activity (Table 1). Together, our findings suggest that the downregulation of *ins-6p::mCherry* in ASI is a specific response to the dauer pheromone cue, whereas the switch in neuronal expression is a distinct response that is specific to the coordinated action of the pheromone and the dauer state.

ins-6 and *daf-28* play only a minor role in regulating lifespan

Since our observations suggested that *ins*-6, *daf-28* and *ins-1* have discrete, non-redundant functions in regulating two developmental programs, we asked whether they also affect lifespan, which is known to be regulated by DAF-2 (Kenyon et al., 1993; Kimura et al., 1997). Unlike *daf-2* reduction-of-function mutants, we found that *ins-6*, *daf-28* or *ins-1* alone had little or no effect on adult lifespan (see Fig. S3 in the supplementary material). Similarly, loss of both *ins-6* and *daf-28* had little effect on the lifespan of animals that did not undergo dauer formation (see Fig. S3B,D in the supplementary material). However, *ins-6*; *daf-28* double mutants that formed transient dauers did live longer than double mutants that never became dauers (see Fig. S3B,D in the supplementary material). This suggests that post-dauer adults are not physiologically the same as well-fed adults that have not undergone dauer arrest. Consistent with this idea, we found that *ins-6p::mCherry* surprisingly persisted in ASJ and was absent in the ASI of post-dauer L4s, young adults and 5-day-old adults (Fig. 5E, Table 1), which is different from the situation for continuously well-fed animals that expressed *ins-6p::mCherry* in ASI and never in ASJ (Fig. 5D, Table 1). Our findings also suggest that, to regulate lifespan, other ILPs with a more primary role than *ins-6*, *daf-28* and *ins-1* in this process are required to modulate DAF-2 signaling.

Table 1. Specific cues have distinct effects on ILP gene expression

		ASIL/R expression				ASJL/R expression			
Condition/stage	Total *n*	None	Weak	Medium	Strong	None	Weak	Medium	Strong
ins-6p::mCherry									
Well-fed									
L1	23	1	0	22	0	23	0	0	0
L2	17	1	0	16	0	17	0	0	0
L3	49	6	0	43	0	49	0	0	0
L4	80	4	0	76	0	80	0	0	0
Adult	56	3	0	53	0	56	0	0	0
Dauer	121	92	29	0	0	1	0	0	120
Post-dauer L4	38	37	1	0	0	0	0	0	38
Post-dauer adult	53	50	3	0	0	2	0	0	51
Pheromone									
L1	9†	1	0	8	0	8	1	0	0
L2d	46‡	8	38	0	0	41	5	0	0
L4	28	10	18	0	0	28	0	0	0
Adult	3†	3	0	0	0	3	0	0	0
Starvation									
L1	51	7	0	44	0	51	0	0	0
L2	21	2	0	19	0	21	0	0	0
L3	10‡	2	0	8	0	10	0	0	0
L4	25	4	0	21	0	25	0	0	0
Adult	2†	0	0	2	0	2	0	0	0
27°C									
L1	28	2	0	26	0	26	2	0	0
L2	16‡	2	0	14	0	16	0	0	0
L3	5†	0	0	5	0	5	0	0	0
L4	26	1	0	25	0	26	0	0	0
daf-2(e1368)									
L1	33‡	3	0	30	0	33	0	0	0
L2	26‡	4	0	22	0	26	0	0	0
L3	11‡	0	0	11	0	11	0	0	0
L4	77‡	6	0	71	0	77	0	0	0
Adult	6†	1	0	5	0	6	0	0	0
Dauer, high pheromone	42‡	36	6	0	0	1	0	0	41
Dauer, low pheromone	31‡	5	1	25	0	24	0	7	0
daf-16(mu86)									
L1	23‡	1	0	22	0	23	0	0	0
L2	18‡	0	0	18	0	18	0	0	0
L3	13‡	0	0	13	0	13	0	0	0
L4	68‡	3	0	65	0	68	0	0	0
Adult	33‡	3	0	30	0	33	0	0	0
Partial dauer	14‡	0	14	0	0	14	0	0	0
ins-1p::cfp									
Well-fed L3	20‡,*	0	0	20	0	0	0	0	20
Dauer	20‡,*\	0	0	20	0	0	0	7	13

ins-6p::mCherry or *ins-1p::cfp* expression in different wild-type and mutant stages that developed under different treatments. The wild-type dauers were induced either by addition of high dauer pheromone levels or high population density. The post-dauers were induced either by shifting dauers to new plates with high food levels and/or lower temperatures. *daf-2* mutants were assayed under well-fed conditions at 20°C, with the exception of dauers, which were induced by overcrowding at 20°C (high pheromone) or by temperature (25°C; low pheromone). *daf-16* mutants were either well-fed or starved at 20°C (partial dauers). The total number of animals assayed for expression in both left and right neurons of ASI (ASIL/R) and ASJ (ASJL/R) comes from three independent transgenic lines, unless indicated otherwise.
*Expression is present in additional head and tail neurons.
†Analyzed only one line.
‡Analyzed only two lines.

DISCUSSION

The large number of ILP genes in *C. elegans* and the spatiotemporal diversity of their expression patterns, which includes partly overlapping subsets of sensory neurons (Li et al., 2003; Pierce et al., 2001), raise the likelihood that different ILPs regulate different processes in response to diverse stimuli. In this study, we show that the ILP regulation of dauer entry versus exit is more complex than previously thought. We observe not only that *daf-28* and *ins-6* have growth-promoting activities that oppose those of *ins-1*, but also that the relative requirement for *daf-28* and *ins-6* switches between dauer entry and exit. Accordingly, our data suggest that ILPs function as a combinatorial code to regulate *C. elegans* development in response to complex sensory cues.

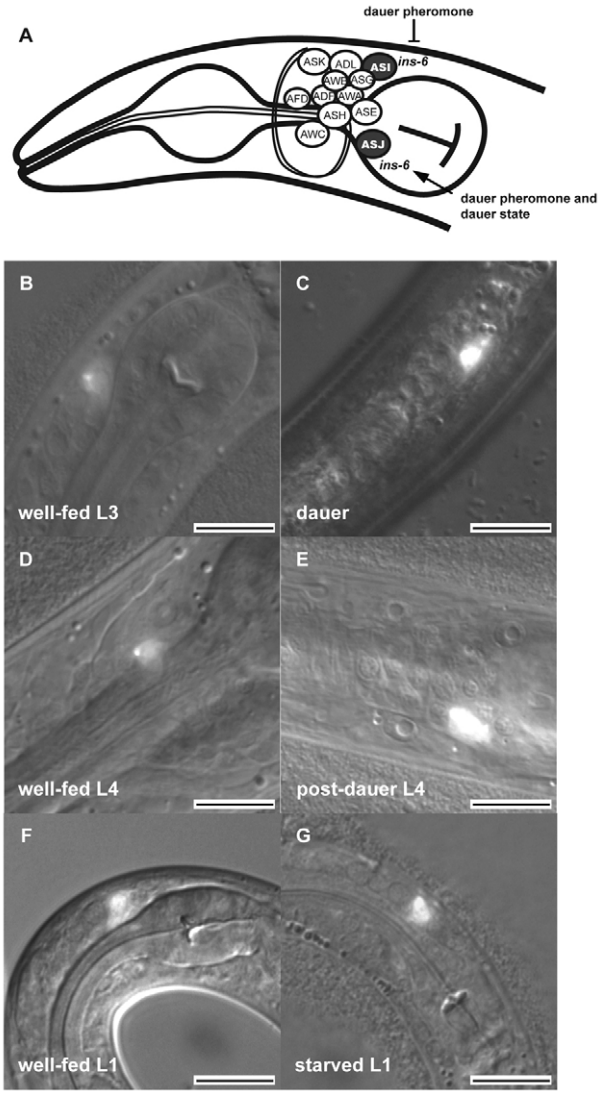

Fig. 5. *ins-6* transcription switches between ASI and ASJ in response to dauer pheromone and dauer arrest. (**A**) The twelve sensory neurons in the *C. elegans* amphid sensory organ (White et al., 1986). The ASI and ASJ neurons are indicated in black. Dauer pheromone inhibits *ins-6* transcription in ASI, whereas both the pheromone and the dauer state activate *ins-6* in ASJ. (**B**,**D**,**F**) *ins-6p::mCherry* is expressed in the ASI of well-fed L3 (B), L4 (D) and L1 (F) larvae. (**C**,**E**) *ins-6p::mCherry* becomes expressed in the ASJ of a dauer larva (C) and remains on in the ASJ of a post-dauer L4 larva (E). (**G**) *ins-6p::mCherry* is unaffected in ASI and is not activated in the ASJ of a starved L1 larva. All animals are oriented with their anterior to the lower left and their dorsal side up. Scale bars: 10 μm.

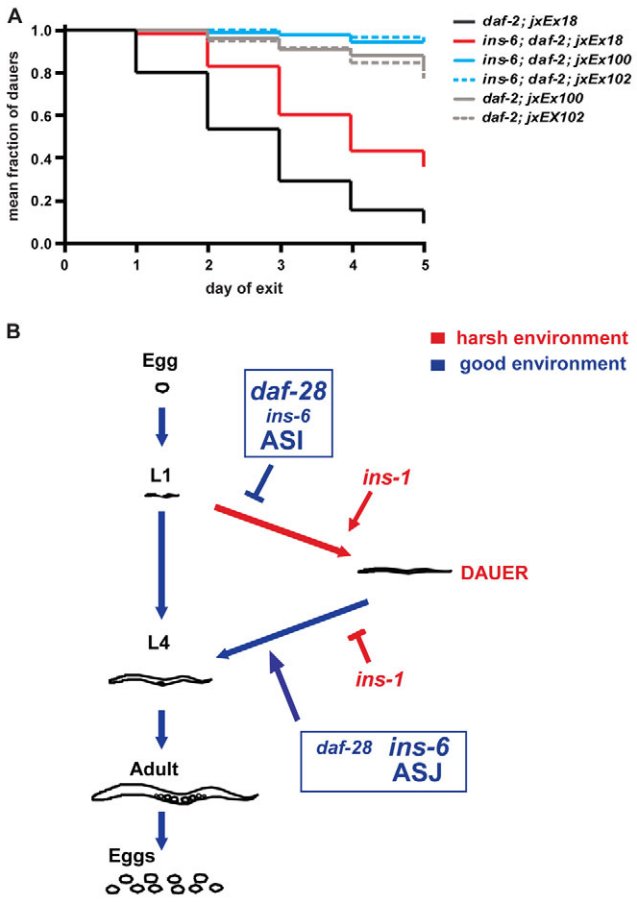

Fig. 6. *ins-6* acts primarily from ASJ to promote dauer exit. (**A**) The rates of dauer exit of *daf-2(e1368)* single and *ins-6; daf-2(e1368)* double mutants, in which ASJ is genetically ablated (*jxEx100* and *jxEx102*). Control lines are *daf-2(e1368)* and *ins-6*; *daf-2(e1368)* mutants that carry the *ofm-1p::gfp* co-injection marker alone (*jxEx18*). The slight difference observed between the dauer exit phenotypes of *daf-2* and *ins-6*; *daf-2* mutants upon ASJ ablation might be due to the incomplete loss of ASJ in all animals, as this neuron can still sometimes be seen in some of the post-dauers (data not shown). (**B**) A model for *daf-28*, *ins-6* and *ins-1* function in dauer regulation. *daf-28* and *ins-6* inhibit dauer entry and promote dauer exit, whereas *ins-1* promotes dauer entry and inhibits dauer exit. Whereas *daf-28* plays a more prominent role in inhibiting dauer entry (depicted with *daf-28* in larger font), *ins-6* has a more primary role in promoting dauer exit (depicted with *ins-6* in larger font). The sensory neurons in which *daf-28* and/or *ins-6* might function to regulate the different developmental switches are also shown.

Individual ILPs encode specific cues to regulate distinct switches in developmental programs

In *C. elegans*, the ratio between food cues and dauer pheromone, which reflect food availability and population density, determines whether L1 larvae will undergo dauer arrest or whether dauers will exit from arrest (Golden and Riddle, 1982; Golden and Riddle, 1984). The perception of the food and pheromone cues by a specific subset of sensory neurons that either inhibit (ASI, ADF and ASG) or promote (ASJ and ASK) dauer entry or promote (ASJ and AWC) or inhibit (IL2) dauer exit (Bargmann and Horvitz, 1991; Kim et al., 2009; Macosko et al., 2009; Ouellet et al., 2008; Schackwitz et al., 1996) in turn regulates the secretion of growth-modulatory signals. For example, low food levels and high dauer pheromone concentrations repress the expression in ASI of the TGFβ *daf-7*, which is required for reproductive development under growth-inducing conditions (Ren et al., 1996; Schackwitz et al., 1996). However, re-introduction of food to a dauer population induces dauer exit and resumption of *daf-7* expression and reproductive development (Ren et al., 1996).

Similarly, food cues activate transcription of the ILP *daf-28* in ASI and ASJ, whereas the dauer pheromone suppresses it in both neurons (Li et al., 2003). However, food levels and dauer

pheromone have different effects on other ILPs (Fig. 5, Table 1). For example, both cues have little or no effect on *ins-1* mRNA levels in ASI and ASJ (Table 1). By contrast, high levels of dauer pheromone specifically repress the transcription of *ins-6* in ASI, whereas food levels do not affect *ins-6* expression in this neuron (Fig. 5, Table 1). Interestingly, other cues derived from dauer arrest-induced physiological changes, together with high pheromone levels, trigger *ins-6* transcription in ASJ (Fig. 5, Table 1), a change in expression that alone is insufficient to promote dauer exit. This suggests that (1) the animal initiates *ins-6* expression in this dauer exit-promoting neuron to facilitate its activation as soon as environmental conditions improve and (2) *ins-6* activity is also regulated post-transcriptionally by other cues, perhaps such as increases in food levels. Thus, it is possible that different sensory cues regulate ILP function not only at the level of transcription but also at the level of translation and/or secretion. Indeed, this might be the case for *ins-1*, for which we observe no transcriptional changes in response to food or pheromone signals (Table 1). The specificity in the effects of different cues on the spatiotemporal expression of *daf-28*, *ins-6* and *ins-1* suggests a mechanism through which these ILPs encode environmental information and consequently regulate dauer entry versus exit.

Our findings also suggest that *ins-6* and *daf-28* encode ligands that have differing requirements in activating the DAF-2 pathway during the two developmental switches, whereas *ins-1* antagonizes the activity of this pathway (Fig. 6B). These ligands might modulate DAF-2 signaling in a context-dependent manner. For example, besides having been shown to act as an antagonist of the pathway in regulating the dauer program (Pierce et al., 2001) (this paper) and food-associated thermotactic behavior (Kodama et al., 2006), *ins-1* can also act like a DAF-2 agonist in salt-chemotactic learning behavior (Tomioka et al., 2006). Thus, to control a particular process, *ins-1* and other ILPs may act from specific neurons to regulate DAF-2 signaling in a specific subset of cells, in either an autocrine or paracrine manner. Previous mosaic analyses of *daf-2* function have already raised the possibility that different cells have different DAF-2 activities (Apfeld and Kenyon, 1998; Wolkow et al., 2000). Yet, none of our experiments has ruled out the possibility that some of these ILPs, which are predicted to have diverse structures [e.g. DAF-28 or INS-6 compared with INS-1 (Pierce et al., 2001)], will bind receptors other than DAF-2.

It should also be noted that, unlike *daf-2* reduction-of-function mutants (Kenyon et al., 1993; Larsen et al., 1995), *ins-6*, *daf-28* and *ins-1*, which function combinatorially to regulate development (Fig. 6B), have little or no effect on lifespan (see Fig. S3 in the supplementary material). Since lifespan is influenced by many types of cues and sensory neurons (Alcedo and Kenyon, 2004; Apfeld and Kenyon, 1999; Lee and Kenyon, 2009; Libert et al., 2007; Poon et al., 2010), it is not surprising that many cue-responsive ILPs would be involved in regulating DAF-2 activity to affect longevity.

ins-6 activity depends on spatial context

The switch in *ins-6* expression from ASI to ASJ upon dauer arrest (Fig. 5, Table 1) suggests that *ins-6* activity, and perhaps that of other ILPs, depends on the spatial context of its expression. Indeed, the expression of *ins-6* in the dauer-inhibiting ASI neurons of well-fed larvae is consistent with *ins-6* function in suppressing entry into dauer arrest (Fig. 6B). Similarly, *ins-6* expression in ASJ upon dauer arrest is in keeping with its role in promoting dauer exit (Fig. 6B), i.e. INS-6 is secreted from ASJ to induce exit.

However, exogenous *ins-6* expression in either ASI or ASJ not only inhibits entry into, but also promotes exit from, dauer (see Figs S1 and S2 and Tables S1 and S2 in the supplementary material). Although this might be due to much higher than endogenous expression of *ins-6*, *daf-2(e1368)* dauers formed under low pheromone still exit from arrest when *ins-6* remains primarily expressed in ASI and does not completely switch to ASJ (Table 1). Nonetheless, high pheromone-induced ASJ-ablated *daf*-2 single-mutant and *ins-6*; *daf-2* double-mutant dauers have very similar exit phenotypes (Fig. 6A and see Table S2 in the supplementary material). Since high pheromone-induced dauers with intact ASJ do exhibit the neuronal switch in *ins-6* expression (Table 1), the ASJ ablation experiment suggests that *ins-6* acts from ASJ under this condition, and not from other neurons, to promote exit.

These observations also suggest that temperature-induced, low pheromone-exposed *daf-2(e1368)* dauers might represent a weaker arrest, thus explaining the absence of the *ins-6* shift from ASI to ASJ (Table 1). By contrast, the high pheromone-induced dauers might reflect a stronger arrest, which induces the full dauer transcriptional program, including the altered *ins-6* expression (Table 1). *ins-6* may become specifically upregulated in ASJ after a strong arrest because ASJ has the receptors that sense such an arrest and the inputs that induce exit from it. Our data further raise the possibility that the circuit that induces exit after a weak arrest, which might or might not be similar to a dauer entry-regulating circuit, is different from the circuit that induces exit after a stronger arrest. Accordingly, INS-6, as well as other ILPs, might act locally as part of different developmental circuits that are remodeled in response to cues, such as the combination of dauer pheromone with the dauer state.

Post-dauer adults are distinct from continuously well-fed adults

Our observation that *ins-6* is expressed in a different sensory neuron in post-dauer adults suggests that these animals might exhibit a different physiology from continuously fed adults. Consistent with this hypothesis, the duration of the dauer state has been shown to correlate positively with the number of reproductive defects in post-dauer adults (Kim and Paik, 2008). Moreover, *ins-6*; *daf-28* post-dauer adults live 11% longer than the continuously fed double mutants (see Fig. S3 in the supplementary material). This is similar to a recent observation that wild-type post-dauer adults live longer than their continuously fed counterparts (Hall et al., 2010). This suggests that (1) the dauer state causes a physiological change that is sufficient to induce a small but significant extension in adult lifespan and (2) this lifespan extension is not necessarily dependent on *ins-6* and *daf-28*.

The relevance of multiple ILPs in other animals

The concept that ILPs encode environmental information to regulate physiology might be true not only for *C. elegans* but also for other animals, such as *Drosophila* or mammals. *Drosophila* has seven known ILPs, *dilp1-7* (*Ilp1-7* – FlyBase), which are expressed in neuronal and/or non-neuronal cells (Brogiolo et al., 2001; Ikeya et al., 2002; Rulifson et al., 2002; Slaidina et al., 2009; Yang et al., 2008). Intriguingly, the neurons that express some of these ILPs send or receive projections from subesophageal ganglion interneurons, which in turn receive information (Melcher and Pankratz, 2005; Rulifson et al., 2002; Yang et al., 2008) from gustatory neurons that innervate chemosensory structures within the fly mouthparts (Scott et al., 2001). In addition, some of these neuronally expressed ILPs (*dilp2*, *dilp3* and *dilp5*) have been

proposed to regulate growth and metabolism in a nutrient level-dependent manner, whereas others do not (Broughton et al., 2008; Grönke et al., 2010; Ikeya et al., 2002; Min et al., 2008; Zhang et al., 2009). Some are also required in other processes, such as in selecting an optimal environment for egg laying, which is *dilp7* dependent (Yang et al., 2008).

By comparison, mammals have seven to ten known members of the insulin/relaxin superfamily that are expressed in non-overlapping cells, including neurons with known sensory-associated functions (Ayer-le Lievre et al., 1991; Bathgate et al., 2002; Liu and Lovenberg, 2008; Meyts et al., 2009; Sherwood, 2004). The roles of insulin, IGF1 and IGF2 in mammalian metabolism, growth, differentiation and lifespan have been studied in great detail (Kenyon, 2005; Nakae et al., 2001; Sherwood, 2004), and some relaxins have been found to regulate reproductive, as well as non-reproductive, processes (Sherwood, 2004). However, the functions of other members of this family are less clear. Since the effects of ILP signaling on physiology (e.g. growth and lifespan) are conserved from worms to mammals (Blüher et al., 2003; Holzenberger et al., 2003; Kenyon et al., 1993; Taguchi et al., 2007), our study raises the possibility that specific subsets of mammalian ILPs also act together to regulate specific processes in response to different sets of environmental cues.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material for this article is available at
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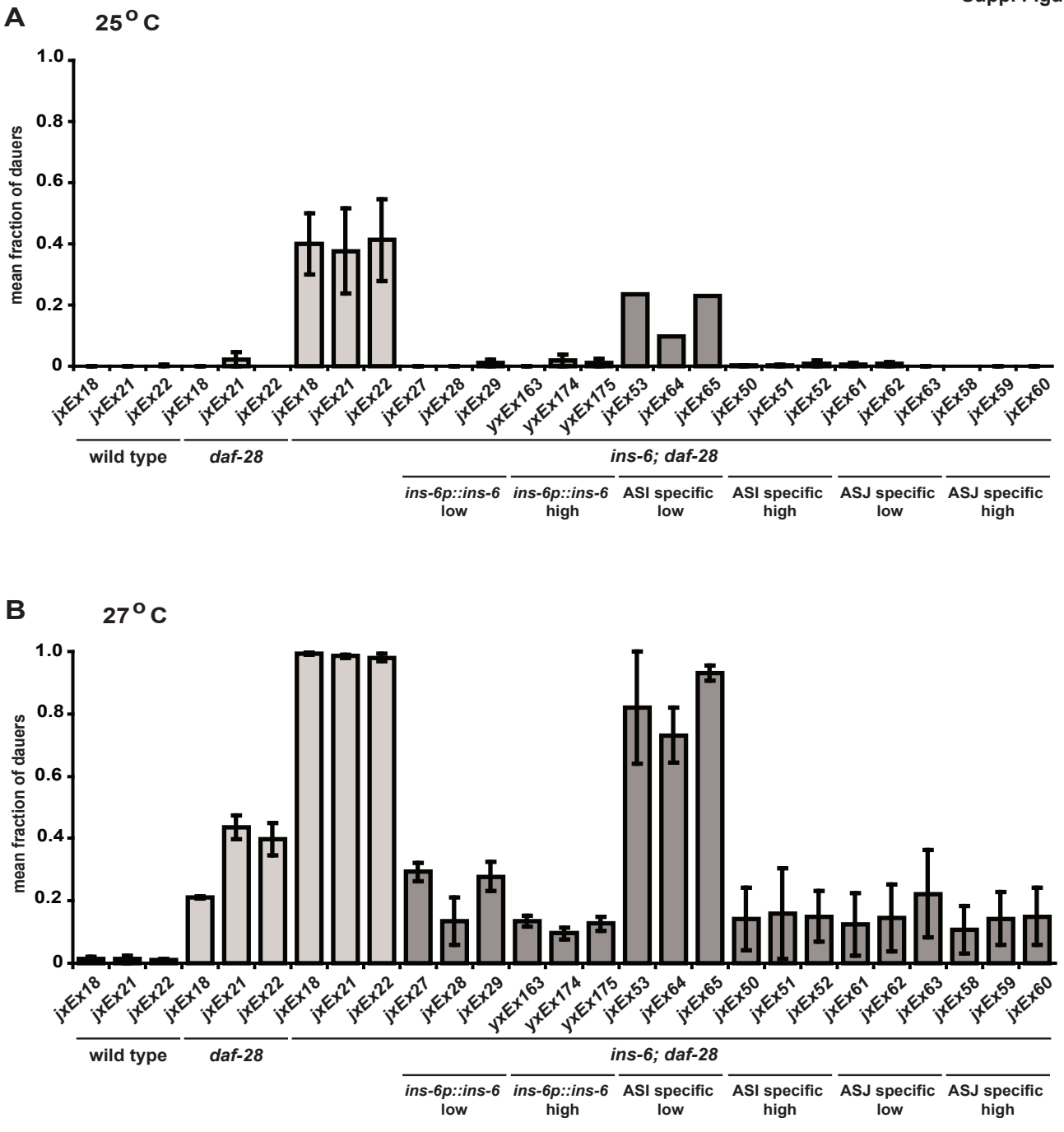
A
25°C
mean fraction of dauers
1.0
0.8
0.6
0.4
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0
jxEx18
jxEx21
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jxEx27
jxEx28
jxEx29
yxEx163
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jxEx65
jxEx50
jxEx51
jxEx52
jxEx61
jxEx62
jxEx63
jxEx58
jxEx59
jxEx60
wild type
daf-28
ins-6; daf-28
ins-6p::ins-6 low
ins-6p::ins-6 high
ASI specific low
ASI specific high
ASJ specific low
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B
27°C
mean fraction of dauers
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jxEx58
jxEx59
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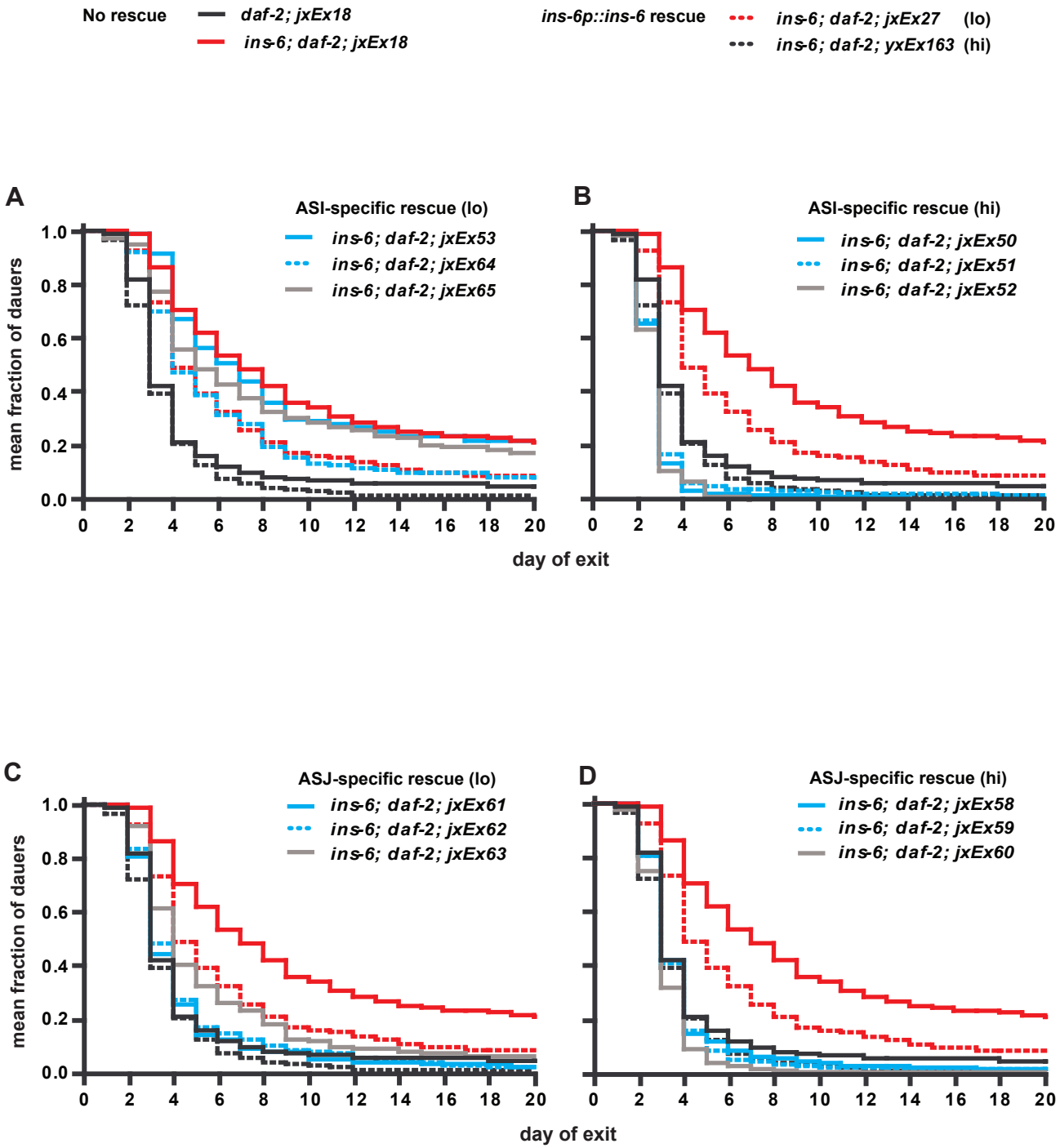
wild type
daf-28
ins-6; daf-28
ins-6p::ins-6 low
ins-6p::ins-6 high
ASI specific low
ASI specific high
ASJ specific low
ASJ specific high


No rescue
daf-2; jxEx18
ins-6; daf-2; jxEx18
ins-6p::ins-6 rescue
ins-6; daf-2; jxEx27 (lo)
ins-6; daf-2; yxEx163 (hi)
A
ASI-specific rescue (lo)
ins-6; daf-2; jxEx53
ins-6; daf-2; jxEx64
ins-6; daf-2; jxEx65
B
ASI-specific rescue (hi)
ins-6; daf-2; jxEx50
ins-6; daf-2; jxEx51
ins-6; daf-2; jxEx52
mean fraction of dauers
day of exit
C
ASJ-specific rescue (lo)
ins-6; daf-2; jxEx61
ins-6; daf-2; jxEx62
ins-6; daf-2; jxEx63
D
ASJ-specific rescue (hi)
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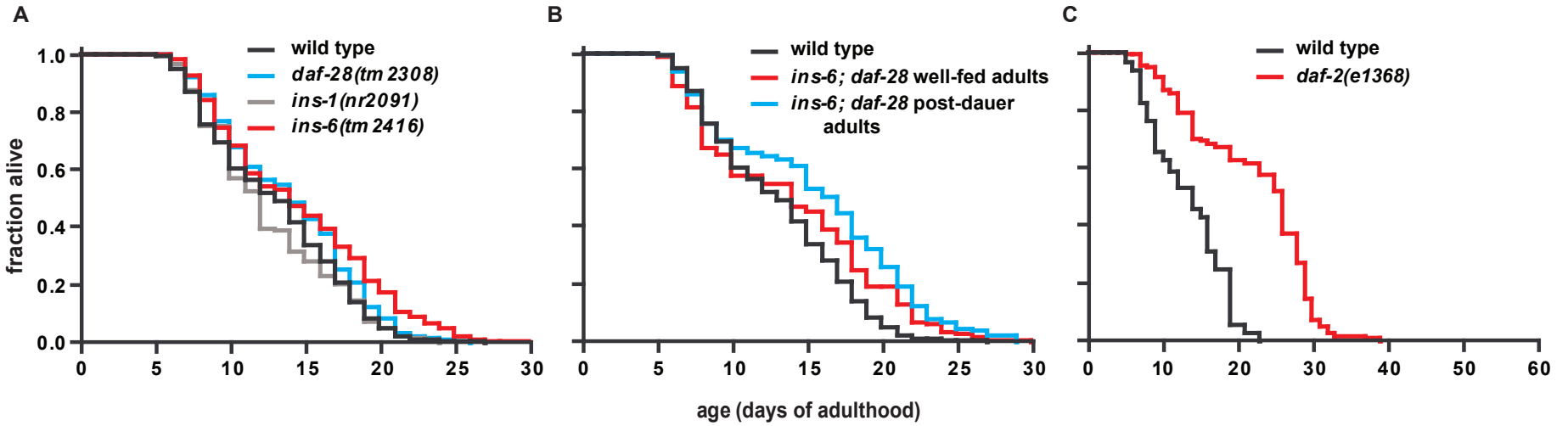
ins-6; daf-2; jxEx59
ins-6; daf-2; jxEx60
mean fraction of dauers
day of exit



D

Table. Adult lifespans of insulin mutants

Strain	Mean Lifespan ± SEM (Days)	75th Percentile (Days)	No. Animals Observed / Total Initial Animals	% Wild Type	*P* Value Against Wild Type (Logrank)	
25°C						
Wild Type	13.0 ± 0.1	17	1177/1389 (10)			
ins-6(tm2416)	14.5 ± 0.3	19	483/597 (6)	+12	< 0.0001	
daf-28(tm2308)	13.9 ± 0.2	18	570/700 (7)	+7	< 0.0001	
ins-1(nr2091)	12.6 ± 0.3	16	279/397 (4)	-3	0.35	
ins-6(tm2416); daf-28(tm2308)						
continuously well-fed adults	13.9 ± 0.4	18	208/299 (3)	+7	< 0.0001	+11 (*P* = 0.01)
post-dauer adults	15.4 ± 0.4	21	207/300 (3)	+19	< 0.0001	
20°C → 25°C						
Wild Type	13.3 ± 0.4	17	170/200 (2)			
ins-6(tm2416)	13.6 ± 0.4	19	150/200 (2)	+3	0.38	
daf-28(tm2308)	12.8 ± 0.5	17	82/100 (1)	-4	0.33	
ins-1(nr2091)	12.2 ± 0.5	16	71/100 (1)	-8	0.03	
daf-2(e1368)	22.1 ± 0.6	29	152/100 (2)	+66	< 0.0001	

Table S1. Dauer entry phenotypes of wild-type and insulin-deficient worms

Strain/treatment	Mean fraction of dauers ± s.e.m.	Total no. of animals observed (no. of trials)	*P*-value against control*	*P*-value against specified groups
25°C				
Wild type*	0.00±0.00	1005 (10)		
ins-1(nr2091)	0.00±0.00	368 (4)	n.s.	
ins-6(tm2416)	0.00±0.00	566 (6)	n.s.	
daf-28(tm2308)	0.02±0.01	705 (7)	0.004	
ins-6; *ins-1*	0.00±0.00	277 (3)	n.s.	
ins-1; *daf-28*	0.04±0.01	494 (5)	0.004	n.s.†
ins-6; *daf-28*	0.36±0.04	1061 (10)	<0.0001	0.0006†
ins-6; *ins-1*; *daf-28*	0.30±0.06	392 (3)	0.002	n.s.‡
20°C				
Wild type	0.00±0.00	608 (6)	n.s.	
*daf-2(e1368)**	0.00±0.00	595 (6)		
daf-2; *daf-28*	0.76±0.08	444 (4)	0.002	
daf-2; *ins-1*; *daf-28*	0.44±0.09	621 (6)	0.004	0.06§
ins-6; *daf-2*; *daf-28*	0.92±0.05	437 (4)	0.004	n.s.§
ins-6; *daf-2*; *ins-1*; *daf-28*	0.93±0.04	419 (4)	0.004	n.s.¶
27°C				
Wild type*	0.01± 0.01	1262 (13)		
ins-1	0.02±0.01	730 (8)	n.s.	
ins-6	0.06±0.02	849 (9)	0.007	
daf-28	0.74±0.04	873 (9)	<0.0001	
ins-6; *ins-1*	0.11±0.05	353 (4)	0.04	
ins-1; *daf-28*	0.79±0.07	605 (6)	0.0003	n.s.†
ins-6; *daf-28*	1.00±0.00	903 (9)	<0.0001	0.0001†
ins-6; *ins-1*; *daf-28*	1.00±0.00	328 (3)	0.004	n.s.‡
22.5°C				
Wild type	0.00±0.00	488 (5)	0.009	
*daf-2**	0.29±0.07	487 (5)		
daf-2; *ins-1*	0.10±0.03	476 (5)	0.03	
ins-6; *daf-2*	0.36±0.08	487 (5)	n.s.	
ins-6; *daf-2*; *ins-1*	0.14±0.03	487 (5)	0.08	0.03**
daf-2; *daf-28*	0.99±0.00	509 (5)	0.009	
daf-2; *ins-1*; *daf-28*	0.97±0.01	510 (5)	0.009	0.05§
ins-6; *daf-2*; *daf-28*	1.00±0.00	503 (5)	0.005	
ins-6; *daf-2*; *ins-1*; *daf-28*	1.00±0.00	299 (3)	0.02	n.s.¶
25°C				
Wild type	0.00±0.00	486 (5)		
*daf-16**	0.00±0.00	501 (5)	n.s.	
ins-6; *daf-28*	0.21±0.05	504 (5)	0.005	0.005††
daf-16; *ins-6*; *daf-28*	0.01±0.01	491 (5)	n.s.	0.008‡
ins-6; *ins-1*; *daf-28*	0.25±0.06	426 (5)	0.005	n.s.‡
daf-16; *ins-6*; *ins-1*; *daf-28*	0.00±0.00	458 (5)	n.s.	0.005‡‡
***ins-6* rescues**				
25°C				
ofm-1p::gfp (25 ng)				
wild type; *jxEx18**	0.00±0.00	594 (5)		
wild type; *jxEx21**	0.00±0.00	477 (5)		
wild type; *jxEx22**	0.00±0.00	477 (5)		
daf-28; *jxEx18*	0.03±0.02	337 (5)	n.s.§§	0.008***
daf-28; *jxEx21*	0.02±0.01	516 (5)	0.05§§	0.009***
daf-28; *jxEx22*	0.01±0.01	414 (4)	n.s.§§	0.01***
ins-6; *daf-28*; *jxEx18*	0.47±0.05	554 (5)	0.005§§	
ins-6; *daf-28*; *jxEx21*	0.31±0.06	559 (5)	0.005§§	
ins-6; *daf-28*; *jxEx22*	0.34±0.06	532 (5)	0.007§§	
Full rescue (low) *ins-6p::ins-6* (2 ng); *ofm-1p::gfp* (25 ng)				

ins-6; *daf-28*; *jxEx27*	0.01±0.00	638 (5)	<0.01[¶¶]	
ins-6; *daf-28*; *jxEx28*	0.01±0.01	682 (5)	<0.01[¶¶]	
ins-6; *daf-28*; *jxEx29*	0.01±0.00	571 (5)	<0.01[¶¶]	
Full rescue (high) *ins-6p::ins-6* (25 ng); *ofm-1p::gfp* (25 ng)				
ins-6; *daf-28*; *yxEx163*	0.00±0.00	175 (2)		
ins-6; *daf-28*; *yxEx174*	0.02±0.02	264 (2)		
ins-6; *daf-28*; *yxEx175*	0.01±0.01	215 (2)		
ASI-specific rescue (low) *str-3p::ins-6* (2 ng); *ofm-1p::gfp* (25 ng)				
ins-6; *daf-28*; *jxEx53*	0.23	47 (1)		
ins-6; *daf-28*; *jxEx54*	0.01	96 (1)		
ins-6; *daf-28*; *jxEx64*	0.10	114 (1)		
ins-6; *daf-28*; *jxEx65*	0.23	105 (1)		
ins-6; *daf-28*; *jxEx66*	0.26	100 (1)		
ASI-specific rescue (high) *str-3p::ins-6* (25 ng); *ofm-1p::gfp* (25 ng)				
ins-6; *daf-28*; *jxEx50*	0.00±0.00	479 (2)		
ins-6; *daf-28*; *jxEx51*	0.00±0.00	300 (2)		
ins-6; *daf-28*; *jxEx52*	0.01±0.01	264 (2)		
ASJ-specific rescue (low) *trx-1p::ins-6* (2 ng); *ofm-1p::gfp* (25 ng)				
ins-6; *daf-28*; *jxEx61*	0.01±0.01	378 (2)		
ins-6; *daf-28*; *jxEx62*	0.01±0.01	284 (2)		
ins-6; *daf-28*; *jxEx63*	0.00±0.00	219 (2)		
ASJ-specific rescue (high) *trx-1p::ins-6* (25 ng); *ofm-1p::gfp* (25 ng)				
ins-6; *daf-28*; *jxEx58*	0.00	134 (1)		
ins-6; *daf-28*; *jxEx59*	0.00±0.00	412 (2)		
ins-6; *daf-28*; *jxEx60*	0.00±0.00	241 (2)		
27°C				
ofm-1p::gfp (25 ng)				
wild type; *jxEx18**	0.01±0.01	320 (3)		
wild type; *jxEx21**	0.01±0.01	312 (3)		
wild type; *jxEx22**	0.01±0.01	345 (3)		
daf-28; *jxEx18*	0.21±0.00	232 (2)	0.08[§§]	0.08***
daf-28; *jxEx21*	0.44±0.04	328 (3)	0.05[§§]	0.05***
daf-28; *jxEx22*	0.40±0.05	333 (3)	0.05[§§]	0.05***
ins-6; *daf-28*; *jxEx18*	0.99±0.00	286 (3)	0.05[§§]	
ins-6; *daf-28*; *jxEx21*	0.98±0.00	292 (3)	0.05[§§]	
ins-6; *daf-28*; *jxEx22*	0.99±0.01	326 (3)	0.05[§§]	
Full rescue (low) *ins-6p::ins-6* (2 ng); *ofm-1p::gfp* (25 ng)				
ins-6; *daf-28*; *jxEx27*	0.29±0.03	360 (3)	<0.05[¶¶]	
ins-6; *daf-28*; *jxEx28*	0.13±0.08	308 (3)	<0.05[¶¶]	
ins-6; *daf-28*; *jxEx29*	0.28±0.05	312 (3)	<0.05[¶¶]	
Full rescue (high) *ins-6p::ins-6* (25 ng); *ofm-1p::gfp* (25 ng)				
ins-6; *daf-28*; *yxEx163*	0.13±0.02	373 (3)	<0.05[¶¶]	
ins-6; *daf-28*; *yxEx174*	0.10±0.02	357 (3)	<0.05[¶¶]	
ins-6; *daf-28*; *yxEx175*	0.13±0.02	344 (3)	<0.05[¶¶]	
ASI-specific rescue (low) *str-3p::ins-6* (2 ng); *ofm-1p::gfp* (25 ng)				

ins-6; *daf-28*; *jxEx53*	0.82±0.18	70 (2)	n.s.[¶¶]	
ins-6; *daf-28*; *jxEx54*	0.12±0.09	303 (3)	<0.05[¶¶]	
ins-6; *daf-28*; *jxEx64*	0.73±0.09	310 (3)	<0.05[¶¶]	
ins-6; *daf-28*; *jxEx65*	0.93±0.02	152 (2)	0.08[¶¶]	
ins-6; *daf-28*; *jxEx66*	0.86±0.09	179 (2)	0.08[¶¶]	
ASI-specific rescue (high) *str-3p::ins-6* (25 ng); *ofm-1p::gfp* (25 ng)				
ins-6; *daf-28*; *jxEx50*	0.14±0.10	339 (3)	<0.05[¶¶]	
ins-6; *daf-28*; *jxEx51*	0.16±0.14	350 (3)	<0.05[¶¶]	
ins-6; *daf-28*; *jxEx52*	0.15±0.08	372 (3)	<0.05[¶¶]	
ASJ-specific rescue (low) *trx-1p::ins-6* (2 ng); *ofm-1p::gfp* (25 ng)				
ins-6; *daf-28*; *jxEx61*	0.12±0.10	325 (3)	<0.05[¶¶]	
ins-6; *daf-28*; *jxEx62*	0.15±0.11	301 (3)	<0.05[¶¶]	
ins-6; *daf-28*; *jxEx63*	0.22±0.14	342 (3)	<0.05[¶¶]	
ASJ-specific rescue (high) *trx-1p::ins-6* (25 ng); *ofm-1p::gfp* (25 ng)				
ins-6; *daf-28*; *jxEx58*	0.11±0.07	301 (3)	<0.05[¶¶]	
ins-6; *daf-28*; *jxEx59*	0.14±0.08	310 (3)	<0.05[¶¶]	
ins-6; *daf-28*; *jxEx60*	0.15±0.09	351 (3)	<0.05[¶¶]	
***daf-28* rescues**				
25°C				
ofm-1p::gfp (25 ng)				
wild type; *jxEx18*	0.00±0.00	315 (3)		
wild type; *jxEx21*	0.00±0.00	320 (3)		
ins-6; *daf-28*; *jxEx18*	0.55±0.04	416 (3)	0.04[§§]	
ins-6; *daf-28*; *jxEx21*	0.30±0.02	347 (3)	0.04[§§]	
Full rescue (low) *daf-28p::daf-28* (2 ng); *ofm-1p::gfp* (25 ng)				
ins-6; *daf-28*; *jxEx107*	0.27±0.05	391 (3)	0.04[†††]	0.05[‡‡‡] n.s.[§§§]
ins-6; *daf-28*; *jxEx108*	0.40±0.02	344 (3)	0.04[†††]	0.05[‡‡‡,§§§]
Full rescue (high) *daf-28p::daf-28* (25 ng); *ofm-1p::gfp* (25 ng)				
ins-6; *daf-28*; *jxEx103*	0.05±0.02	379 (3)	0.04[†††]	0.05[‡‡‡,§§§]
ins-6; *daf-28*; *jxEx105*	0.15±0.07	352 (2)	0.05[†††]	0.08[‡‡‡,§§§]
27°C				
ofm-1p::gfp (25 ng)				
wild type; *jxEx18*	0.04±0.03	255 (3)		
wild type; *jxEx21*	0.04±0.03	282 (3)		
daf-28; *jxEx18*	0.74±0.08	257 (3)	0.02[§§]	
daf-28; *jxEx21*	0.82±0.08	308 (3)	0.02[§§]	
Full rescue (low) *daf-28p::daf-28* (2 ng); *ofm-1p::gfp* (25 ng)				
daf-28; *jxEx112*	0.67±0.14	353 (3)	0.03[†††]	n.s.[¶¶¶]
daf-28; *jxEx108*	0.68±0.06	352 (3)	0.03[†††]	n.s.[¶¶¶]
Full rescue (high) *daf-28p::daf-28* (25 ng); *ofm-1p::gfp* (25 ng)				
daf-28; *jxEx110*	0.86±0.05	174 (2)	0.06[†††]	n.s.[¶¶¶]
daf-28; *jxEx111*	0.77±0.07	186 (2)	0.06[†††]	n.s.[¶¶¶]
daf-28; *jxEx103*	0.59±0.21	299 (2)	0.06[†††]	n.s.[¶¶¶]
daf-28; *jxEx105*	0.73±0.15	221 (2)	0.06[†††]	n.s.[¶¶¶]

We assayed wild-type, mutant and rescued worms in parallel in independent trials at different temperatures and show statistics from the cumulative experiments. We used the Wilcoxon Mann-Whitney rank sum test to determine the statistical significance of the differences among the groups.
*The control to which the different worms were compared in each trial.
†Compared with *daf-28(tm2308)* mutants.
‡Compared with *ins-6(tm2416)*; *daf-28(tm2308)* mutants.
§Compared with *daf-2(e1368)*; *daf-28(tm2308)* mutants.
¶Compared with *ins-6(tm2416)*; *daf-2(e1368)*; *daf-28(tm2308)* mutants.
**Compared with *ins-6(tm2416)*; *daf-2(e1368)* mutants.
††Compared with wild type.
‡‡Compared with *ins-6(tm2416)*; *daf-28(tm2308)*; *ins-1(nr2091)* mutants.
§§Compared with wild type carrying the corresponding transgene.
¶¶Compared with *ins-6*; *daf-28*; *jxEx18*, *ins-6*; *daf-28*; *jxEx21* or *ins-6*; *daf-28*; *jxEx22*.
***Compared with *ins-6*; *daf-28* mutants carrying the corresponding transgene.
†††Compared with wild type carrying *jxEx18* or *jxEx21*.
‡‡‡Compared with *ins-6*; *daf-28*; *jxEx18*.
§§§Compared with *ins-6*; *daf-28*; *jxEx21*.
¶¶¶Compared with *daf-28*; *jxEx18* or *daf-28*; *jxEx21*.
n.s., not significant ($P>0.1$). See the Fig. S1 legend concerning the lack of rescue in some animals carrying low levels of the ASI-specific *ins-6* expression construct.

Table S2. The different roles of different ILPs in regulating dauer exit

Strain/treatment	No. of animals observed/total animals	No. of trials	*P*-value against control (log-rank)	*P*-value against specified groups (logrank)	Rescue effect
25°C					
daf-2(e1368)	438/599	6			
ins-6(tm2416); *daf-2(e1368)*	324/578	6	<0.0001*	<0.0001†	
daf-2(e1368); *ins-1(nr2091)*	480/588	6	0.0001*		
daf-2(e1368); *daf-28(tm2308)*	407/587	6	0.0001*	<0.0001‡	
ins-6; *daf-2*; *ins-1*	375/583	6	<0.0001*		
daf-2; *ins-1*; *daf-28*	495/579	6	0.001*		
ins-6; *daf-2*; *daf-28*	194/596	6	<0.0001*	<0.0001§	
ins-6; *daf-2*; *ins-1*; *daf-28*	293/605	6	<0.0001*	<0.0001¶	
***ins-6* rescues**					
25°C					
ofm-1p::gfp (25 ng)					
daf-2; *jxEx18*	632/739	8			
daf-2; *jxEx21*	409/516	6			
daf-2; *jxEx22*	448/591	6			
ins-6; *daf-2*; *jxEx18*	484/815	8	<0.0001**		
ins-6; *daf-2*; *jxEx21*	353/592	6	<0.0001††		
ins-6; *daf-2*; *jxEx22*	342/605	6	<0.0001‡‡		
Full rescue (low)					
ins-6p::ins-6 (2 ng); *ofm-1p::gfp* (25 ng)					
ins-6; *daf-2*; *jxEx27*	541/835	8	<0.0001**,††,‡‡	<0.0001§§,¶¶,***	+**,††,‡‡
ins-6; *daf-2*; *jxEx28*	632/804	8	<0.0001**,†† <0.05‡‡	<0.0001§§,¶¶,***	+**,†† ++‡‡
ins-6; *daf-2*; *jxEx29*	472/698	7	<0.0001**,††,‡‡	<0.0001§§,¶¶,***	+**,††,‡‡
Full rescue (high)					
ins-6p::ins-6 (25 ng); *ofm-1p::gfp* (25 ng)					
ins-6; *daf-2*; *yxEx163*	677/809	8	n.s.** <0.02†† <0.0001‡‡	<0.0001§§,¶¶,***	++** +++††,‡‡
ins-6; *daf-2*; *yxEx174*	698/817	8	n.s.** <0.02†† <0.0001‡‡	<0.0001§§,¶¶,***	++** +++††,‡‡
ins-6; *daf-2*; *yxEx175*	578/684	7	n.s.** <0.005†† <0.0001‡‡	<0.0001§§,¶¶,***	++** +++††,‡‡
ASI-specific rescue (low)					
str-3p::ins-6 (2 ng); *ofm-1p::gfp* (25 ng)					
ins-6; *daf-2*; *jxEx53*	121/215	3	<0.0001**,††,‡‡	n.s.§§,¶¶,***	–**,††,‡‡
ins-6; *daf-2*; *jxEx54*	168/257	3	<0.0001**,††,‡‡	n.s.§§,¶¶,***	–**,††,‡‡
ins-6; *daf-2*; *jxEx64*	168/213	2	<0.0001**,†† n.s.‡‡	<0.0001§§,¶¶,***	+**,†† ++‡‡
ins-6; *daf-2*; *jxEx65*	164/228	2	<0.0001**,†† 0.0005‡‡	<0.05§§ n.s.¶¶,***	+** –††,‡‡
ins-6; *daf-2*; *jxEx66*	133/214	2	<0.0001**,††,‡‡	n.s.§§,¶¶,***	–**,††,‡‡
ASI-specific rescue (high)					
str-3p::ins-6 (25 ng); *ofm-1p::gfp* (25 ng)					
ins-6; *daf-2*; *jxEx50*	345/352	3	<0.0001**,††,‡‡	<0.0001§§,¶¶,***	+++**,††,‡‡
ins-6; *daf-2*; *jxEx51*	354/361	3	<0.0001**,††,‡‡	<0.0001§§,¶¶,***	+++**,††,‡‡
ins-6; *daf-2*; *jxEx52*	327/333	3	<0.0001**,††,‡‡	<0.0001§§,¶¶,***	+++**,††,‡‡
ASJ-specific rescue (low)					
trx-1p::ins-6 (2 ng); *ofm-1p::gfp* (25 ng)					
ins-6; *daf-2*; *jxEx61*	182/208	2	n.s.**,†† 0.0002‡‡	<0.0001§§,¶¶,***	++**,†† +++‡‡
ins-6; *daf-2*; *jxEx62*	185/205	2	n.s.** <0.05†† <0.05‡‡	<0.0001§§,¶¶,***	++** +†† +++‡‡
ins-6; *daf-2*; *jxEx63*	167/197	2	<0.0001** n.s.‡‡	<0.0001§§,¶¶,***	+**,†† ++‡‡
ASJ-specific rescue (high)					
trx-1p::ins-6 (25 ng); *ofm-1p::gfp* (25 ng)					

ins-6; *daf-2*; *jxEx58*	278/288	3	n.s.**,†† <0.0001‡‡	<0.0001§§,¶¶,***	++**,†† +++‡‡
ins-6; *daf-2*; *jxEx59*	298/319	3	<0.05** n.s.†† <0.0001‡‡	<0.0001§§,¶¶,***	+++** ++†† +++‡‡
ins-6; *daf-2*; *jxEx60*	302/316	3	<0.0001**,‡‡ <0.05††	<0.0001§§,¶¶,***	+++**,††,‡‡
***daf-28* rescues**					
25°C					
ofm-1p::gfp (25 ng)					
daf-2; *jxEx21*	207/316	3			
daf-2; *jxEx22*	188/307	3			
daf-2; *daf-28*; *jxEx21*	169/302	3	0.01††		
daf-2; *daf-28*; *jxEx22*	90/298	3	<0.0001‡‡		
Full rescue (low)					
daf –28p::daf-28 (2 ng); *ofm-1p::gfp* (25 ng)					
daf-2; *daf-28*; *jxEx108*	150/307	3	<0.0001†† 0.03‡‡	n.s.††† <0.0001‡‡‡	–†† +‡‡
daf-2; *daf-28*; *jxEx114*	105/197	3	0.006†† n.s.‡‡	n.s.††† <0.0001‡‡‡	–†† ++‡‡
Full rescue (high)					
daf –28p::daf-28 (25 ng); *ofm-1p::gfp* (25 ng)					
daf-2; *daf-28*; *jxEx105*	183/268	3	n.s.†† 0.002‡‡	0.0006††† <0.0001‡‡‡	++†† +++‡‡
daf-2; *daf-28*; *jxEx116*	182/263	3	n.s.†† 0.003‡‡	0.001††† <0.0001‡‡‡	++†† +++‡‡
Genetic ASJ ablations					
25°C					
Intact ASJ controls					
ofm-1p::gfp (25 ng)					
daf-2; *jxEx18*	90/106	1			
ins-6; *daf-2*; *jxEx18*	62/104	1	<0.0001**		
Ablated ASJ					
trx-1p::ICE (100 ng); *ofm-1p::gfp* (25 ng)					
daf-2; *jxEx100*	19/103	1	<0.0001**		
daf-2; *jxEx102*	13/62	1	<0.0001**		
ins-6; *daf-2*; *jxEx100*	5/90	1	<0.0001**	0.009§§§	
ins-6; *daf-2*; *jxEx102*	5/100	1		0.002¶¶¶	

We analyzed the rates of dauer exit of *daf-2(e1368)* mutants in the presence or absence of specific insulins at 25°C and show the statistics from the cumulative experiments. We used the log-rank test to determine the statistical significance of the differences among the groups.
*Compared with *daf-2(e1368)* mutants.
†Compared with *ins-6(tm2416)*; *daf-2(e1368)*; *ins-1(nr2091)* mutants.
‡Compared with *daf-2(e1368)* ; *ins-1(nr2091)*; *daf-28(tm2308)* mutants.
§Compared with *ins-6(tm2416)*; *daf-2(e1368)* ; *ins-1(nr2091)*; *daf-28(tm2308)* mutants.
¶Compared with *ins-6(tm2416)*; *daf-2(e1368)*; *daf-28(tm2308)* mutants.
**Compared with *daf-2*; *jxEx18* animals.
††Compared with *daf-2*; *jxEx21* animals.
‡‡Compared with *daf-2*; *jxEx22* animals.
§§Compared with *ins-6*; *daf-2*; *jxEx18* animals.
¶¶Compared with *ins-6*; *daf-2*; *jxEx21* animals.
***Compared with *ins-6*; *daf-2*; *jxEx22* animals.
†††Compared with *daf-2*; *daf-28*; *jxEx21* animals.
‡‡‡Compared with *daf-2*; *daf-28*; *jxEx22* animals.
§§§Compared with *daf-2*; *jxEx100* animals.
¶¶¶Compared with *daf-2*; *jxEx102* animals.
–, no rescue; +, partial rescue; ++, full rescue; +++, over rescue.
n.s., not significant since (P>0.05). In the ASJ-ablated animals, most of the larvae remain in the dauer stage, which accounts for the low number of animals observed as having exited into the L4 stage (second column).