Insulin-like peptides encode sensory information to regulate *C. elegans* development

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A. List of abbreviations

Less frequently used abbreviations are defined upon their first use in the text.

C. elegans	*Caenorhabditis elegans*
cDNA	Complementary deoxyribonucleic acid
CFP	Cyan fluorescent protein
cGMP	Cyclic guanosine monophosphate
daf-16	*daf-16(mu86)I*
daf-2	*daf-2(e1368)III*
daf-28(sd)	*daf-28(sa191)V*
daf-28; daf-28(lf)	*daf-28(tm2308)V*
DNA	Deoxyribonucleic acid
Drosophila	*Drosophila melanogaster*
FOXO	Forkhead box O subclass
gf	Gain-of-function
GFP	Green fluorescent protein
GPCR	G-protein coupled receptor
ICE	Interleukin-1 converting enzyme
IGF	Insulin-like growth factor
ILP	Insulin-like peptide
ins-1	*ins-1(nr2091)IV*
ins-18	*ins-18(tm339)I*
ins-6	*ins-6(tm2416)II*
ins-7	*ins-7(tm1907)IV*
L1-4	Larval stages 1-4
L2d	Predauer stage
lf	Loss-of-function
ORF	Open reading frame
PCR	Polymerase chain reaction
PI3	Phosphoinositide 3
RNA	Ribonucleic acid
RNAi	RNA interference
sd	Semidominant
TGF	Transforming growth factor

B. Summary

The sensory system senses and conveys information about an animal's complex environment to induce the optimal physiological and behavioral responses that are necessary for survival. Sensory information is transduced within neurons and downstream target tissues through a variety of molecular signaling pathways. One such pathway is the insulin-like signaling pathway, which is not only a key regulator of growth and metabolism in many species but also of other biological processes that are influenced by sensory inputs.

In the nematode worm *C. elegans*, the insulin-like pathway is part of the signaling network that mediates the sensory influence on development and lifespan. *C. elegans* is predicted to have 40 genes that encode insulin-like peptides (ILPs), many of which are expressed in sensory neurons and interneurons, as well as in other tissues. Thus, insulin-like peptides are likely candidates to regulate *C. elegans* physiology in response to environmental cues by modulating the activities of the affected sensory circuits and/or their target tissues.

During the first larval stage, an important developmental decision is made between reproductive growth and a larval arrest program, also known as the dauer program. In response to harsh environmental cues, worms enter the stress-resistant dauer (endurance) stage, from which they exit when environmental cues again favor reproductive development. This developmental decision is known to be mediated not only by specific sensory neurons but also by insulin-like signaling. Considering the complexity of the sensory cues that regulate this developmental switch and the number of

ILPs that are expressed in different sensory neurons, I hypothesize that ILPs encode sensory information to regulate *C. elegans* development.

For my thesis, I have tested this hypothesis by focusing on three ILPs, *daf-28*, *ins-6* and *ins-1*, which have been implicated in the regulation of dauer arrest through gain-of-function and RNA-interference analyses. Since these previous studies have only indirectly examined ILP function, which prevents the direct comparison of the relative contributions of each ILP in regulating this process, I have used deletion mutants, in which ILP function is specifically and completely eliminated. Such an approach has allowed me to examine directly and define the precise functions of these ILPs in regulating the different steps of the developmental switch in response to specific sensory cues.

In contrast to the earlier studies, I show that these ILPs have distinct, non-redundant functions in controlling this switch in development. While *ins-1* is necessary for dauer arrest, *daf-28* and *ins-6* are required for reproductive growth. I find that *daf-28* has a major function in inhibiting dauer entry, while *ins-6* has only a minor role in this process. However, the relative importance of these two ILPs is reversed in the regulation of dauer exit: *ins-6* now has a major function in promoting dauer exit, whereas *daf-28* has only a minor role.

To regulate the developmental switch, these ILPs generate precise responses to dauer-inducing sensory cues, like low food availability or high levels of a pheromone mixture that signals overcrowding. While *daf-28* expression has been shown to be downregulated by either high pheromone or low food levels in sensory neurons that regulate the switch, I do not observe any such regulation for *ins-1* expression in the same

neurons. At the same time, I also find that during reproductive growth *ins-6* is expressed in sensory neurons (ASI) that inhibit dauer entry, whereas *ins-6* transcription shifts during dauer arrest to another pair of sensory neurons (ASJ) that promote dauer exit, a change that persists in post-dauer adults. I further show that *ins-6* expression in ASI is specifically downregulated by the dauer pheromone mixture and not by food levels, whereas the switch in expression to ASJ requires both the dauer pheromones and the full induction of the dauer program. Thus, the specificity in the stimulus regulation of *ilp* expression in sensory neurons suggests a mechanism through which these ILPs encode sensory information and regulate development in a combinatorial fashion.

1. Introduction

1.1 *C. elegans* as a model for the environmental influence on development

During the course of evolution, animals were bound to develop strategies to endure harsh environmental conditions. The nematode worm *C. elegans* can adjust its physiology in response to the surroundings, in order to survive periods of starvation and harsh conditions, and to delay reproduction until conditions improve. Under favorable conditions, newly-hatched *C. elegans* develop through four larval stages (L1-L4) to become reproductive adults (Figure 1.1). However, when resources become scarce, *C. elegans* can enter an alternative developmental program, the so-called dauer program [Figure 1.1; (Cassada and Russell, 1975)]. Dauers are developmentally arrested, but highly stress-resistant alternative third-stage larvae [L3; (Cassada and Russell, 1975; Riddle et al, 1981)], which can endure harsh conditions and survive starvation for longer periods of time. L1-stage worms enter the dauer program in response to cues that signal overcrowding and decreasing food levels (Golden and Riddle, 1982; Golden and Riddle, 1984). In contrast, a complete lack of food causes larval arrest at all stages, and is followed by starvation and death within days.

Prior to entering the dauer stage, worms enter the pre-dauer L2 (L2d) stage [Figure 1.1; (Riddle and Albert, 1997)]. During L2d, the final decision is made either to enter the dauer stage or to resume reproductive growth and develop into an L3 larva, which is dependent on whether or not the conditions remain adverse (Riddle and Albert, 1997). In contrast, L2 worms, which are distinct from L2d worms, are unable to enter the dauer program when conditions become unfavorable, since the dauer decision has to be initiated by the L1 stage (Riddle, 1988). The decision between the alternative developmental programs is determined by environmental conditions that are detected by the *C. elegans* sensory system

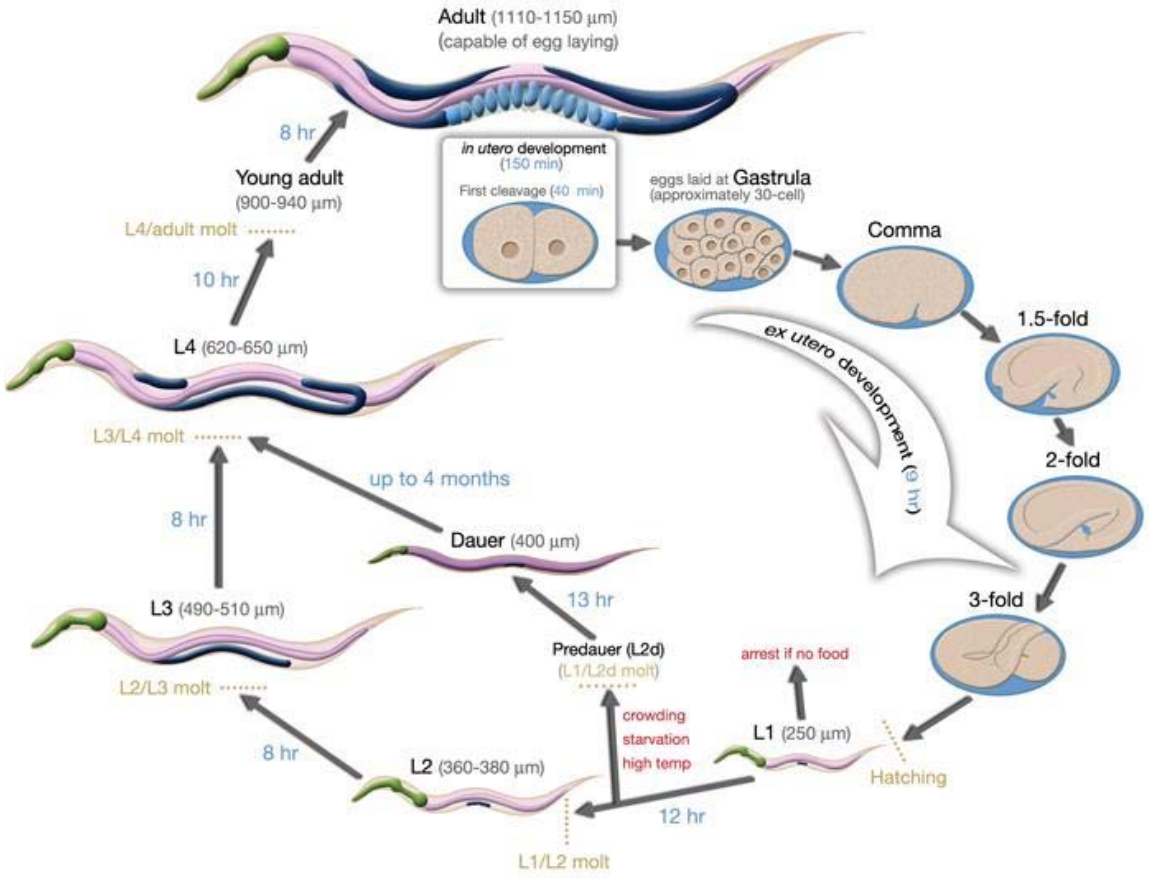

Figure 1.1: The C. elegans life cycle. In response to the environmental conditions, worms can switch between the different developmental programs, reproductive growth and dauer arrest. Under good environmental conditions, worms develop through four larval stages into reproductive adults. Under harsh environmental conditions, worms can enter an alternative developmental program and become dauers. Dauers are developmentally arrested and highly stress resistant, and thus survive periods of harsh conditions. Once the conditions are again favorable for reproductive development, worms exit from the dauer stage to the L4 stage to become reproductive adults. Dauer arrest is regulated by the ratio of dauer pheromone to food, and is also induced by high temperature. In addition, downregulation of insulin-like signaling induces dauer arrest (Image taken from Altun and Hall, 2009).

(Bargmann and Horvitz, 1991a; Schackwitz et al., 1996). Similarly, the decision to exit from or remain within the dauer program is determined by environmental conditions that are also perceived by specific sensory neurons (Bargmann and Horvitz, 1991a).

1.2 Dauer arrest

1.2.1 Dauer morphology and metabolism

The dauer differs in several ways from the L3 larva (Cassada and Russell, 1975; Riddle et al., 1981; Riddle, 1988; Sulston, 1988; Vowels and Thomas, 1992; Wadsworth and Riddle, 1989). Its mouth is plugged by a cuticular block. Thus, the dauer cannot feed, but survives by metabolizing internal resources stored in numerous fat storage vesicles, which are visible as dark spots under the microscope and results in an overall dark appearance. The dauer pharynx is remodeled, in that the lumen of both the anterior and posterior bulbs is largely reduced, and the pharyngeal ability to pump, which is important for digestion, is also completely or largely inhibited. Likewise, the dauer's intestinal lumen is reduced, while its gonad is arrested in an L2-like state. Moreover, dauers are protected by a thick dauer cuticle, which has alae structures distinct from those of the L1 and adult, the only other developmental stages that have cuticular alae. The dauer's plugged mouth, reduced pumping and dauer-specific cuticle hinder dessication and confer resistance against toxic substances, *e.g.*, detergents that solubilize membrane structures. Furthermore, the dauer locomotion is strongly reduced: periods of lethargus are interrupted by periods of fast locomotion, which are triggered, for instance, by mechanical stimulation, possibly as a defensive response against predation.

Reproductively growing worms shift their metabolism between the L1 and L2 stages: L1 worms use fats from lipid stores to generate carbohydrates via the glyoxylate cycle, while L2 and older animals generate energy through increased tricarboxylic acid (TCA) cycle activity during aerobic respiration. During the dauer program, worms metabolize their lipid reserves like L1 worms and do not shift towards aerobic respiration (Wadsworth and Riddle, 1989; Burnell et al., 2005). Dauers are relatively transcriptionally inactive, but upregulate

certain stress response genes, *e.g.*, *hsp-90* (Dalley and Golomb, 1992; Snutch and Baillie, 1983). Moreover, upregulation of enzymes, such as superoxide dismutases and catalase (Larsen, 1993; Vanfleteren and De Vreese, 1995), renders dauers more resistant to metabolic stress.

At present, the actual lifespan of individual dauers before they die from starvation remains unclear. However, if conditions become favorable again for reproductive development, dauers do exit into the last larval stage (L4) to develop further into reproductive adults [Figure 1.1; (Riddle and Albert, 1997)]. This exit from the dauer stage is triggered within an hour of accessing food. Then, feeding is resumed after 2-3 hours, which is followed by a molt into the L4 stage after about 10 hours. Since post-dauer adults live a normal lifespan, or even have a slightly prolonged lifespan, the dauer stage is considered to be "non-aging".

1.2.2 Environmental cues regulating dauer arrest

Dauer arrest is regulated by a mixture of dauer pheromones, food quantity and quality, and ambient temperature (Golden and Riddle, 1984a). The dauer pheromone mixture is continuously secreted by worms; thus, its concentration indicates population density (Golden and Riddle, 1982). It consists of at least three different ascarosides, which are glycolipids containing the sugar ascarylose (Butcher et al, 2007; Jeong et al, 2005). The molecular identity of the first dauer pheromone, the ascaroside (-)-6-(3,5-dihydroxy-6-methyltetrahydropyran-2-yloxy) heptanoic acid, was discovered in 2005 (Jeong et al., 2005). Several structurally related ascaroside-derivatives, also with dauer pheromone activity, which differ in the side-chain residues on the ascarylose, were identified later (Butcher et al, 2007). These distinct dauer pheromones have different potencies in inducing dauer arrest or dauer

recovery (Butcher et al. 2007, Butcher et al., 2008). At least some of these dauer pheromones have also recently been shown to bind and activate two pheromone receptors, the G protein-coupled receptors (GPCRs) SRBC-64 and SRBC-66, which are expressed in one set of dauer-promoting sensory neurons (Kim et al., 2009). However, the loss of both *srbc-64* and *srbc-66* does not completely prevent worms from entering the dauer program (Kim et al., 2009). Since there are at least four ascarosides that are known to play a role in dauer formation (Butcher et al, 2007; Butcher et al, 2008; Jeong et al., 2005) and there are other sensory neurons that are believed to sense this mixture (Bargmann and Horvitz, 1991a; Schackwitz et al., 1996), it is possible that other dauer pheromone-sensing GPCRs expressed in other sensory neurons remain to be discovered.

The absolute levels of the dauer pheromone mixture and food do not determine the induction of the dauer program, but rather it is the ratio of pheromones to food that does so (Golden and Riddle, 1982; Golden and Riddle, 1984a; Golden and Riddle, 1984b). Indeed, a high concentration of dauer pheromones, together with low food, promotes dauer entry (Golden and Riddle, 1982). Besides these two cues, dauer formation can also be induced by high temperature (Ailion and Thomas, 2000; Golden and Riddle, 1984a). However, of the different dauer-regulating cues, the pheromone cue is the most potent, since it can overcome both food and temperature cues: high concentrations of the pheromone mixture can induce dauer arrest at low temperatures or even in the presence of enough food.

1.3 Sensory influence on dauer arrest

1.3.1 The *C. elegans* chemosensory system

Through its chemosensory system, which is a large component of its nervous system, *C. elegans* can detect environmental cues (Figure 1.2). These cues are either volatile or water-soluble chemicals, and signal the environmental conditions, such as food quantity and quality or population density. For example, volatile organic compounds, which are breakdown products of bacterial metabolism, can signify the type and concentration of bacteria, the main *C. elegans* food source. The perception of these chemosensory cues regulates not only *C. elegans* behavior, like chemotaxis towards food cues, but also physiology and development, like the developmental decision between reproductive growth and dauer arrest. Chemosensory signals are detected by the following *C. elegans* sensory neurons: the amphid neurons in the head, the phasmid neurons in the tail, the IL2 neurons within the inner labial organs, and the oxygen sensing neurons [(Figure 1.2A); (Ward et al., 1975; Ware et al., 1975)]. In response to different cues, these chemosensory neurons produce and secrete neuropeptides and other signals, which might act on neighboring neurons as part of different circuits, or on distant neurons and other tissues as hormones.

The subset of sensory neurons that specifically regulate dauer arrest is located in the amphid sensory organ (Figure 1.2B). There are eleven bilateral pairs of chemosensory neurons (ADF, ADL, ASE, ASG, ASH, ASI, ASJ, ASK, AWA, AWB and AWC) plus one bilateral pair of thermosensory neurons (AFD) found within the amphid organ. Within this organ, the cilia of the amphid sensory neurons are either directly exposed to the environment through the amphid pore formed by a glial socket cell or end within the glial sheath cell. The

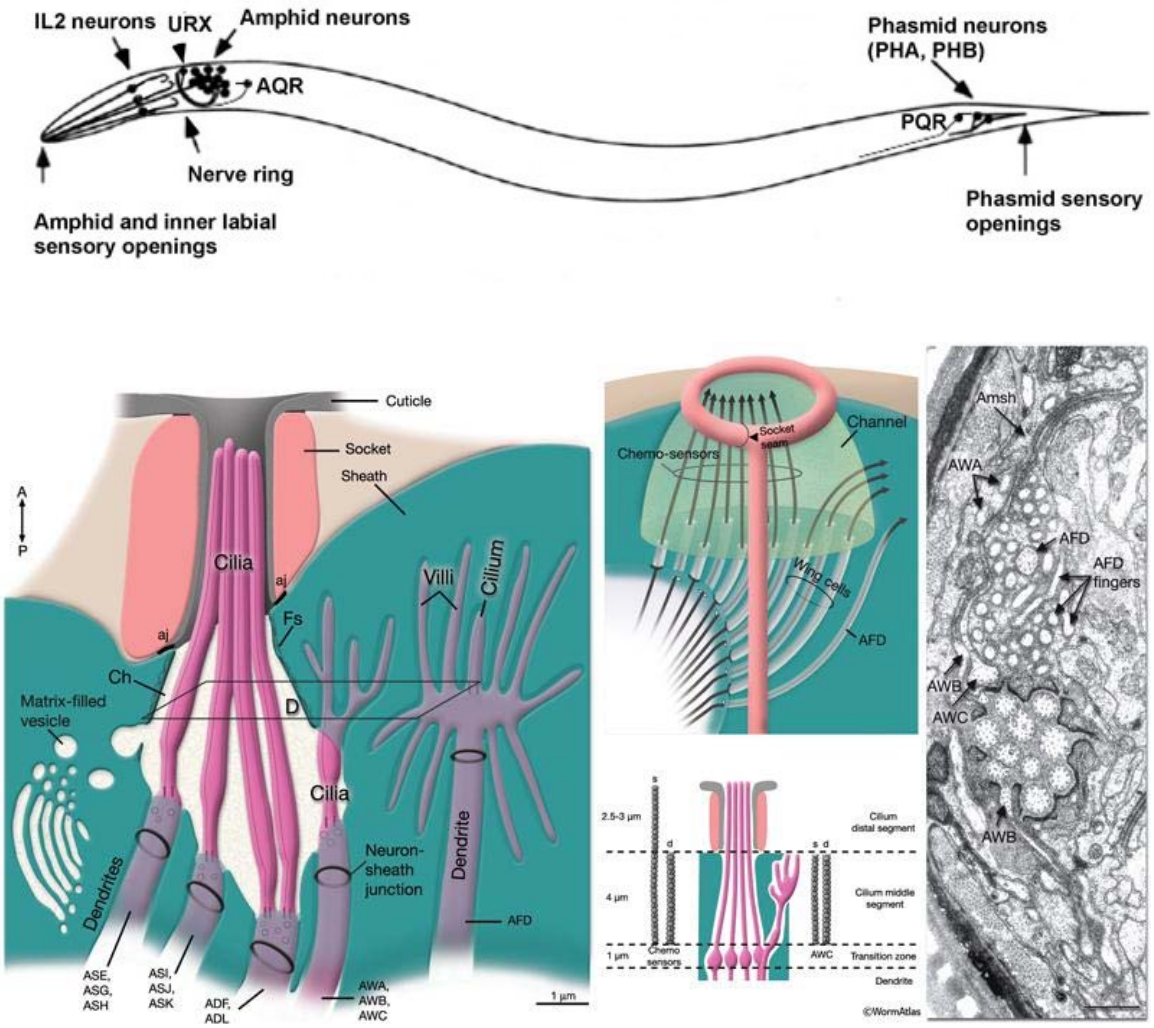

Figure 1.2: The *C. elegans* chemosensory system. A) *C. elegans* chemosensory neurons. There are eleven pairs of amphid chemosensory neurons and six chemosensory neurons in the inner labial organ (IL2 neurons) in the worm's head and two pairs of phasmid chemosensory neurons in the tail, which are all directly or indirectly exposed to the environment. The oxygen sensing neurons AQR, PQR and URX are not exposed to the environment, but sense internal oxygen levels. The axons of many of these neurons project into the nerve ring, where they make synaptic connections with other neurons. B) Left: Schematic drawing of the sensory cilia of the amphid neurons. The cilia of the amphid neurons either end within the amphid pore, which is formed by a glial socket cell, and are directly exposed to the environment, or end within a glial sheath cell and sense cues that enter by diffusion (see middle panels). Right: Cross-section of an electromicroscopy image of sensory cilia within the amphid sheath (Image taken from Bargmann, 2006; Altun and Hall, 2009).

neurons with ciliated endings that are directly exposed to the environment are known to sense water-soluble cues, like amino acids or salts (Bargmann and Horvitz, 1991b). In contrast, the neurons with ciliated endings within the sheath cell have been shown to detect volatile cues, which enter by diffusion (Bargmann et al, 1993). Moreover, the cilia of neurons that sense water-soluble cues have a single (ASE, ASG, ASH, ASI, ASJ and ASK) or a dual (ADF and

ADL) tubular structure [(Figure 1.2B); White et al., 1986]. On the other hand, the cilia of neurons that sense volatile cues differ in morphology, in that they are more of a wing-like structure (AWA, AWB and AWC; Figure 1.2B).

A subset of the soluble cue-sensing cilia (ADL, ADF, ASH, ASI, ASJ and ASK) are also known to take up fluorescent dyes directly from the environment (Herman, 1984; Perkins et al., 1986). Thus, mutations in these cilia can be identified by their dye-filling (Dyf) phenotype, which means that these cilia have defects in their structure or in the connection between the cilia and the amphid sensory pore (Culotti and Russell, 1978; Lewis and Hodgkin, 1977). These defects impair chemosensation, since chemoreceptors located in the cilia are unable to transmit the correct sensory information (Culotti and Russell, 1978; Lewis and Hodgkin, 1977).

C. elegans chemoreceptors are mainly localized to the cilia and not found in the axons or dendrites. The amphid sensory neurons express specific chemoreceptors that recognize specific cues (Sengupta et al., 1996; Troemel et al., 1995). Many candidate *C. elegans* chemosensory receptors are GPCRs, which are distinct from the classical GPCRs that recognize other ligands, *e.g.*, neuropeptides, within the sensory system (Bargmann, 1998). One prominent class of GPCR chemoreceptors are distantly related to the rhodopsin receptor (Troemel et al., 1995). These chemoreceptors are also preferentially expressed in single neurons or subsets of neurons (Chen et al., 2005; Colosimo et al., 2004; McCarroll et al., 2005; Troemel et al., 1995). Moreover, unlike mammalian olfactory neurons that express one receptor per neuron (Malnic et al., 1999; Serizawa et al., 2000), one *C. elegans* chemosensory neuron can express several different chemosensory receptors (Troemel et al., 1995), which is more similar to mammalian gustatory cells (Adler et al., 2000; Hoon et al, 1999).

C. elegans has twenty different Gα subunits and two Gβ and Gγ subunits that are possible components of the heterotrimeric G-protein signaling downstream of GPCRs (Jansen

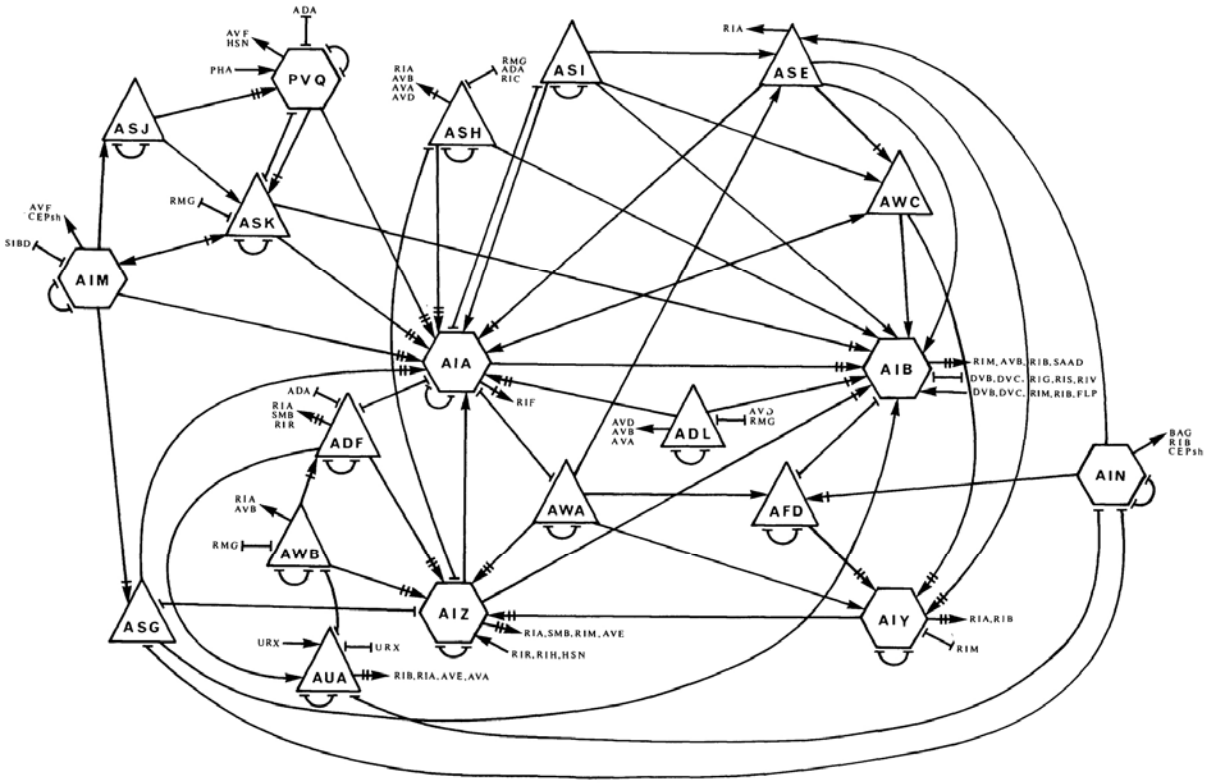

FIGURE 21. (a) Circuitry associated with amphids.

Figure 1.3. **The circuitry associated with the amphid sensory neurons**. Triangles represent sensory neurons, whereas hexagons represent interneurons. Chemical synapses from one neuron to another are shown as arrows, while gap junctions are shown as "T's". The prominence of the synaptic connection is shown as a cross-hatch on the arrow. Thus, a higher number of cross-hatches denote a higher degree of prominence for the synapse. The presence of a gap junction between individual pairs of neurons is also shown, *e.g.*, the gap junction between the left ASI and the right ASI neurons (Image taken from White et al, 1986).

et al., 1999). Fourteen of the Gα subunits are nematode-specific Gαi-like subunits expressed in subsets of sensory neurons. Two of these, *gpa-2* and *gpa-3*, seem to be involved in the induction of dauer arrest in response to the dauer pheromones (Zwaal et al., 1997). G protein signaling activates downstream signaling mechanisms, such as the production of the cGMP second messenger, which can function to open cGMP-gated ion channels that eventually lead to secretion of signals, like neurotransmitters, that can act on other cells (Bargmann, 2006).

The amphid sensory neurons also differ from each other by their connectivities [Figure 1.3; (White et al., 1986)]. Some sensory neurons synapse to other sensory neurons, and neurons of certain pairs also form gap junctions with each other [(Figure 1.3); (White et al., 1986)]. The postsynaptic partners of different amphid sensory neurons overlap

considerably; but the sensory neurons that sense soluble cues synapse more extensively to certain interneurons, whereas sensory neurons that sense volatile cues synapse more to other interneurons [Figure 1.3; (White el al., 1986)].

1.3.2 Sensory influence on dauer entry and dauer exit

Interestingly, neuronal ablation studies revealed that a subset of amphid sensory neurons controls dauer entry and exit (Figure 1.4): ASI and ADF, and to a minor extent ASG, inhibit dauer entry, while ASJ, and to a minor extent ASK, promote it (Bargmann and Horvitz, 1991a; Schackwitz et al., 1996). In addition, ASJ has a second function, which is to promote dauer exit (Bargmann and Horvitz, 1991a). Thus, these neurons might directly sense the dauer-regulating pheromone and food cues or they might be activated by other sensory neurons that sense these cues.

Some of the dauer-regulating neurons also have additional functions. Some of them have been implicated in the regulation of adult physiology. ASI and other amphid sensory neurons take part in regulating intestinal fat storage (Ashrafi et al., 2003: Ogg et al. 1997; Sze et al., 2000; Thomas et al. 1993). ASI, ASG, ASJ and ASK also influence adult lifespan: ablation of ASI or ASG increases lifespan, which is suppressed by ASJ or ASK ablation (Alcedo and Kenyon, 2004). Moreover, adult lifespan is shortened by AWA and AWC, which act in parallel to the other neurons in their effects on lifespan (Alcedo and Kenyon, 2004).

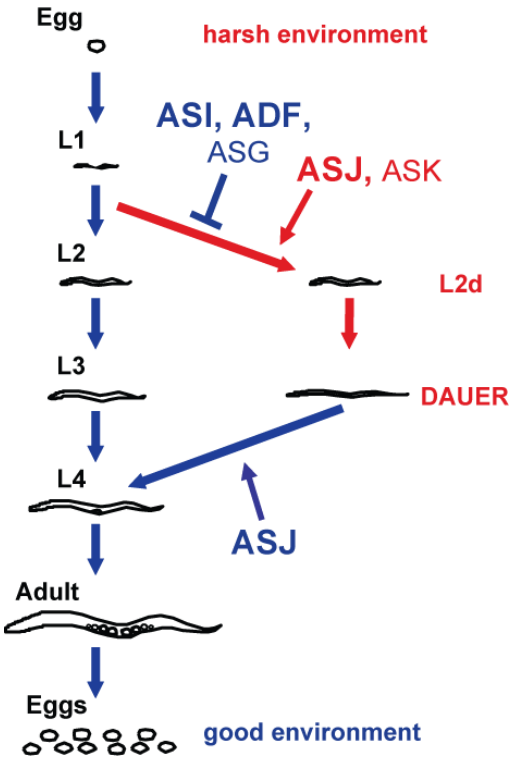

Figure 1.4: Specific functions of amphid sensory neurons in dauer entry and dauer exit. The amphid sensory neurons ASI and ADF, and to a minor extent ASG, inhibit dauer entry, whereas ASJ, and to a minor extent ASK, promote dauer entry. The ASJ neurons have a second function in promoting dauer exit (Bargmann and Horvitz, 1991a; Schackwitz et al., 1996).

1.4 Molecular signaling pathways regulating dauer arrest

There are at least four distinct pathways that regulate dauer arrest: an insulin-like pathway, a TGF-β-like pathway, a guanylate cylase pathway and a steroid hormone signaling pathway [Figure 1.5; (Gerisch et al., 2001; Gottlieb and Ruvkun, 1994; Jia et al., 2002; Riddle et al., 1981; Thomas et al., 1993; Vowels and Thomas, 1992)]. The genes that regulate the dauer program have been identified in forward and reverse genetic screens. The dauer phenotypes caused by mutations in any of these genes are classified as Daf-c for dauer-formation constitutive and Daf-d for dauer-formation defective (Riddle et al., 1981).

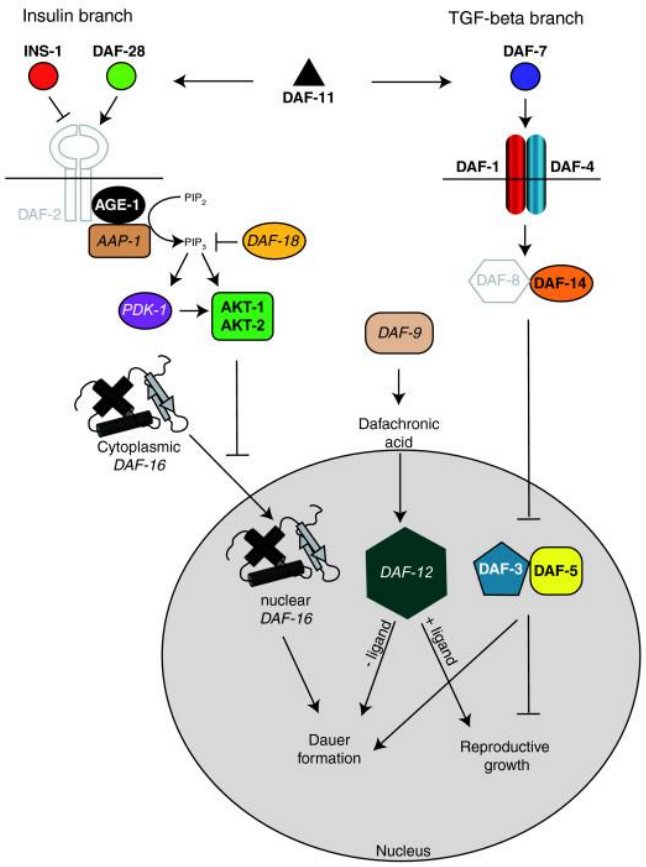

Figure 1.5: The dauer-regulating pathways. The insulin-like/DAF-2, TGF-β-like/DAF-7 and guanylate cyclase/DAF-11 signaling pathways are thought to act in parallel in the regulation of dauer arrest. However, both DAF-11 and DAF-7 can regulate the expression of *daf*-28, and DAF-11 can regulate the expression of *daf-7*. Thus, both the DAF-11 and DAF-7 pathways can also act upstream of the insulin pathway. The steroid hormone/DAF-12 signaling pathway is thought to act more proximal in dauer regulation (Li et al., 2003; Image taken from Stetina et al., 2007).

1.4.1 The insulin-like pathway

The downregulation of insulin-like signaling in *C. elegans* (Figure 1.6) induces dauer arrest (Gottlieb and Ruvkun, 1994; Riddle and Albert, 1997; Thomas et al., 1993), as well as promotes adult longevity (Kenyon et al., 1993; Larsen et al., 1995). While strong impairment of the pathway induces constitutive dauer arrest, a weaker downregulation of insulin-like signaling only leads to transient dauer arrest (Patel et al., 2008). In addition, of all Daf-c alleles that have been isolated so far, only strong mutant alleles that affect the insulin-like signaling pathway arrest non-conditionally as dauers and are unable to exit from the dauer

stage (Gems et al., 1998). Together these observations suggest that insulin-like signaling not only controls the dauer entry, but also the dauer exit decision.

The insulin-like pathway is also named the DAF-2 pathway after the worm insulin/IGF receptor ortholog DAF-2 (Kimura et al., 1997), a receptor tyrosine kinase. In addition to dauer and longevity regulation, DAF-2 signaling is also required for several other processes, such as embryonic and larval development, reproduction, fat storage, salt chemotaxis learning, and stress responses (Ashrafi et al., 2003; Gems et al., 1998; Lee et al., 2003; Murphy et al., 2003; Tomioka et al., 2006). DAF-2 activity is thought to be controlled by insulin-like ligands. Upon activation, the DAF-2 receptor negatively regulates the FOXO transcription factor DAF-16 through a conserved signaling cascade (Figure 1.6): DAF-2 activates the AGE-1 phosphoinositide 3 (PI3)-kinase, which activates the PI3-dependent kinase PDK-1 and the protein kinase B orthologs AKT-1 and AKT-2 (Kimura et al., 1997; Morris et al., 1996; Paradis and Ruvkun, 1998; Paradis et al., 1999). DAF-16 is phosphorylated by the *C. elegans* AKT-1/AKT-2 kinases, and subsequently sequestered to the cytoplasm (Hertweck et al., 2004; Lee et al., 2001; Lin et al., 1997; Lin et al., 2001; Ogg et al., 1997), which requires the 14-3-3-like proteins PAR-5 and FTT-2 (Berdichevsky et al., 2006; Li et al., 2007). Furthermore, the E3 ubiquitin ligase RLE-1 marks cytoplasmic DAF-16 for proteasomal degradation (Li et al., 2007). The serum glucocorticoid kinase, SGK-1, which forms a complex with the AKT kinases also phosphorylates DAF-16 in a PDK-1-dependent manner (Brunet et al. 2001; Hertweck et al., 2004). On the other hand, the PI3-phosphatase DAF-18 antagonizes AGE-1, and thereby counteracts DAF-16 inhibition (Gil et al., 1999; Mihaylova et al., 1999; Ogg and Ruvkun, 1998; Rouault et al., 1999).

There is genetic evidence for other outputs from DAF-2 in parallel to AGE-1 and the canonical downstream pathway (Inoue and Thomas, 2000a; Paradis and Ruvkun, 1998; Paradis et al., 1999). In addition to a DAF-16-independent output, there is also another signal

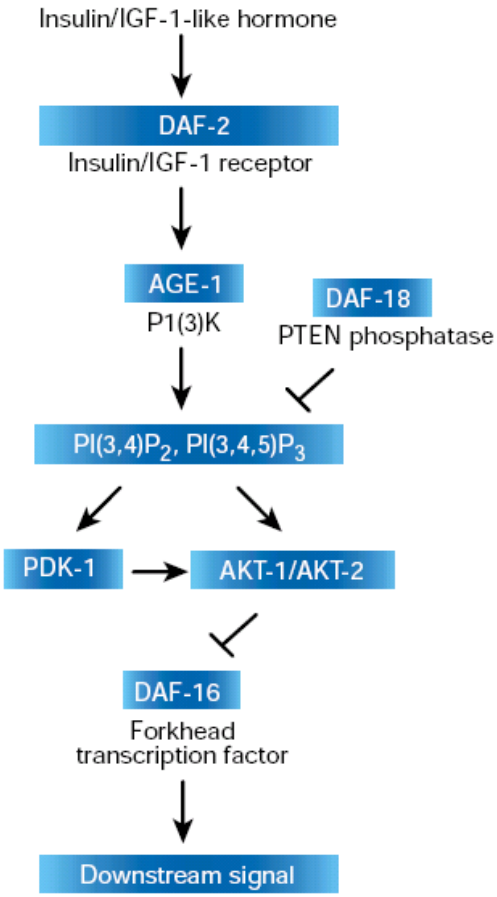

Figure 1.6: The DAF-2 signaling pathway. Insulin-like peptides are thought to modulate the activity of DAF-2, the *C. elegans* insulin/IGF-1 receptor homologue. DAF-2 activation leads to activation of AGE-1/PI3-kinase and PDK-1/3-phosphoinositide-dependent kinase, which in turn activate the PKB homologues AKT-1 and AKT-2. AKT-1/AKT-2 phosphorylate and thereby inhibit the FOXO transcription factor DAF-16, which regulates dauer arrest and longevity genes. Inhibition of DAF-16 by DAF-2 signaling prevents dauer arrest and shortens lifespan (Image taken from Guarente and Kenyon, 2000).

coming from DAF-2 that renders DAF-16 partially inactive, even if its AKT-1/AKT-2-dependent phosphorylation or its cytoplasmic sequestration is inhibited (Berdichevsky et al., 2006; Hertweck et al., 2004; Lin et al., 2001).

Accordingly, the inhibition of DAF-16 transcriptional activity promotes reproductive growth and shortens adult lifespan (Kenyon et al., 1993; Riddle et al., 1981). In turn, active nuclear DAF-16 promotes dauer arrest during the L1 stage and increased adult lifespan (Henderson and Johnson, 2001; Kenyon et al., 1993; Lee et al., 2001; Lin et al, 2001; Riddle and Albert, 1997) by regulating target genes that include stress-response and metabolic genes (Lee et al, 2003; Murphy et al, 2003). Moreover, loss of *daf-16* largely, if not completely,

suppresses the growth arrest or lifespan extension seen in *daf-2* mutants (Kenyon et al., 1993; Larsen et al., 1993; Riddle et al., 1981; Vowels and Thomas, 1992).

DAF-2 and AGE-1 act non-autonomously to inhibit dauer arrest or longevity, as demonstrated by mosaic analysis (Apfeld and Kenyon, 1998) and tissue-specific rescue (Wolkow et al., 2000) of either gene function. Furthermore, DAF-2 seems to function largely in neurons to control both dauer arrest and adult lifespan (Apfeld and Kenyon, 1998; Wolkow et al., 2000). On the other hand, neuronal DAF-16 seems to control dauer arrest more than longevity, while intestinal DAF-16 seems to have a stronger role in lifespan (Libina et al., 2003). The temporal role for insulin-like signaling in dauer arrest and longevity also differs: DAF-2 acts in larvae to regulate dauer arrest, with little effect on lifespan; yet DAF-2 function in adults is more than sufficient to affect lifespan (Dillin at el., 2002).

The insulin-like ligands

To date, forty *C. elegans* genes have been predicted to encode insulin-like ligands for the DAF-2 receptor [Figure 1.7; (Li et al., 2003; Pierce et al., 2001); see www.wormbase.org]. The predicted insulin-like peptides (ILPs) have helical segments that correspond to the A and B chains of the human insulin. At least some of these ILPs likely modulate DAF-2 activity, and thereby regulate the developmental decision between reproductive growth and dauer arrest, as well as adult lifespan. So far, there is no direct evidence that any of the ILPs act on the DAF-2 receptor. However, one ILP, INS-6, has been shown to bind and activate the human insulin receptor *in vitro* and to have an IGF-like fold by NMR analyses (Hua et al., 2003). Notably, many of the *ilp* genes are expressed in neurons, including sensory neurons and interneurons, in addition to non-neuronal tissues (Li et al., 2003; Pierce et al., 2001).

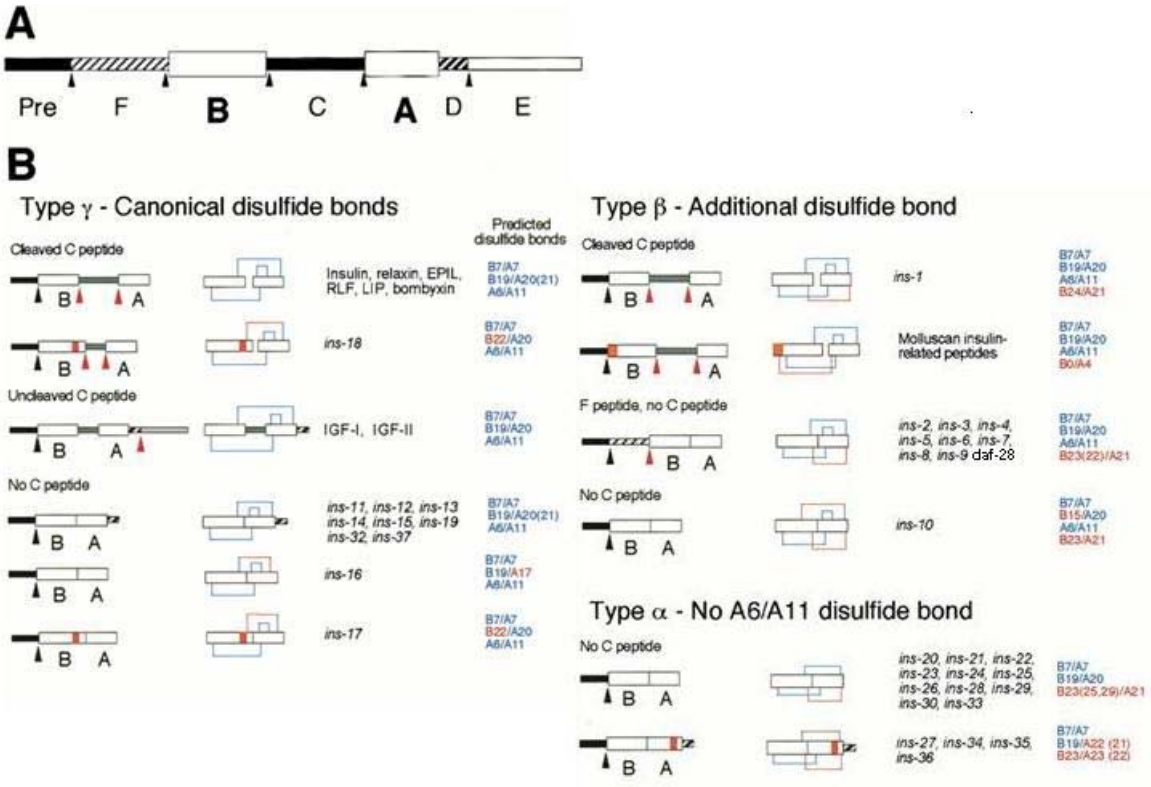

Figure 1.7: The insulin-like genes. A) Predicted proteolytic cleavage sites (arrows) and peptide chains of *C. elegans* insulin-like peptides. B) Three classes of predicted insulin-like peptides: The γ-type insulin-like peptides have three disulfide bonds in the canonical positions, like human insulin-like proteins. The β-type insulin-like peptides have a fourth disulfide bond in addition to the three canonical ones. This group includes the insulin-like peptides *ins-1, ins-6* and *daf-28*. The α-type insulin-like peptides are lacking one disulfide bond in a canonical position, but have another in a non-canonical position (Image taken from Pierce et al., 2001).

The forty *ilp* genes have been sorted into four classes according to the predicted tertiary structures of the corresponding peptides [Figure 1.7; (Pierce et al., 2001)]. The γ-type ILPs contain three disulfide bonds in conserved positions as in human insulin or IGFs. In comparison, both α- and *β*-type ILPs have an additional disulfide bond in a non-canonical position. However, the α-type ILPs lack the canonical disulfide bond within the A chain of the peptide. Moreover, the fourth ILP class is predicted to encode a protein with three alternating sets of B-A chains, which is unlike the first three classes of ILPs, and is represented by only one member, *ins-31* (Pierce et al., 2001).

Two ILPs, the *β*-type *ins-1* and the γ-type *ins-18*, are the only *C. elegans* ILPs predicted to have a cleavable C peptide between the A and B chains, and would thus be most similar in structure to human insulin (Pierce et al, 2001). Furthermore, the *β*-type ILPs

contain a group of proteins with a so-called F peptide between the signal peptide and the B chain, which could be removed during processing (Figure 1.7). This F peptide subclass of the *β*-type ILPs includes *daf-28*, *ins-4*, *ins-6* and *ins-7*. Some of these ILPs have been implicated in the regulation of dauer arrest and/or lifespan by gain-of-function and RNA interference studies: the γ-type *ins-18* and the *β*-type *ins-1*, *ins-4, ins-6, ins-7* and *daf-28* (Li et al., 2003; Malone and Thomas, 1996; Murphy et al, 2003; Pierce et al., 2001). A semi-dominant gain-of-function allele (*sa191*) of *daf-28*, the first ILP to be described in *C. elegans*, causes 100% transient dauer arrest at 25°C and a lifespan extension of ~10% (Malone and Thomas, 1994; Malone and Thomas, 1996). Since overexpression of *ins-4*, *ins-6* or *daf-28* reduces dauer arrest in the *daf-28(sa191)* background, these ILPs might act at least partly redundantly with each other in inhibiting dauer arrest (Li et al., 2003). On the other hand, *ins-7* appears to act independently of this group in inhibiting dauer arrest and/or longevity (Li et al., 2003; Murphy et al., 2003). In contrast, overexpression of *ins-1* or *ins-18* has the opposite effect on dauer arrest and/or lifespan: their overexpression enhanced dauer arrest and, in the case of *ins-1*, slightly extended lifespan (Pierce et al., 2001). Thus, INS-1 and INS-18 might antagonize DAF-2 directly, or competitively inhibit the function of other agonists for the DAF-2 receptor.

1.4.2 The TGF-β-like pathway

Another signaling pathway that regulates dauer arrest is the DAF-7 TGF-β-like pathway, which acts to suppress dauer arrest at least partly in parallel to the insulin-like signaling pathway [Figure 1.5; (Malone et al., 1996; Vowels and Thomas, 1992)]. DAF-7 is a TGF-β-like ligand (Ren et al., 1996; Schackwitz et al., 1996), which binds and activates the serine/threonine kinase receptors DAF-1 (TGF-β type I-like receptor; Georgi et al., 1990) and

DAF-4 (TGF-β type II-like receptor; Estevez et al., 1993). Upon activation of the receptors, the SMAD transcription factors DAF-8 and DAF-14 are activated by phosphorylation and translocate to the nucleus, where they inhibit dauer arrest (Inoue and Thomas, 2000b). However, the other SMAD transcription factor, DAF-3, which is inactivated by TGF-ß signaling activity, promotes dauer arrest by acting with the SNO/SKI protein DAF-5 (Da Graca et al., 2004; Patterson et al., 1997).

daf-7 is primarily expressed in ASI (Ren et al., 1996; Schackwitz et al., 1996), one of the amphid sensory neurons that inhibit dauer arrest (Bargmann and Horvitz, 1991a). *daf-7* is also repressed under harsh environmental conditions, like low food availability or high population density (Ren et al, 1996). In contrast, the other members of the pathway are widely expressed (Da Graca et al., 2004; Gunther et al., 2000; Inoue and Thomas, 2000b; Patterson et al., 1997; Tewari et al., 2004).

Loss of function in *daf-7* causes a temperature-sensitive dauer arrest, which is suppressed by mutations in *daf-3* or *daf-5*, but not by mutations in *daf-16* (Malone et al., 1996; Vowels and Thomas, 1992). Conversely, mutations in *daf-2* that promote dauer arrest are not suppressed by loss of *daf-3* or *daf-5* activity, but suppressed by lack of *daf-16* function (Malone et al., 1996; Vowels and Thomas, 1992). Together these data suggest that the DAF-7 and DAF-2 pathways act in parallel (Figure 1.5). However, later studies show that the expression of a DAF-2 ligand, the ILP *daf-28*, also requires the activity of *daf-7* (Li et al., 2003). Thus, this suggests that these two pathways have complex interactions in their regulation of the dauer program: they act not only in parallel but also as part of a linear pathway to control this process.

1.4.3 The guanylate cyclase pathway

DAF-11, a transmembrane guanylate cyclase, acts in the *C. elegans* sensory system (Birnby et al., 2000) to suppress dauer arrest and modulate chemosensation (Vowels and Thomas, 1994). The dauer-regulating G protein GPA-2 has been proposed to act upstream of DAF-11 (L'Etoile and Bargmann, 2000), while the other dauer-regulating G protein GPA-3 has been shown to act in parallel (Birnby et al., 2000). DAF-11 suppresses dauer arrest most likely by providing cGMP to activate a cGMP-gated cation channel, which consists of the essential α-subunit TAX-4 and a β-subunit TAX-2 (Coburn and Bargmann, 1996; Coburn et al., 1998; Komatsu et al., 1996). TAX-4 and TAX-2 are localized to the cilia of several sensory neurons and also functions in chemosensation (Coburn and Bargmann, 1996; Komatsu et al., 1996). Activation of the TAX-4/TAX-2 channel allows the influx of sodium and calcium ions (Komatsu et al., 1999), which leads to cellular depolarization and secretion of different signals (Salio et al, 2006; Strand, 1999). The TAX-4/TAX-2 channel is also regulated by additional enzymes that control cGMP levels, such as other transmembrane guanylate cyclases or an unknown cGMP-degrading phosphodiesterase (Bargmann, 2006*;* L'Etoile and Bargmann, 2000).

DAF-11 might mediate the dauer-arrest promoting signal from the neuron ASJ, since ablation of ASJ suppresses the *daf-11* Daf-c phenotype (Coburn et al., 1998). The dauer-arrest promoting signal from the ASJ neuron might, therefore, be constitutively active in *daf-11* mutants.

Genetic evidence suggests that DAF-11 functions in parallel to the DAF-2 and DAF-7 pathways (Thomas et al., 1993). However, DAF-11 is also likely to be upstream of both pathways (Figure 1.5): (i) DAF-11 promotes *daf-7* expression in the ASI neurons, and

thereby suppresses dauer arrest (Murakami et al., 2001); and (ii) DAF-11 promotes the expression of the ILP *daf-28* in sensory neurons (Li et al., 2003). Thus, all three pathways provide a complex molecular network that regulates the switch between reproductive growth and dauer arrest.

1.4.4 The steroid hormone-like pathway

A steroid hormone-like pathway functions downstream of the other dauer-regulating pathways [Figure 1.5; (Albert and Riddle, 1988; Gerisch et al., 2001; Jia et al., 2002; Riddle et al., 1981; Thomas et al., 1993]: DAF-9, a cytochrome P450-like steroid hydroxylase (Gerisch et al., 2001; Jia et al., 2001), generates inhibitory ligands for the nuclear hormone receptor DAF-12 (Antebi et al. 2000), which promotes dauer arrest in its ligand-free form (Ludewig et al., 2004). Consequently, the binding of the inhibitory ligands to DAF-12 suppresses dauer arrest and promotes reproductive growth. These inhibitory ligands are the cholesterol derivatives Δ^4- and Δ^7- dafachronic acid (Motola et al., 2006).

The substrates for DAF-9 are provided by the Rieske oxygenase DAF-36 (Rottiers et al., 2006): the DAF-36 metabolites that are made in the intestine are likely transported by cholesterol transporting proteins to the DAF-9 expressing tissues, such as the XXX neuroendocrine cells, which synthesize the dafachronic acids (Gerisch et al., 2001; Gerisch et al., 2007; Jia et al., 2002).

Besides regulating dauer arrest, DAF-12 affects lifespan (Gerisch et al., 2001; Larsen et al., 1995). The ligand-bound form of DAF-12 shortens lifespan, while its ligand-free form lengthens lifespan (Ludewig et al., 2004). The DAF-12 pathway also acts together with the DAF-2 pathway (Gerisch et al., 2001; Larsen et al., 1995). Indeed, loss of *daf-12* suppresses the lifespan extension seen in *daf-2* mutants (Gerisch et al., 2001; Larsen et al., 1995).

Table 1.1: Expression patterns of some *ilp* genes

Gene	Stages*			Tissues			
	Embryo	Larvae	Adult	Nerve ring#	Sensory neurons$	Other neurons$	Non-neuronal tissues**
ins-1	4	L1–L4	+	●●●	a	v, la, t, p	vm, in
ins-2	b, 2, 4	L1–L3	−	●●●	a, l	v, t	ph, vul
ins-3	4	L1–L4	+	●●	a, l	v, la, d	
ins-4	2, 4	L1–L4	+	●●	a, l	v, d, t	h
ins-5	2, 4	L1–L4	+	●●	a, l	v, la, t	vul
ins-6	2, 4	L1–L4	+	●	a, l	v, t	
ins-7	4	L1–L4	+	●●	a, l	v, t	
ins-8	4	L1–L4	+	●●	a, l	v, t	vul
ins-9		L1–L4	+	●	a		
ins-11	4	L1–L4	+	●	l	v, t	
ins-18	4	L1–L4	+	●●	a	v, t, p	in
ins-21	4	L1–L4	+	●	a	v, t	
ins-22	4	L1–L4	+	●●●	a, l	v, la, t	
ins-23	4	L1–L4	+	●	a, l	v	

*Stages: b, bean-stage embryo; 2, twofold elongated embryo; 4, fourfold elongated embryo; L1, first larval stage; L2, second larval stage; L3, third larval stage; L4, fourth larval stage.
#Indicates expression in more than eight (●●●), in 4–8 (●●), or in two (●) bilaterally symmetric pairs of neurons.
$Neurons: a, amphid sensory neurons; l, labial neurons; v, ventral nerve cord neurons; la, neurons with lateral processes and/or cell bodies; d, dorsal nerve cord neurons, observed by dorsal and/or circumferential processes; t, tail neurons; p, pharyngeal neurons.
**Non-neuronal tissues: vm, vulval muscle; in, intestine; ph, pharynx; vul, vulva; h, hypodermis.

Table 1.1: *ilp* gene expression patterns. Transcriptional GFP reporter constructs of the *ilp* genes were analyzed for their expression patterns. *Many ilp* genes are expressed in sensory head neurons, among other neurons and tissues. The expression patterns are partly overlapping and partly unique to specific *ilp* genes or subsets of *ilp* genes (modified from Pierce et al., 2001).

1.5 Insulin-like signaling mediates the sensory influence on development

Insulin-like signaling is one of the pathways that transform sensory information into physiological responses. There is a number of genetic evidence that suggest that insulin-like signaling acts in the sensory system to control dauer arrest, as well as adult longevity (Apfeld and Kenyon, 1998; Libina et al., 2003; Wolkow et al., 2000). Consistent with this idea, the

DAF-2 receptor has been shown to act in several head neurons, as well as in the intestine (Apfeld and Kenyon, 1998; Wolkow et al., 2000). At the same time, many of the *ilp* genes are expressed in overlapping subsets of sensory neurons and/or interneurons, as well as other tissues [Table 1.1; (Kodama et al., 2006; Li et al., 2003; Pierce et al., 2001)]. Moreover, some *ilp* genes are expressed in subsets of the dauer- and longevity-regulating sensory neurons in the amphid organs: *ins-1* is expressed in several amphid sensory neurons, among them the dauer-regulating neurons ASI, ADF, ASG and ASJ, as well as several other neurons and other tissues (Kodama et al., 2006; Tomioka et al., 2006). *daf-28* is also expressed mainly in the ASI and ASJ neurons and is downregulated in these neurons upon starvation, overcrowding or pheromone treatment of the animals (Li et al., 2003). Since ASI neurons might inhibit dauer entry by secreting dauer inhibitory signals, the above observation suggests that ILPs function in sensory neurons to control dauer arrest, and possibly other physiological processes in response to environmental conditions. Likewise, *Drosophila* ILPs have been shown to regulate physiology and behavior in response to environmental cues (Ikeya et al., 2002; Yang et al., 2008).

2. Scope of the thesis

Considering the pleiotropic activities of the insulin signaling pathway in response to a diverse range of sensory cues and the diversity in the *C. elegans ilp* expression patterns, which include the sensory system (Gems et al., 1998; Kodama et al., 2006; Lee et al., 2003; Li et al., 2003; Murphy et al., 2003; Pierce et al., 2001; Tomioka et al., 2006), I hypothesized that ILPs encode sensory information to regulate physiology. Accordingly, I focused on investigating how C. *elegans* ILPs mediate the sensory influence on development. Specifically, I asked how ILPs regulate the important developmental decision between reproductive growth and dauer arrest in response to different sets of environmental cues.

Some of the *ilp* genes have previously been implicated in the regulation of dauer arrest (Malone et al., 1996; Murphy et al., 2003; Li et al., 2003; Pierce et al., 2001). However, these earlier studies involved indirect manipulations of ILP function through gain-of-function and RNA interference (RNAi) methods, which might have interfered with the endogenous activities of other dauer-regulating ligands. For example, the gain-of-function mutation, *daf-28(sa191)*, impairs a proteolytic cleavage site, which has been proposed to sequester and reduce the activity of an ILP-processing peptidase (Li et al., 2003). Thus, the protein encoded by *daf-28(sa191)* might interfere with the processing and subsequent function of a whole group of peptide precursors, which would include *β*-type ILPs, since overexpression of wild-type *daf-28* or other *β*-type ILPs can rescue the mutation (Li et al., 2003). At the same time, overexpression of ILPs or RNAi assays against specific ILPs could also hinder the functions of other ILP ligands. In addition, many of the *ilp* genes are expressed in neurons (Li et al., 2003; Pierce et al., 2001) and RNAi is known to work less efficiently in these cells (Tavernarakis et al., 2000). Thus, these previous approaches do not allow the direct analysis

of each ILP function or the relative contribution of each ILP to each step of the developmental switch.

In order to define the exact functions of different ILPs, I analyzed the deletion mutations available for the *ilps* already implicated in dauer regulation: *daf-28*, *ins-6* and *ins-1* (Li et al., 2003; Pierce et al., 2001). Unlike the previous studies, I find that these three ILPs have distinct and non-redundant functions in the regulation of dauer entry and dauer exit. Like *daf-28* and *ins-1, ins-6* is expressed in the amphid sensory neurons that regulate the dauer program. Again, like *daf-28*, but unlike *ins-1*, *ins-6* transcription is regulated in response to distinct, dauer-regulating cues. However, unlike *daf-28*, induction of the dauer program leads to a switch in *ins-6* expression from one pair of amphid sensory neurons to another. Thus, the specificity in the spatiotemporal regulation of *ilp* expression in specific sensory neurons by different sensory cues suggests how these ILPs might encode specific environmental conditions, and subsequently elicit the appropriate physiological and developmental responses.

3. Results

3.1 Insulin-like peptides act as part of a sensory code to regulate *C. elegans* development

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3.1.1 Abstract

C. elegans has 40 putative insulin-like genes, many of which are expressed in sensory neurons and interneurons. This raises an intriguing possibility that insulin-like peptides (ILPs) encode environmental complexity to regulate worm physiology. Here we show that specific ILPs act combinatorially in response to distinct sensory cues to regulate the switch between two developmental programs, reproductive growth versus dauer arrest. One ILP, *ins-1*, ensures dauer arrest under harsh environments. Two other ILPs ensure reproductive growth under good conditions. While *daf-28* plays a more primary role in inhibiting dauer entry, *ins-6* has a more significant role in promoting dauer exit. Interestingly, the switch into the dauer program also shifts *ins-6* transcriptional expression from a set of dauer-inhibiting sensory neurons to another set known to stimulate dauer exit. Together our data suggest that specific ILPs generate precise responses to dauer-inducing cues, like pheromones, and food levels to control development through stimulus-regulated expression in different neurons.

3.1.2 Introduction

The environment has long been known to influence animal physiology. For example, the olfactory composition of the environment can affect the release of hormones necessary for the development and function of the mammalian reproductive system (Yoon et al., 2005). In *C. elegans*, the nature of its environment also 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, 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; Riddle et al., 1981; Golden and Riddle, 1982; Golden and Riddle, 1984).

The entry into the dauer program is regulated by specific neurons that sense not only the decrease in food cues but also the increase in levels of a pheromone mixture (Bargmann and Horvitz, 1991; Schackwitz et al., 1996; Kim et al., 2009) of glycosides (Jeong et al., 2005; Butcher et al., 2007), which is secreted by each animal throughout its life (Golden and Riddle, 1982; Golden and Riddle, 1984). A large increase in the dauer pheromone mixture in the environment, which signifies overcrowding and thus low food availability, is sufficient to induce animals to switch from reproductive growth to dauer arrest (Golden and Riddle, 1984). On the other hand, the exit from the dauer program into the last larval stage (L4), prior to becoming fertile adults, is promoted by a subsequent improvement in the environment (Golden and Riddle, 1984), *e.g.*, an increase in food levels, which is also sensed by neurons (Bargmann and Horvitz, 1991).

One of the pathways known to mediate the sensory influence on *C. elegans* development is the insulin/IGF DAF-2 pathway (Riddle et al., 1981; Vowels and Thomas, 1992; Kimura et al., 1997). Mutations that downregulate the insulin/IGF receptor ortholog DAF-2 (Kimura et al., 1997) lead to dauer arrest, which requires the activity of the FOXO transcription factor *daf-16* (Riddle et al., 1981; Vowels and Thomas, 1992; Gottlieb and Ruvkun, 1994; Lin et al., 1997; Ogg et al., 1997). Moreover, while 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 the dauer program but also when they should exit from the program.

While 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 ILPs [(Pierce et al., 2001; Li et al., 2003); see www.wormbase.org, release WS207]. Some of the ILPs, like INS-1, INS-6 and DAF-28, have already been implicated in regulating the switch between reproductive development and dauer arrest through gain-of-function or RNA-mediated interference studies (Malone et al., 1996; Pierce et al., 2001; Li et al., 2003; Murphy et al., 2003). Many of the ILPs are also expressed in overlapping subsets of sensory neurons and/or interneurons, including the sensory neurons that regulate dauer formation (Pierce et al., 2001; Li et al., 2003; Kodama et al., 2006). Thus, the complexity of the sensory cues that induce this developmental switch and the different *ilp* expression patterns raise an intriguing possibility that ILPs encode sensory information to regulate development, and perhaps other physiological responses. Indeed, some of the *Drosophila* ILPs have recently been shown to mediate different processes (Ikeya et al., 2002; Yang et al., 2008; Zhang et al., 2009), which could also be regulated by a variety of sensory inputs. Consistent with the above hypothesis, the mammalian ILPs of the insulin/relaxin superfamily are also expressed in non-overlapping

cells, some of which are neurons with known sensory-associated functions (Ayer-le Lievre et al., 1991; Bathgate et al., 2002; Sherwood, 2004; Liu and Lovenberg, 2008; Meyts et al., 2009).

Here we have tested the hypothesis that *C. elegans* ILPs encode environmental information to control development. We have analyzed the functions of specific ILPs in regulating entry into and exit from the dauer state and find that the ILPs *daf-28*, *ins-6* and *ins-1* act combinatorially to determine the switch between developmental programs. We also show that environmental information is encoded by 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.

3.1.3 Results

daf-28 Has a More Prominent Role Than *ins-6* in Inhibiting Dauer Entry

The loss-of-function mutations of many specific ILPs have never been tested for phenotypes in dauer formation, although previous RNA-mediated interference or gain-of-function experiments suggested that at least some of the ILPs, *e.g.*, *daf-28*, *ins-6* and *ins-1*, play a role in this process (Malone et al., 1996; Pierce et al., 2001; Li et al., 2003; Murphy et al., 2003). However, these earlier studies involved indirect manipulations of ILP function that do not allow the direct comparison of the relative contributions of specific ILPs in dauer formation. Thus, to test directly the role of each ILP in this process, we studied deletion mutants, in which gene function is completely and specifically eliminated. We focused on examining the dauer program phenotypes of 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 (Figures 1A and 1B; Table S1). In contrast, the *daf-28*

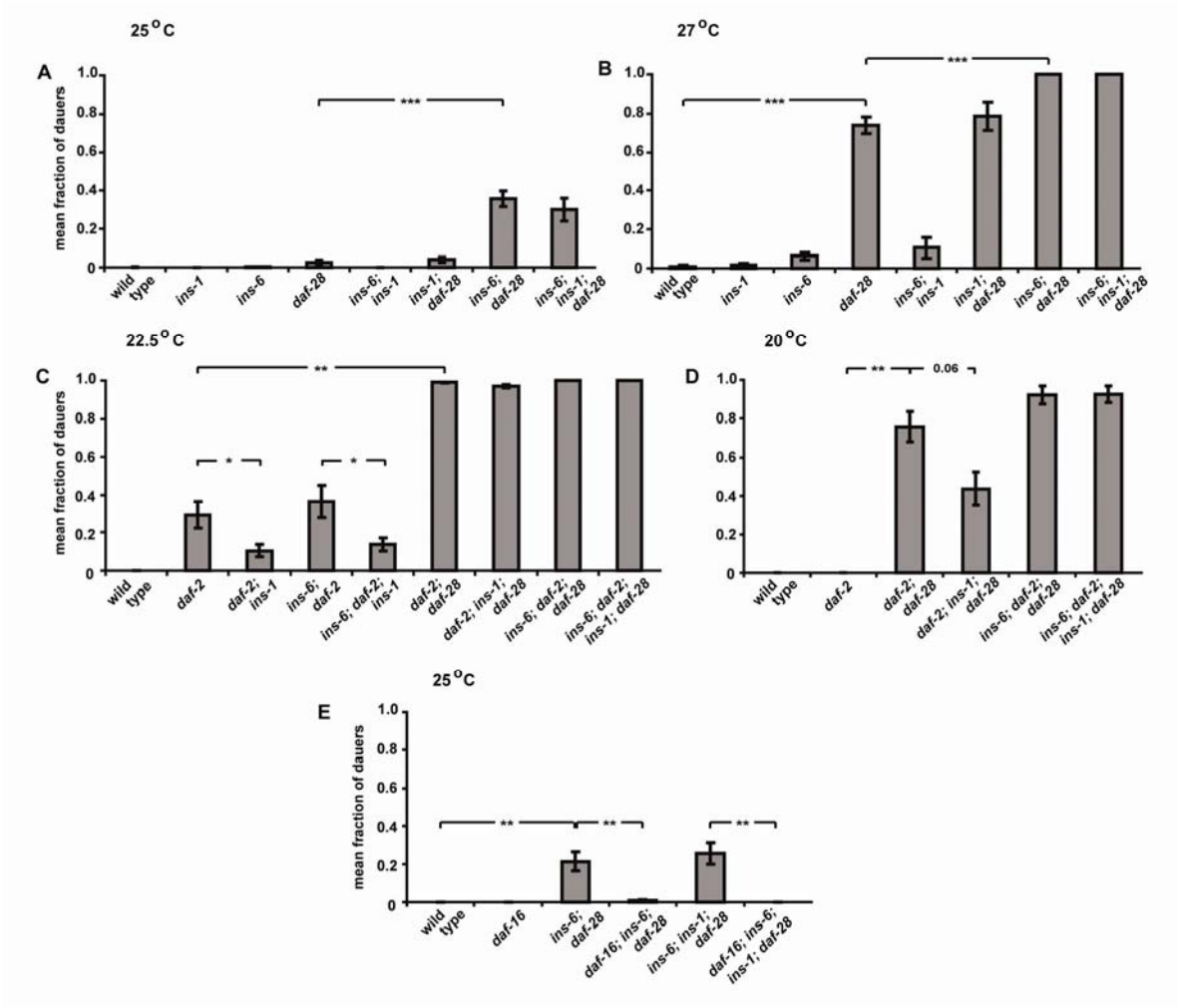


Figure 1. *daf-28* acts with *ins-6* to inhibit dauer entry, whereas *ins-1* promotes it. (A-B) The mean fractions of wild-type and insulin-deletion mutant worms that form dauers are shown at the indicated temperature. Each mean ± SEM includes at least three independent trials of approximately 100 worms. The detailed statistical comparisons between the dauer entry phenotypes of different genotypes under different conditions in these and subsequent analyses can be found in Table S1. In this and later panels, * indicates $P \leq 0.05$; **, $P \leq 0.01$; and ***, $P \leq 0.001$. (C-D) The effect of different insulin deletions on dauer entry in a *daf-2(e1368)* mutant background at 22.5°C (C) and 20°C (D). (E) The dauer entry phenotype of *ins-6*; *daf-28* deletion mutants is suppressed by the *daf-16(mu86)* mutation at 25°C.

deletion mutants formed few dauers at 25°C and a much larger fraction of dauers at 27°C (Figures 1A and 1B; Table S1). In addition, loss of *ins-6* enhanced the dauer entry phenotype of *daf-28* deletion mutants at both temperatures, while removing *ins-1* did not (Figures 1A and 1B; Table S1). 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 with overexpression of wild-type genomic *daf-28* or *ins-6* (Li et al., 2003). Furthermore, by directly comparing null mutants, we 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, while the *ins-6* deletion had little or no effect (Figures 1C and 1D; Table S1). 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 phenotype of *ins-6*; *daf-28* double mutants is suppressed by loss of *daf-16* (Figure 1E; Table S1). Thus, this 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

A previous study suggested a role for *ins-1* in dauer formation (Pierce et al., 2001). By using *ilp* deletion mutants in our study, 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 (Figure 1C; Table S1). Likewise, loss of *ins-1* decreased the number of dauers formed by *ins-6*; *daf-2(e1368)* mutants (Figure 1C; Table S1). On the other hand, an *ins-1* deletion can only suppress the dauer entry phenotype of the *daf-2*; *daf-28* double mutants at a lower temperature, 20°C (Figure 1D; Table S1), which is a weaker dauer-inducing condition, and not at 22.5°C (Figure 1C; Table S1), a stronger dauer-inducing condition. Consistent with these observations, loss of *ins-1*

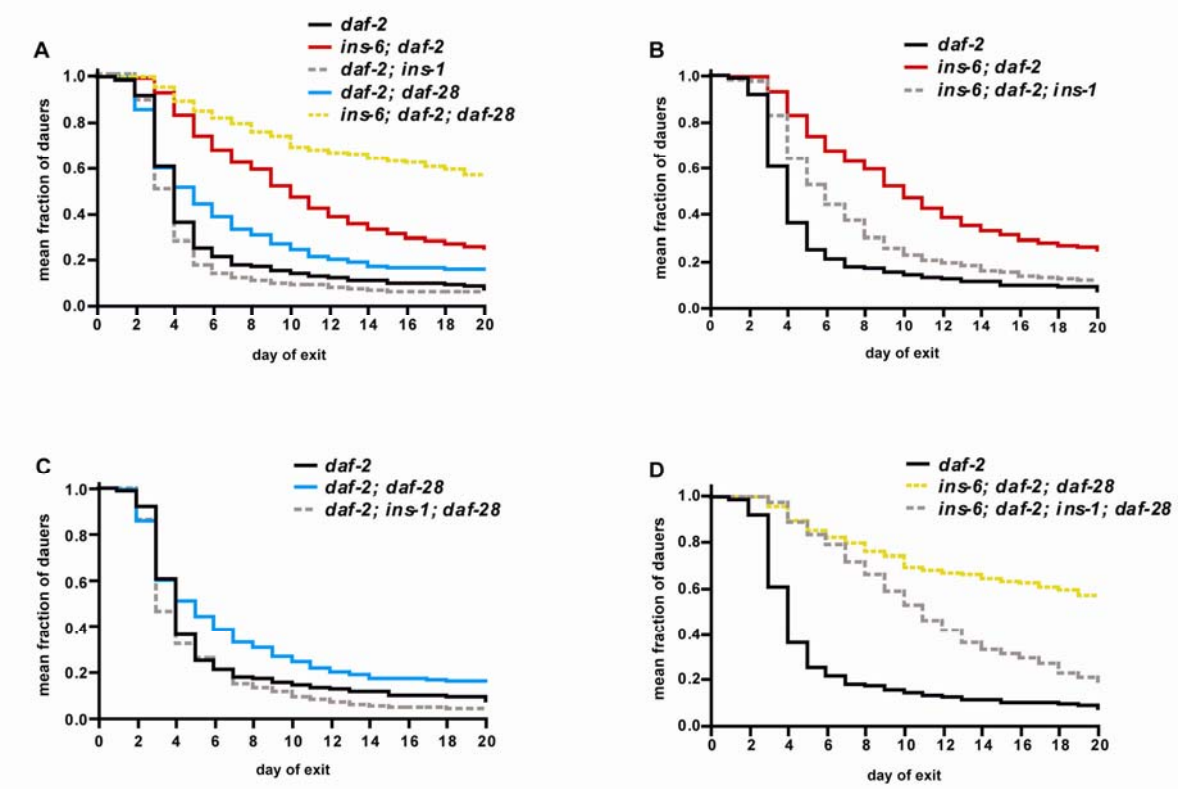


Figure 2. *ins-6* acts with *daf-28* to promote dauer exit, whereas *ins-1* inhibits it. (A-D) The rates of dauer exit at 25°C of animals carrying different combinations of insulin deletions in a *daf-2(e1368)* mutant background. Each curve represents cumulative data from six independent trials. All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

also had no effect on dauer entry in *ins-6; daf-2(e1368); daf-28* triple mutants, which all form dauers at both temperatures (Figures 1C and 1D; Table S1)**.** Thus, these findings suggest that, in contrast to *daf-28* and *ins-6*, *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). At the same time, 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, *daf-2(e1370)* mutants, which have a strong reduction in *daf-2* function, arrest constitutively as dauers at 25°C, while *daf-2(e1368)* mutants, which have a weaker reduction in receptor activity, arrest as transient dauers that exit after a few days (Gems et al., 1998). To determine whether the same insulin-like genes that control dauer entry also regulate dauer exit, we analyzed the effects of loss of *ins-1*, *ins-6* and/or *daf-28* on dauer exit of *daf-2(e1368)* mutants, which all form dauers at 25°C. We found that deletion of *ins-6* in *daf-2* mutants strongly inhibits dauer exit, but removal of *daf-28* only slightly delayed it (Figure 2A; Table S2). In addition, removal of both *ins-6* and *daf-28* in *daf-2* mutants caused the greatest delay in dauer exit (Figure 2A; Table S2). Thus, these data indicate (i) that both ILPs act together to promote dauer exit; and (ii) that while *daf-28* plays a more primary role in inhibiting dauer entry, *ins-6* has a more significant role in promoting dauer exit.

The differences in the relative importance of *ins-6* in dauer entry versus dauer exit could be reflected by the levels of *ins-6* required to rescue the dauer entry phenotype compared to the levels needed to rescue the dauer exit phenotype. We found that a low level of wild-type *ins-6* activity is sufficient to rescue the dauer entry phenotype of the *ins-6*; *daf-28* double mutants back to that of the *daf-28* single mutants (Figures 3A and 3B; Table S1). On the other hand, we found that the dauer exit phenotype of *ins-6*; *daf-2* double mutants can only be fully rescued to the dauer exit phenotype of *daf-2* single mutants with a high level of wild-type *ins-6* activity (Figures 4A and 4B; Table S2). It should also be noted that high levels of *ins-6* activity do not completely rescue the phenotype of *ins-6*; *daf-28* double mutants back to wild type (Figure 3B; Table S1), which again suggests that these ILPs do not act completely redundantly with each other.

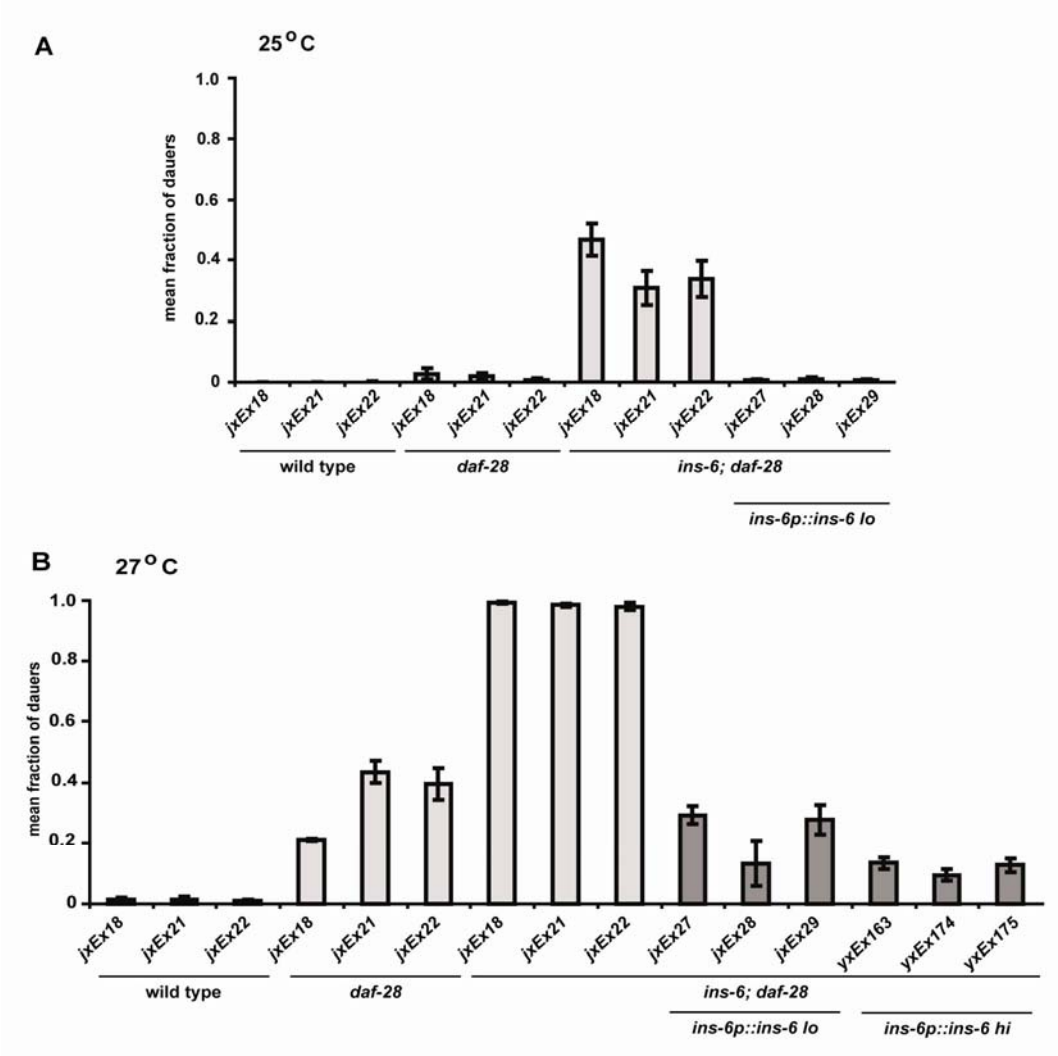


Figure 3. Inhibition of dauer entry requires low levels of *ins-6* activity. (A-B) The *ins-6*; *daf-28* mutants that were rescued with low (2 ng/µl; *jxEx27*, *jxEx28* or *jxEx29*) or high levels of *ins-6* activity (25 ng/µl; *yxEx163*, *yxEx174* or *yxEx175*) are compared to wild type or insulin-deletion mutants that carry the *ofm-1p::gfp* coinjection marker alone (*jxEx18*, *jxEx21* or *jxEx22*). The mean fractions of dauers are shown at 25°C (A) or 27°C (B; see Table S1 for the statistical analyses of these experiments).

ins-1 Inhibits Dauer Exit

Overexpression of *ins-1* was previously shown to increase dauer formation (Pierce et al., 2001). However, it is unclear from this former study whether *ins-1* only regulates dauer entry or dauer exit or both. Since we already showed that *ins-1* does promote dauer entry (Figures 1C and 1D; Table S1), 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 (Figure 2A; Table S2),

enhanced dauer exit in all other *daf-2* mutants that are lacking *ins-6* and/or *daf-28* (Figures 2B-2D; Table S2). Thus, this suggests that *ins-1* not only plays a role in dauer entry but also in dauer exit and that the wild-type function of *ins-1* is to ensure dauer arrest under harsh environmental conditions.

ins-6 Expression Switches Between Two Sensory Neurons to Control Dauer Entry versus Dauer Exit

The switch between reproductive growth and dauer arrest is regulated by specific sensory neurons (Bargmann and Horvitz, 1991; Schackwitz et al., 1996) that have been shown to express some *ilp* genes (Pierce et al., 2001; Li et al., 2003). The sensory neurons ADF, ASI and ASG inhibit dauer entry (Bargmann and Horvitz, 1991), while the sensory neurons ASJ and ASK promote it (Schackwitz et al., 1996; Kim et al., 2009). Furthermore, ASJ has a second function in that it senses other cues to stimulate dauer exit (Bargmann and Horvitz, 1991).

Although *daf-28* is expressed in ASI and ASJ neurons of well-fed animals and is downregulated in both neurons by low food availability, a dauer pheromone mixture or entry into the dauer program (Li et al., 2003), the cells from which *ins-1* or *ins-6* might act to regulate this developmental switch remain 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*, we observed that the switch in developmental programs had little or no effect on the expression of *ins-1* in ASI and ASJ (Table 1), which is based on a *cfp* transcriptional reporter fused to the *ins-1* 5' and 3' *cis* regulatory sequences.

On the other hand, we found that *ins-6* expression, which is based on an *mCherry* transcriptional reporter fused to the upstream and downstream regulatory regions of *ins-6* (*ins-6p::mCherry*), is restricted to the ASI neurons of well-fed larvae and adults (Figure 5).

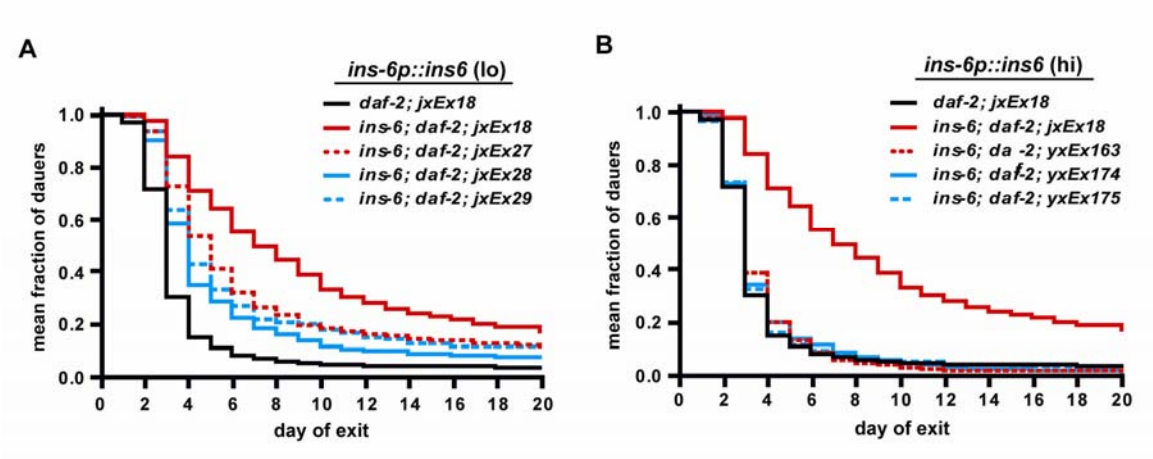


Figure 4. Unlike the case for dauer entry, higher levels of *ins-6* activity are required to promote dauer exit. (A-B) The rates of dauer exit of *ins-6*; *daf-2(e1368)* mutants that were rescued with low (*jxEx27*, *jxEx28* or *jxEx29*; A) or high (*yxEx163*, *yxEx174* or *yxEx175*; B) *ins-6* activity are compared to those of *daf-2(e1368)* or *ins-6*; *daf-2(e1368)* mutants that carry the *ofm-1p::gfp* coinjection marker alone (*jxEx18*). Each curve represents the cumulative data from at least seven independent 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. See Table S2 for the complete statistical analyses of these experiments, and a comparison of the rescue lines with two additional control lines.

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 is determined with a *gfp* or an *mCherry* transcriptional reporter fused only to the *ins-6* upstream regulatory region. This suggests that sequences downstream of *ins-6* contain element(s) that repress its expression in other neurons.

Interestingly, the switch into the dauer program shifts *ins-6p::mCherry* transcription from ASI to ASJ (Figures 5B and 5C; Table 1). In addition, as the animals start to exit from dauer, ASJ expression of *ins-6p::mCherry* appears to become even stronger (data not shown) in response to improved environmental conditions. This activation of *ins-6* in ASJ is also observed in worms carrying a transcriptional reporter fused only to the *ins-6* upstream regulatory region (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. However, overexpression of *ins-6* from either ASI or ASJ is sufficient to rescue both the dauer entry phenotype of *ins-6*; *daf-28* mutants and the dauer exit phenotype of *ins-6*; *daf-2* mutants (Figures S1 and S2).

Moreover, *ins-6p::mCherry* surprisingly continues to be expressed in the ASJ neurons and is absent in the ASI neurons of post-dauer L4s, young adults and five-day-old adults (Figure 5E; Table 1), which is dissimilar from continuously well-fed L4 larvae and adults that express *ins-6p::mCherry* in ASI and never in ASJ (Figure 5D; Table 1). Thus, post-dauer animals appear physiologically distinct from animals that never underwent the dauer program.

The Dauer Pheromone and the Dauer Program Have Distinct Effects on *ins-6* Expression

ins-6p::mCherry expression changes between developmental programs that are triggered by distinct sets of sensory cues. 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, which is a mixture of glycosides (Jeong et al., 2005; Butcher et al., 2007), downregulated *ins-6p::mCherry* in 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 ASJ of the different well-fed larvae and adults that were analyzed (Table 1). Surprisingly, induction of the dauer program by shifting *daf-2(e1368)* mutants to 25^{o}C, but under low pheromone levels, was also insufficient in fully activating *ins-6p::mCherry* in ASJ (Table 1). We found that the switch in *ins-6p::mCherry* from ASI to ASJ is 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

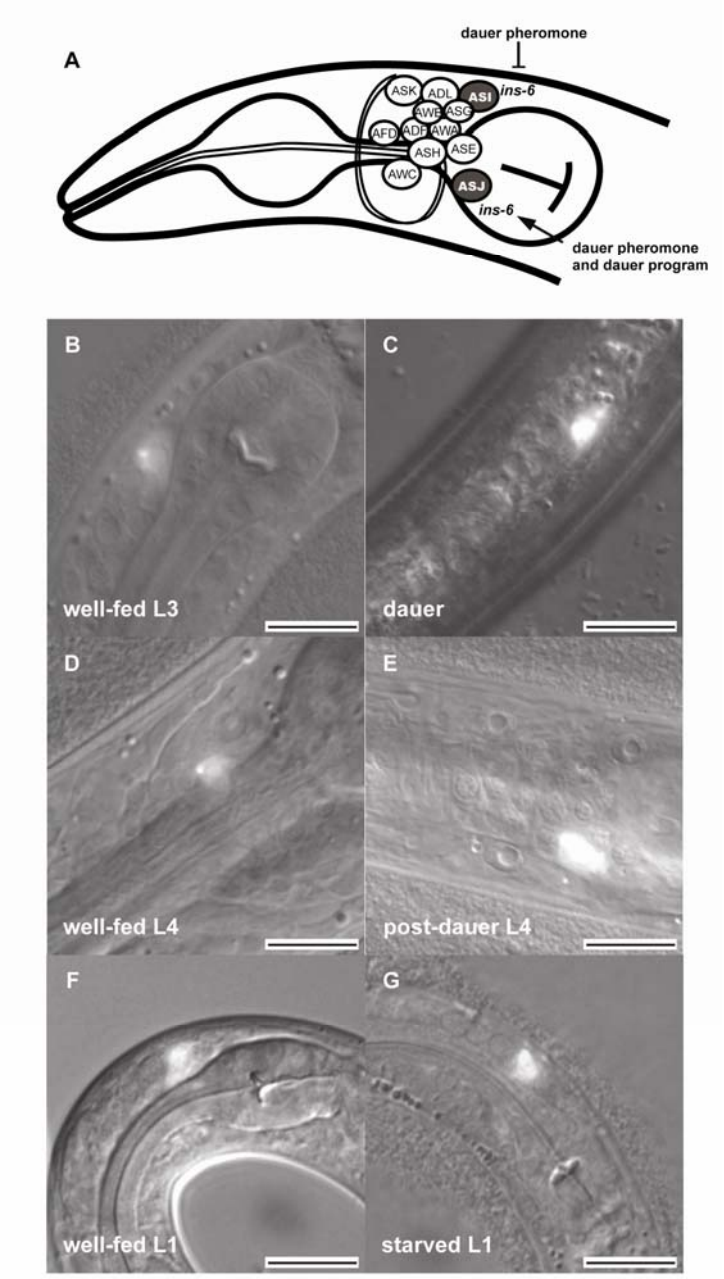


Figure 5. *ins-6* transcription switches between ASI and ASJ sensory neurons in response to the dauer pheromone and the dauer program. (A) A schematic diagram of the twelve sensory neurons in the *C. elegans* amphid sensory organ (White et al., 1986). The ASI and ASJ neurons are indicated in dark grey. The dauer pheromone mixture inhibits *ins-6* transcription in ASI, whereas both the pheromone and the dauer program activate *ins-6* in ASJ. (B, D, F) *ins-6p::mCherry* is expressed in the ASI neuron of well-fed L3 (B), L4 (D) and L1 (F) larvae. (C, E) *ins-6p::mCherry* becomes expressed in the ASJ neuron of a dauer larva (C) and remains on in ASJ of a post-dauer L4 larva (E). (G) In contrast, *ins-6p::mCherry* is unaffected in ASI and is not activated in ASJ of a starved L1 larva. All animals are oriented with their anterior to the lower left and their dorsal side up. The scale bar represents 10 μm in length.

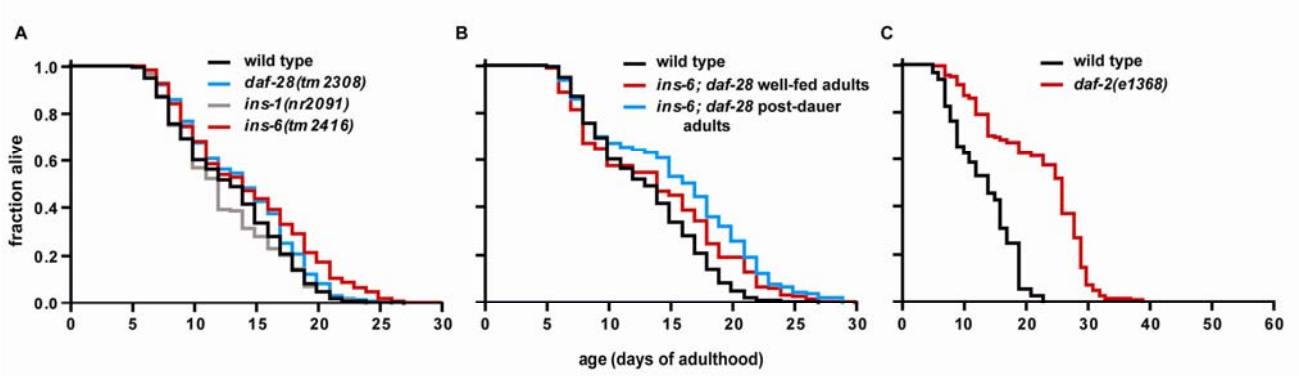
Table 1. *ilp* expression

Condition / Stage		**ASIL/R** expression				**ASJL/R** expression			
ins-6p::mCherry	Total *N*	none	weak	medium	strong	none	weak	medium	strong
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 [a]	1	0	8	0	8	1	0	0
L2d	46 [b]	8	38	0	0	41	5	0	0
L4	28	10	18	0	0	28	0	0	0
Adult	3 [a]	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 [b]	2	0	8	0	10	0	0	0
L4	25	4	0	21	0	25	0	0	0
Adult	2 [a]	0	0	2	0	2	0	0	0
27°C									
L1	28	2	0	26	0	26	2	0	0
L2	16 [b]	2	0	14	0	16	0	0	0
L3	5 [a]	0	0	5	0	5	0	0	0
L4	26	1	0	25	0	26	0	0	0
daf-2(e1368)									
L1	33 [b]	3	0	30	0	33	0	0	0
L2	26 [b]	4	0	22	0	26	0	0	0
L3	11 [b]	0	0	11	0	11	0	0	0
L4	77 [b]	6	0	71	0	77	0	0	0
Adult	6 [a]	1	0	5	0	6	0	0	0
Dauer									
High pheromone	42 [b]	36	6	0	0	1	0	0	41
Low pheromone	31 [b]	5	1	25	0	24	0	7	0
daf-16(mu86)									
L1	23 [b]	1	0	22	0	23	0	0	0
L2	18 [b]	0	0	18	0	18	0	0	0
L3	13 [b]	0	0	13	0	13	0	0	0
L4	68 [b]	3	0	65	0	68	0	0	0
Adult	33 [b]	3	0	30	0	33	0	0	0
Partial dauer	14 [b]	0	14	0	0	14	0	0	0
ins-1p::cfp									
Well-Fed L3	20 [b*]	0	0	20	0	0	0	0	20
Dauer	20 [b*]	0	0	20	0	0	0	7	13

Table 1. Specific cues have distinct effects on *ilp* expression. *ins-6p::mCherry* or *ins-1p::cfp* expression in different stages of wild-type and mutant worms that developed under different treatments (see Experimental Procedures). The wild-type dauers listed were induced either by the addition of high concentrations of dauer pheromone 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 dauer larvae, which were induced by overcrowding at 20°C (high pheromone) or by temperature (25°C; low pheromone). *daf-16* mutants that were assayed 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. The symbols indicate the following: a, analyzed only one line; b, analyzed only two lines; and *, expression is present in additional head and tail neurons.

density (Table 1). Since we also never observed *ins-6p::mCherry* expression in ASJ of partial dauers, our data suggest that the combined activities of the dauer program with the dauer pheromone, which presumably induces a stronger arrest, are required for this shift in expression (Table 1).

In addition, we found that starvation alone or high temperature (27°C) has little or no effect on *ins-6p::mCherry* expression, in comparison to well-fed worms maintained at 20°C (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 more specific response to the dauer pheromone cue, whereas the switch in neuronal expression is another response distinct to the coordinated action of the pheromone and the dauer program.



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	

Figure 6. *ins-6* and *daf-28* have little effect on lifespan, whereas dauer-induced changes have a more significant effect. (A) The effects of *ins-6*, *daf-28* or *ins-1* on lifespan. (B) The lifespan of continuously well-fed or post-dauer *ins-6*; *daf-28* mutant adults is compared to that of well-fed wild-type adults. (C) The effect on lifespan of *daf-2(e1368)* mutants. Since *daf-2* mutants undergo dauer formation at 25°C, wild type and mutant animals were grown at 20°C until the first day of adulthood, when the worms were shifted to 25°C to initiate lifespan analyses. (D) The statistics for the cumulative lifespan data. The 75th percentile is the age when the fraction of worms alive in each group falls below 0.25. The first number in the fourth column is the number of observed deaths, whereas the second number is the total number of worms in each experiment, including worms that were censored during the assay. Worms that crawled off the plate, exploded or bagged were censored at the time of the event, but were incorporated into the data set until the censor date to avoid loss of information. The numbers in parentheses in the fourth column indicate the number of trials performed. The % difference between wild type and mutants is indicated in the fifth column and *P* values were determined using the logrank test. The % difference between continuously fed and post-dauer *ins-6*; *daf-28* mutants is also shown.

ins-6 and *daf-28* Play Only a Minor Role in Regulating Lifespan

Since our observations suggest that these three *ilps* have distinct, non-redundant functions in regulating two developmental programs, we then asked whether they also affect lifespan, which is known to be regulated by the DAF-2 pathway (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 has little or no effect on adult lifespan (Figure 6). Similarly, loss of both *ins-6* and *daf-28* has little effect on the lifespan of animals that did not undergo dauer formation (Figures 6B and 6D). However, we observed that *ins-6*; *daf-28* double mutants that formed transient dauers do live longer than double mutants that never became dauers (Figures 6B and 6D). This is consistent with the idea that post-dauer adults are physiologically distinct from continuously well-fed adults, as demonstrated by the switch in *ins-6* expression that persists in post-dauer animals. Together these findings suggest that other *ilps* are required to modulate the DAF-2 signaling cascade to affect lifespan and that these *ilps* may also have a more primary role than *ins-6*, *daf-28* and *ins-1* in this process.

3.1.4 Discussion

The large number of *ilp* genes in *C. elegans* and the spatiotemporal diversity of their expression patterns, which include different subsets of sensory neurons [(Pierce et al., 2001; Li et al., 2003); see www.wormbase.org, release WS207], raise the likelihood that these genes may regulate different processes in response to different stimuli. Since some of these genes share overlapping expression patterns, the resulting ligands may act not only individually but also combinatorially on their receptor, DAF-2, in different subsets of cells. In this study, we show that some of these ILPs function as a combinatorial code to regulate *C. elegans* developmental programs in response to a complex set of sensory cues.

ILPs Encode Environmental Information to Regulate *C. elegans* Physiology

The development of many animals is subject to their environment. In *C. elegans*, food availability can determine whether L1 larvae will undergo the developmental dauer arrest program (Golden and Riddle, 1982; Golden and Riddle, 1984). The ratio between levels of food cues and that of the dauer pheromone mixture reflects the food supply for a given worm population density (Golden and Riddle, 1982; Golden and Riddle, 1984). The perception of these cues by a specific subset of sensory neurons that either inhibit dauer entry (ASI, ADF and ASG), promote dauer entry (ASJ and ASK) or promote dauer exit (ASJ) (Bargmann and Horvitz, 1991; Schackwitz et al., 1996; Kim et al., 2009; Macosko et al., 2009) in turn regulates the secretion of growth-modulatory signals. For example, low food levels and high concentrations of dauer pheromone repressed the expression in ASI of the TGF-β *daf-7*, which is required for reproductive development under growth-inducing conditions (Ren et al., 1996; Schackwitz et al., 1996). At the same time, re-introduction of food to a dauer population induces dauer exit and resumption of *daf-7* expression and reproductive development (Ren et al., 1996).

Like *daf-7*, food cues are required for the transcription of the ILP *daf-28* in ASI and ASJ, whereas the dauer pheromone mixture suppresses it in both neurons (Li et al., 2003). On the other hand, we show that food cues and dauer pheromone have different effects on other ILPs (Figure 5; Table 1). We find that food levels and dauer pheromone have little or no effect on the transcription of the ILP *ins-1* in ASI and ASJ (Table 1). In contrast, we observe that high levels of dauer pheromone specifically repress the transcription of the ILP *ins-6* in ASI, while food cues have little or no effect on its expression in this neuron (Figure 5; 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 function combinatorially to regulate development. Consistent with their role in ensuring reproductive growth (Figures 1-4; Tables S1 and S2), *daf-28* (Li et al., 2003) and *ins-6* (Figure 5) are expressed in the dauer entry-inhibiting neuron ASI (Bargmann and Horvitz, 1991) in response to high food and/or low population density signals. Interestingly, other cues derived from dauer program-induced physiological changes, together with high pheromone levels, trigger *ins-6* transcription in ASJ (Figure 5; Table 1), a change in expression that alone is insufficient to promote dauer exit. This suggests that (i) the animal initiates *ins-6* expression in this dauer exit-promoting neuron to facilitate its activation as soon as environmental conditions improve and (ii) *ins-6* activity is also regulated post-transcriptionally by other cues, like, perhaps, by 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).

Our study also shows that *ins-6* expression shifts from ASI to ASJ only under strong dauer-arrest conditions. We find that this shift is less evident in temperature-induced, low pheromone-exposed *daf-2(e1368)* dauers (Table 1), which exit after a few days (Figure 2) and thus may represent a weaker dauer-arrest condition. It is likely that a strong, but not a weak, dauer arrest induces the full dauer transcriptional program, such as the altered *ins-6* expression. It is also possible that the sensory receptors required to induce *ins-6* activity after a strong arrest are present only in ASJ but not in ASI, although ASI does contain receptors that sense improved environmental conditions as *daf-7* expression recovers in this neuron with the addition of food (Ren et al., 1996). Alternatively, *ins-6* might act locally as part of different developmental circuits that are remodeled in response to cues, like the combination of dauer pheromone with the dauer program.

Our findings suggest that *ins-6* and *daf-28* encode ligands that act combinatorially through the DAF-2 receptor, while *ins-1* antagonizes the activity of this pathway (Figures 1 and 2; Tables S1 and S2). These ILPs may act as long-range signals, like hormones, or they may act as short-range signals within neuronal circuits. Furthermore, these ligands may 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 dauer formation [(Pierce et al., 2001) and this paper] and the worm's food-associated thermotactic behavior (Kodama et al., 2006), *ins-1* can also act like an agonist of DAF-2 in the worm's 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. Indeed, previous mosaic analyses of *daf-2* function already raised the possibility that different cells have different DAF-2 activities, such as in the case of regulating dauer entry versus dauer exit (Apfeld and Kenyon, 1998; Wolkow et al., 2000). On the other hand, none of our experiments have ruled out the possibility that some of these ILPs, which are predicted to

have diverse structures, *e.g.*, *daf-28* or *ins-6* in contrast to *ins-1* (Pierce et al., 2001), will bind receptors other than DAF-2.

Finally, it should 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 coordinately to regulate developmental programs, appear to have little or no effect on lifespan (Figure 6). Considering that lifespan is influenced by many types of cues and sensory neurons (Apfeld and Kenyon, 1999; Alcedo and Kenyon, 2004; Libert et al., 2007; Lee and Kenyon, 2009), it is therefore not surprising that many cue-responsive ILPs would also be involved in regulating DAF-2 activity to affect longevity.

Post-Dauer Adults Are Distinct from Continuously Well-Fed Adults

Our observation that, in contrast to continuously fed adults, *ins-6* is expressed from a different sensory neuron in post-dauer adults suggests that the two types of animals could exhibit different physiology. Consistent with this hypothesis, a previous finding has shown that the duration of the dauer state correlates positively with the number of reproductive defects in post-dauer adults and correlates negatively with their brood size (Kim and Paik, 2008). Moreover, we find that *ins-6*; *daf-28* post-dauer adults lived 11% longer than the continuously fed double mutant animals (Figure 6). This is similar to a recent observation that wild-type post-dauer adults are longer-lived than their continuously fed counterparts (Hall et al., 2010). This suggests that (i) the dauer state causes a physiological change that is sufficient to induce a small but significant extension in adult lifespan and (ii) this lifespan extension is not necessarily dependent on *ins-6* and *daf-28*. At the moment, the exact physiological change required for this increase in lifespan remains unknown.

The Relevance of Multiple ILPs in Other Animals

The concept that ILPs combinatorially encode environmental information to regulate physiology might be true not only for *C. elegans* but also for other animals, like *Drosophila* or mammals. *Drosophila* has seven known ILPs, *dilp1* through *dilp7*, which are expressed in neuronal and/or non-neuronal cells (Brogiolo et al., 2001; Ikeya et al., 2002; Rulifson et al., 2002; Yang et al., 2008; Slaidina et al., 2009). Interestingly, the neurons that express some of these ILPs send or receive projections from subesophageal ganglion interneurons, which in turn can receive information (Rulifson et al., 2002; Melcher and Pankratz, 2005; Yang et al., 2008) from gustatory neurons that innervate chemosensory structures within the fly mouthparts (Scott et al., 2001). In addition, some of these neuronal-expressed ILPs, *dilp2*, *dilp3* and *dilp5*, have been proposed to regulate growth and metabolism in a nutrient level-dependent manner, whereas others do not (Ikeya et al., 2002; Broughton et al., 2008; Zhang et al., 2009). In contrast, some are required in other processes, like selecting an optimal environment for egg laying, which is *dilp7*-dependent (Yang et al., 2008). Together these data raise the likelihood that *Drosophila* ILPs also act coordinately to encode environmental information to regulate different aspects of physiology.

Mammals have seven to ten known members of the insulin/relaxin superfamily that are also expressed in neuronal and/or non-neuronal tissues (Ayer-le Lievre et al., 1991; Liu and Lovenberg, 2008; Meyts et al., 2009). The roles of insulin, IGF-I and IGF-II in mammalian metabolism, growth, differentiation and lifespan have been studied in great detail (Nakae et al., 2001; Sherwood, 2004; Kenyon, 2005). A subgroup of mammalian relaxins has also 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 (Kenyon et al., 1993; Bluher et al., 2003; Holzenberger et al., 2003; Taguchi et

al., 2007), our study raises the intriguing possibility that specific subsets of mammalian ILPs also act together to regulate specific processes in response to different sets of environmental cues.

3.1.5 Experimental Procedures

Worm Strains and Culture

All worm mutants used in this study were backcrossed six times to our lab wild-type (N2) strain before any phenotypic analysis was performed. Worms were continuously fed *E. coli* OP50 for at least two generations before each assay.

Transgenic Worms

***ins-6* rescue lines.** We generated several independent rescue lines using standard methods and the *ofm-1p::gfp* (Miyabayashi et al., 1999) coinjection marker (injected at 25 ng/µl). For controls, we generated wild-type and mutant worms that carry the *ofm-1p::gfp* coinjection marker alone.

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 backbone (Invitrogen, UK). We injected this construct at two different doses (2 ng/µl and 25 ng/µl) into wild type or *ins-6(tm2416)* mutants. We then crossed the resulting extrachromosomal arrays into (i) the *ins-6(tm2416)*; *daf-28(tm2308)* mutant background to assay for rescue of the *ins-6*-dependent dauer entry phenotype; or (ii) the *ins-6(tm2416)*; *daf-2(e1368)* mutant background to test for rescue of the *ins-6*-dependent dauer exit phenotype.

To rescue the *ins-6* mutant phenotypes through ASI-specific expression of *ins-6*, we drove *ins-6* transcription from the promoter of the ASI chemosensory receptor *str-3* (Peckol

et al., 2001). We generated a plasmid construct (pQZ33) in the pPD95.77 vector backbone (gift of A. Fire), in which the *ins-6* cDNA is flanked by the 3.1–kb *str-3* promoter (gift of C. Bargmann) and by the 0.7–kb 3' UTR of *unc-54*. To rescue the *ins-6* mutant phenotypes through ASJ-specific expression of *ins-6*, we constructed a similar plasmid (pQZ35), which in this case drove *ins-6* transcription from the 1–kb promoter of *trx-1* (gift of P. Swoboda). The thioredoxin *trx-1* is specifically expressed from the ASJ neurons (Miranda-Vizuete et al., 2006). We then introduced either construct at two different doses (2 ng/μl and 25 ng/μl) into *ins-6(tm2416)*; *daf-2(e1368)* or *ins-6(tm2416)*; *daf-28(tm2308)* mutants.

***ilp* expression lines**. To determine the expression pattern of *ins-1*, 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 the *ins-1* stop codon. In addition, the 0.8–kb sequence of the largest intron, which may contain regulatory elements required for expression, is fused downstream of the 3' *cis* sequences. pQZ6 is injected into wild-type worms at a concentration of 100 ng/μl with the *ofm-1p::gfp* coinjection marker (injected at 25 ng/μl). Three independent lines were recovered, which show identical patterns of *cfp* expression.

To determine the expression pattern of *ins-6*, we constructed a transcriptional *ins-6p::mCherry* reporter (pQZ10) by using the Gateway system. The *mCherry* in pQZ10 is flanked by the 1.7–kb sequence upstream of the *ins-6* start codon and by the 2.0–kb sequence downstream the *ins-6* stop codon. pQZ10 is also injected into wild-type worms at 100 ng/μl with the *ofm-1p::gfp* coinjection marker. Three lines were recovered, which have the same *mCherry* expression pattern.

Dauer Entry Assays

The worms were grown at 25°C and allowed to lay eggs at this temperature for 3-7 h. The eggs were then allowed to develop either at 25°C or 27°C and scored ~24 h 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 were counted. Animals carrying transgenes or the *daf-2* or *daf-16* mutation were grown at 20°C and allowed to lay eggs at the same temperature for 3-7 h. The eggs were then allowed to develop either at the same temperature or shifted to 22.5°C, 25°C or 27°C, where the fraction of dauers and L4s/adults were counted at 48 h after the egg-laying midpoint. To compare the different genotypes, we used the Wilcoxon Mann-Whitney rank sum test as implemented in the coin package (Hothorn et al., 2008) of the R statistical software (Team, 2009).

Dauer Exit Assays

The eggs that were laid for 3-7 h by worms grown at 20°C were shifted to 25°C. All dauers that were formed 48 h after the egg-laying midpoint were then collected and scored daily for dauer exit. We used the 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 logrank test.

Lifespan Analyses

Lifespan was measured at 25°C, using worms that were grown at the same temperature, unless stated otherwise. All assays were initiated on the first day of adulthood. To avoid progeny contamination within the assay, we transferred the worms daily to new plates during their reproductive period. We also used the JMP 5.1 software to determine mean lifespan and

to perform statistical comparisons across the different genotypes. *P* values were determined by the logrank test.

Analyses of *ilp* Expression in Response to Environmental Cues

Dauer pheromone. To assess the effect of dauer pheromone on *ilp* expression, we placed about 100 embryos on 3.5-cm nematode-growth (NG) agar plates (Brenner, 1974), to which 100-200 μl of crude dauer pheromone mixture [prepared according to (Thomas et al., 1993)] and 50 μl of the *E. coli* OP50 food source were added. The concentration of the crude dauer pheromone mixture used in these assays caused about 45%-55% of wild-type L1 larvae to form dauers at 25°C. We monitored the *ilp* expression of the different larvae and adults that subsequently developed on these plates.

Starvation. The effect of low food availability on *ilp* expression was tested through several methods. First, we compared the *ilp* expression of well-fed larvae and young adults to 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 (i) were placed directly on NG agar plates in the absence of food, which caused the animals to undergo L1 arrest; or (ii) 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* expression within a day and sometimes for several days afterwards.

Temperature. To determine the influence of temperature, the *ilp* expression of animals that developed at 20°C were compared to those of age-matched animals that developed at 27°C from eggs that were laid at 20°C.

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3.1.7 Supplementary Material

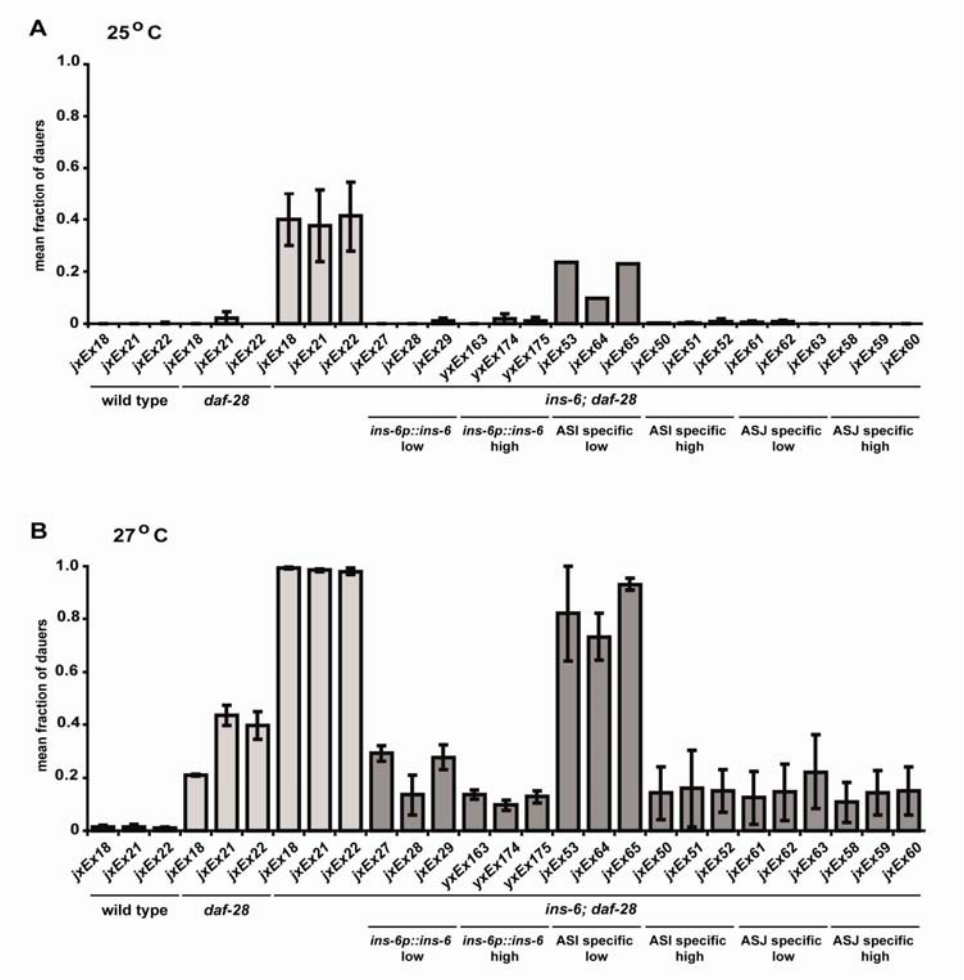

Figure S1. Overexpression of *ins-6* from ASI or ASJ neurons is sufficient to rescue the dauer entry phenotype of the *ins-6* mutation. The *ins-6*; *daf-28* mutants that carry low (2 ng/µl) or high levels (25 ng/µl) of the *ins-6* cDNA expressed under (i) the ASI-specific *str-3* promoter or (ii) the ASJ-specific *trx-1* promoter are compared to wild type or insulin-deletion mutants that carry the *ofm-1p::gfp* coinjection marker alone (*jxEx18*, *jxEx21* or *jxEx22*). The mean fractions of dauers are indicated at 25°C (A) or 27°C (B). See Table S1 for the statistical analyses of these experiments. The lack of rescue in animals carrying 2ng/µl of the *str-3p::ins-6* construct might be explained by the absence of *ins-6* expression. We found that *str-3* is a much weaker promoter than *trx-1*. The *gfp* expression in ASI of animals carrying 25 ng/µl of *str-3p::gfp* is comparable to the *gfp* expression in ASJ of animals carrying 2 ng/µl of *trx-1p::gfp* (data not shown). Consistent with this, animals carrying 25 ng/µl of *trx-1p::gfp* have the strongest *gfp* expression, and animals carrying 2 ng/µl of *str-3p::gfp* have very weak or no *gfp* expression (data not shown).

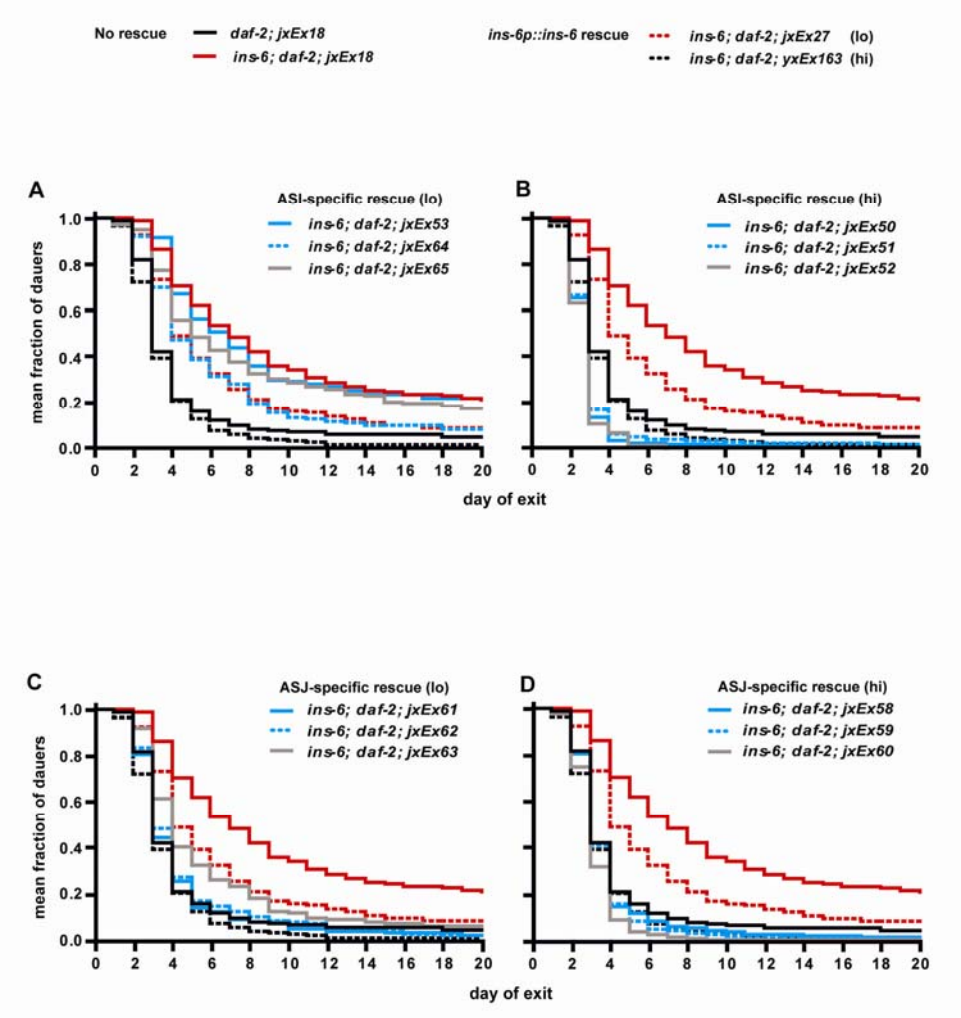

Figure S2. Overexpression of *ins-6* from ASI or ASJ neurons can rescue the dauer exit phenotype of the *ins-6* mutation. The *ins-6*; *daf-2(e1368)* mutants that carry low (A, C) or high levels (B, D) of the ASI-specific (A, B) or ASJ-specific (C, D) *ins-6* expression construct are compared to *daf-2(e1368)* or *ins-6*; *daf-2(e1368)* mutants that carry the *ofm-1p::gfp* coinjection marker alone (*jxEx18*). They are also compared to *ins-6*; *daf-2(e1368)* mutants that are rescued with low or high levels of *ins-6* expressed under its own promoter. See Table S2 for the statistical comparisons between the rates of dauer exit at 25°C of these different groups of animals.

Supplementary Table 1. Dauer entry phenotypes of wild type and insulin-deficient worms

Strain/Treatment	Mean Fraction of Dauers ± SEM (%)	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. [a]
ins-6; daf-28	0.36 ± 0.04	1061 (10)	< 0.0001	0.0006 [a]
ins-6; ins-1; daf-28	0.30 ± 0.06	392 (3)	0.002	n.s. [b]
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 [c]
ins-6; daf-2; daf-28	0.92 ± 0.05	437 (4)	0.004	n.s. [c]
ins-6; daf-2; ins-1; daf-28	0.93 ± 0.04	419 (4)	0.004	n.s. [d]
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. [a]
ins-6; daf-28	1.00 ± 0.00	903 (9)	< 0.0001	0.0001 [a]
ins-6; ins-1; daf-28	1.00 ± 0.00	328 (3)	0.004	n.s. [b]
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 [e]
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 [c]
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. [d]
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 [f]
daf-16; ins-6; daf-28	0.01 ± 0.01	491 (5)	n.s.	0.008 [b]
ins-6; ins-1; daf-28	0.25 ± 0.06	426 (5)	0.005	n.s. [b]
daf-16; ins-6; ins-1; daf-28	0.00 ± 0.00	458 (5)	n.s.	0.005 [g]

Strain/Treatment	Mean Fraction of Dauers ± SEM (%)	Total No. of Animals Observed (No. of Trials)	*P* Value Against Control*	*P* Value Against Specified Groups
25°C				
ofm-1p::gfp (25ng)				
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. [h]	0.008 [j]
daf-28; jxEx21	0.02 ± 0.01	516 (5)	0.05 [h]	0.009 [j]
daf-28; jxEx22	0.01 ± 0.01	414 (4)	n.s. [h]	0.01 [j]
ins-6; daf-28; jxEx18	0.47 ± 0.05	554 (5)	0.005 [h]	
ins-6; daf-28; jxEx21	0.31 ± 0.06	559 (5)	0.005 [h]	
ins-6; daf-28; jxEx22	0.34 ± 0.06	532 (5)	0.007 [h]	
full rescue (low)				
ins-6p::ins-6 (2ng)*;* *ofm-1p::gfp* (25ng)				
ins-6; daf-28; jxEx27	0.01 ± 0.00	638 (5)	< 0.01 [i]	
ins-6; daf-28; jxEx28	0.01 ± 0.01	682 (5)	< 0.01 [i]	
ins-6; daf-28; jxEx29	0.01 ± 0.00	571 (5)	< 0.01 [i]	
full rescue (high)				
ins-6p::ins-6 (25ng)*;* *ofm-1p::gfp* (25ng)				
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 (2ng)*;* *ofm-1p::gfp* (25ng)				
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 (25ng)*;* *ofm-1p::gfp* (25ng)				
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 (2ng)*;* *ofm-1p::gfp* (25ng)				
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 (25ng)*;* *ofm-1p::gfp* (25ng)				
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)		

Strain/Treatment	Mean Fraction of Dauers ± SEM (%)	Total No. of Animals Observed (No. of Trials)	*P* Value Against Control*	*P* Value Against Specified Groups
27°C				
ofm-1p::gfp (25ng)				
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 [h]	0.08 [j]
daf-28; jxEx21	0.44 ± 0.04	328 (3)	0.05 [h]	0.05 [j]
daf-28; jxEx22	0.40 ± 0.05	333 (3)	0.05 [h]	0.05 [j]
ins-6; daf-28; jxEx18	0.99 ± 0.00	286 (3)	0.05 [h]	
ins-6; daf-28; jxEx21	0.98 ± 0.00	292 (3)	0.05 [h]	
ins-6; daf-28; jxEx22	0.99 ± 0.01	326 (3)	0.05 [h]	
full rescue (low)				
ins-6p::ins-6 (2ng)*;*				
ofm-1p::gfp (25ng)				
ins-6; daf-28; jxEx27	0.29 ± 0.03	360 (3)	< 0.05 [i]	
ins-6; daf-28; jxEx28	0.13 ± 0.08	308 (3)	< 0.05 [i]	
ins-6; daf-28; jxEx29	0.28 ± 0.05	312 (3)	< 0.05 [i]	
full rescue (high)				
ins-6p::ins-6 (25ng)*;*				
ofm-1p::gfp (25ng)				
ins-6; daf-28; yxEx163	0.13 ± 0.02	373 (3)	< 0.05 [i]	
ins-6; daf-28; yxEx174	0.10 ± 0.02	357 (3)	< 0.05 [i]	
ins-6; daf-28; yxEx175	0.13 ± 0.02	344 (3)	< 0.05 [i]	
ASI-specific rescue (low)				
str-3p::ins-6 (2ng)*;*				
ofm-1p::gfp (25ng)				
ins-6; daf-28; jxEx53	0.82 ± 0.18	70 (2)	n.s. [i]	
ins-6; daf-28; jxEx54	0.12 ± 0.09	303 (3)	< 0.05 [i]	
ins-6; daf-28; jxEx64	0.73 ± 0.09	310 (3)	< 0.05 [i]	
ins-6; daf-28; jxEx65	0.93 ± 0.02	152 (2)	0.08 [i]	
ins-6; daf-28; jxEx66	0.86 ± 0.09	179 (2)	0.08 [i]	
ASI-specific rescue (high)				
str-3p::ins-6 (25ng)*;*				
ofm-1p::gfp (25ng)				
ins-6; daf-28; jxEx50	0.14 ± 0.10	339 (3)	< 0.05 [i]	
ins-6; daf-28; jxEx51	0.16 ± 0.14	350 (3)	< 0.05 [i]	
ins-6; daf-28; jxEx52	0.15 ± 0.08	372 (3)	< 0.05 [i]	
ASJ-specific rescue (low)				
trx-1p::ins-6 (2ng)*;*				
ofm-1p::gfp (25ng)				
ins-6; daf-28; jxEx61	0.12 ± 0.10	325 (3)	< 0.05 [i]	
ins-6; daf-28; jxEx62	0.15 ± 0.11	301 (3)	< 0.05 [i]	
ins-6; daf-28; jxEx63	0.22 ± 0.14	342 (3)	< 0.05 [i]	
ASJ-specific rescue (high)				
trx-1p::ins-6 (25ng)*;*				
ofm-1p::gfp (25ng)				
ins-6; daf-28; jxEx58	0.11 ± 0.07	301 (3)	< 0.05 [i]	
ins-6; daf-28; jxEx59	0.14 ± 0.08	310 (3)	< 0.05 [i]	
ins-6; daf-28; jxEx60	0.15 ± 0.09	351 (3)	< 0.05 [i]	

Supplementary 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 (Logrank)	*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[a]	< 0.0001 [b]	
daf-2(e1368); ins-1(nr2091)	480/588	6	0.0001[a]		
daf-2(e1368); daf-28(tm2308)	407/587	6	0.0001[a]	< 0.0001 [c]	
ins-6; daf-2; ins-1	375/583	6	< 0.0001[a]		
daf-2; ins-1; daf-28	495/579	6	0.001[a]		
ins-6; daf-2; daf-28	194/596	6	< 0.0001[a]	< 0.0001 [d]	
ins-6; daf-2; ins-1; daf-28	293/605	6	< 0.0001[a]	< 0.0001 [e]	
25°C					
ofm-1p::gfp (25ng)					
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[f]		
ins-6; daf-2; jxEx21	353/592	6	< 0.0001[g]		
ins-6; daf-2; jxEx22	342/605	6	< 0.0001[h]		
full rescue (low)					
ins-6p::ins-6 (2ng)*; ofm-1p::gfp* (25ng)					
ins-6; daf-2; jxEx27	541/835	8	< 0.0001[f, g, h]	< 0.0001[i, j, k]	+ [f, g, h]
ins-6; daf-2; jxEx28	632/804	8	< 0.0001[f, g] < 0.05 [h]	< 0.0001[i, j, k]	+ [f, g] ++ [h]
ins-6; daf-2; jxEx29	472/698	7	< 0.0001[f, g, h]	< 0.0001[i, j, k]	+ [f, g, h]
full rescue (high)					
ins-6p::ins-6 (25ng)*; ofm-1p::gfp* (25ng)					
ins-6; daf-2; yxEx163	677/809	8	n.s. [f] < 0.02 [g] < 0.0001 [h]	< 0.0001[i, j, k]	++ [f] +++ [g, h]
ins-6; daf-2; yxEx174	698/817	8	n.s. [f] < 0.02 [g] < 0.0001[h]	< 0.0001[i, j, k]	++ [f] +++ [g, h]
ins-6; daf-2; yxEx175	578/684	7	n.s. [f] < 0.005 [g] < 0.0001[h]	< 0.0001[i, j, k]	++ [f] +++ [g, h]
ASI-specific rescue (low)					
str-3p::ins-6 (2ng)*; ofm-1p::gfp* (25ng)					
ins-6; daf-2; jxEx53	121/215	3	< 0.0001[f, g, h]	n.s. [i, j, k]	– [f, g, h]
ins-6; daf-2; jxEx54	168/257	3	< 0.0001[f, g, h]	n.s. [i, j, k]	– [f, g, h]
ins-6; daf-2; jxEx64	168/213	2	< 0.0001[f, g] n.s.[h]	< 0.0001[i, j, k]	+ [f, g] ++ [h]
ins-6; daf-2; jxEx65	164/228	2	< 0.0001[f, g] 0.0005 [h]	< 0.05 [i] n.s. [j, k]	+ [f] – [g, h]
ins-6; daf-2; jxEx66	133/214	2	< 0.0001[f, g, h]	n.s. [i, j, k]	– [f, g, h]

Strain/Treatment	No. of Animals Observed/ Total Animals	No. of Trials	*P* Value Against Control (Logrank)	*P* Value Against Specified Groups (Logrank)	Rescue Effect
ASI-specific rescue (high)					
str-3p::ins-6 (25ng)*; ofm-1p::gfp* (25ng)					
ins-6; daf-2; jxEx50	345/352	3	$< 0.0001^{f, g, h}$	$< 0.0001^{ijk}$	$+++^{f, g, h}$
ins-6; daf-2; jxEx51	354/361	3	$< 0.0001^{f, g, h}$	$< 0.0001^{ijk}$	$+++^{f, g, h}$
ins-6; daf-2; jxEx52	327/333	3	$< 0.0001^{f, g, h}$	$< 0.0001^{ijk}$	$+++^{f, g, h}$
ASJ-specific rescue (low)					
trx-1p::ins-6 (2ng)*; ofm-1p::gfp* (25ng)					
ins-6; daf-2; jxEx61	182/208	2	$\text{n.s.}^{f, g}$ 0.0002^{h}	$< 0.0001^{ijk}$	$++^{f, g}$ $+++^{h}$
ins-6; daf-2; jxEx62	185/205	2	n.s.^{f} $< 0.05^{g}$ $< 0.05^{h}$	$< 0.0001^{ijk}$	$++^{f}$ $+^{g}$ $+++^{h}$
ins-6; daf-2; jxEx63	167/197	2	$< 0.0001^{f}$ n.s.^{h}	$< 0.0001^{ijk}$	$+^{f, g}$ $++^{h}$
ASJ-specific rescue (high)					
trx-1p::ins-6 (25ng)*; ofm-1p::gfp* (25ng)					
ins-6; daf-2; jxEx58	278/288	3	$\text{n.s.}^{f, g}$ $< 0.0001^{h}$	$< 0.0001^{ijk}$	$++^{f, g}$ $+++^{h}$
ins-6; daf-2; jxEx59	298/319	3	$< 0.05^{f}$ n.s.^{g} $< 0.0001^{h}$	$< 0.0001^{ijk}$	$+++^{f}$ $++^{g}$ $+++^{h}$
ins-6; daf-2; jxEx60	302/316	3	$< 0.0001^{f, h}$ $< 0.05^{g}$	$< 0.0001^{ijk}$	$+++^{f, g, h}$

Supplementary Table Legends

Table S1. The dauer entry phenotypes of wild type and insulin-deficient worms. We assayed wild-type, mutant and rescued worms in parallel in independent trials at different temperatures and we 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 following indicate: *, the control to which the different worms were compared in each trial; [a], compared to *daf-28(tm2308)* mutants; [b], compared to *ins-6(tm2416)*; *daf-28(tm2308)* mutants; [c], compared to *daf-2(e1368)*; *daf-28(tm2308)* mutants; [d], compared to *ins-6(tm2416)*; *daf-2(e1368)*; *daf-28(tm2308)* mutants; [e], compared to *ins-6(tm2416)*; *daf-2(e1368)* mutants; [f], compared to wild type; [g], compared to *ins-6(tm2416)*; *daf-28(tm2308)*; *ins-1(nr2091)* mutants; [h], compared to wild type carrying the corresponding transgene; [i], compared to *ins-6*; *daf-28*; *jxEx18*, *ins-6*; *daf-28*; *jxEx21* or *ins-6*; *daf-28*; *jxEx22*; [j], compared to *ins-6*; *daf-28* mutants carrying the corresponding transgene; and n.s., not significant since $P > 0.1$. See the Figure S1 legend about 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 insulin-like genes in regulating dauer exit. We analyzed the rates of dauer exit of *daf-2(e1368)* mutants in the presence or absence of specific insulins at 25^{o}C and show the statistics from the cumulative experiments. We used the logrank test to determine the statistical significance of the differences among the groups. The following indicate: [a], compared to *daf-2(e1368)* mutants; [b], compared to *ins-6(tm2416)*; *daf-2(e1368)*; *ins-1(nr2091)* mutants; [c], compared to *daf-2(e1368)* ; *ins-1(nr2091)*; *daf-28(tm2308)* mutants; [d], compared to *ins-6(tm2416)*; *daf-2(e1368)* ; *ins-1(nr2091)*; *daf-28(tm2308)* mutants; [e], compared to *ins-6(tm2416)*; *daf-2(e1368)*; *daf-28(tm2308)* mutants; [f],

compared to *daf-2*; *jxEx18* animals; [g], compared to *daf-2*; *jxEx21* animals; [h], compared to *daf-2*; *jxEx22* animals; [i], compared to *ins-6*; *daf-2*; *jxEx18* animals; [j], compared to *ins-6*; *daf-2*; *jxEx21* animals; [k], compared to *ins-6*; *daf-2*; *jxEx22* animals; –, no rescue; +, partial rescue; ++, full rescue; +++, over rescue; and n.s., not significant since $P > 0.05$.

3.2 Further characterization of *ins-1, ins-6* and *daf-28* activities with other *ilps* in dauer arrest and lifespan

3.2.1 Abstract

In this section, I show that *ins-6*, *daf-28* and *ins-1* act combinatorially with other ILPs to regulate dauer arrest. I also identify a second function for ASI in the regulation of the dauer program: *ins-6* can act from ASI not only to inhibit dauer entry but also to promote dauer exit. Finally, I show that *ilps* have distinct and non-redundant functions in regulating both the dauer program and lifespan.

3.2.2 Introduction

In the preceding manuscript, I have shown that *daf-28*, *ins-1* and *ins-6* encode sensory information to regulate *C. elegans* developmental programs. However, my studies also suggest that other ILPs are required to regulate this process, as well as lifespan. Loss of both *daf-28* and *ins-6* are not sufficient to induce dauer arrest to the same degree as *daf-2* mutants, while loss of *ins-1* is also insufficient to rescue the dauer phenotype of *daf-2* mutant worms (see section 3.1). At the same time, *daf-28*, *ins-1* and *ins-6* have little or no effect on *C. elegans* lifespan (see section 3.1), unlike *daf-2* reduction-of-function mutants (Kenyon et al, 1993).

Here, I investigated *ins-6* and *daf-28* interactions, as well as those of *ins-1*, with other *ilps* in regulating the dauer program or lifespan. I analyzed the dauer or lifespan effect of the deletion of *ins-6* or *ins-1* in the background of a gain-of-function mutation in

daf-28, *sa191* (Malone et al., 1996), which has been proposed to affect the function of several ILPs (Li et al., 2003). I also analyzed the direct role of two other ILPs, *ins-7* and *ins-18*, which have been implicated in either dauer arrest or lifespan regulation through RNA interference (RNAi) or overexpression studies, or through deletion mutant analyses in the presence of a drug that can inhibit DNA synthesis both in the worm and its bacterial food source (Pierce et al., 2001; Murphy et al., 2003; Murphy et al., 2007).

3.2.3 Results and Discussion

The gain-of-function *daf-28(sa191)* mutation enhances the effect of loss of *ins-6*, *ins-1* or *ins-18* on dauer entry

The *daf-28(sa191)* point mutation has been reported to induce dauer entry at 20°C and higher temperatures (Malone et al., 1996). The transient dauer arrest phenotype of *daf-28(sa191)* at 25°C is completely rescued by the overexpression of wild-type *daf-28* or *ins-4*, but only partly rescued by the overexpression of wild-type *ins-6* (Li et al., 2003). This implicates *daf-28, ins-4* and *ins-6* in the inhibition of dauer entry. It has been proposed that the semi-dominant gain-of-function effect of the *daf-28(sa191)* mutation is caused by an interference with the processing of other *β*-type ILPs (Li et al., 2003). The *daf-28(sa191)* mutation causes an R to C substitution at a predicted proteolytic cleavage site, which is likely necessary for the processing of DAF-28 (Li et al., 2003). Thus, the mutation might inhibit the proteolytic cleavage and correct processing of DAF-28, which might be less or completely inactive in its propeptide form. Moreover, the protease might be sequestered by the mutation and inhibited from processing other peptides (Li et al., 2003). Since the predicted cleavage site in *daf-28* is also present in other members of the

β-type ILPs, like *ins-4* and *ins-6*, the dauer entry phenotype of *daf-28(sa191)* might be due to the impaired function not only of *daf-28* but also of other dauer-entry inhibiting ILPs.

I found that loss of *ins-6* increases dauer entry, while loss of *ins-1* decreases dauer entry in the *daf-28(sa191)* background at 20°C and 22.5°C (Figures IA and IB; Table I), which confirm my previous observations that dauer entry is inhibited by wild-type *ins-6* and promoted by wild-type *ins-1*. I also found that the *ins-18(tm339)* deletion mutation partly suppresses dauer entry of *daf-28(sa191)*, but less so than the deletion of *ins-1(nr2091)* (Figures IA and IB; Table I). Conversely, overexpression of *ins-18* has been reported to increase dauer arrest in a *daf-2* mutant background, but again less so than overexpression of *ins-1* (Pierce et al., 2001). Together these data suggest that, like *ins-1*, the wild-type function of *ins-18* is to promote dauer entry, although perhaps *ins-18* has a weaker role than *ins-1* in this process.

Interestingly, I also found that loss of *ins-7* alone, by using the same allele, *tm1907*, that has been proposed to enhance dauer formation of *daf-2*-RNAi-treated worms (Murphy et al., 2007), has no effect on dauer entry at 25°C (Table I). This is also in contrast to the previously observed increase in dauer formation of *ins-7(tm1907)* single mutants at 27°C (Murphy et al., 2007). It is possible that *ins-7* acts like *ins-6*, in that its effect on dauer entry is only observable at higher temperatures or in combination with other *ilp* deletions.

I observed that *daf-28(sa191)* causes more dauer arrest than *daf-28(tm2308)* at all temperatures, *e.g.*, at 25°C (Figure IC; Table I), which supports the hypothesis that the *daf-28(sa191)* phenotype is caused not only by the elimination of *daf-28* function.

Interestingly, the dauer arrest phenotype of the *daf-28(sa191)* mutant is stronger than that of the *daf-2(e1368)* mutant (Figure IB; Table I), but weaker than that of the stronger *daf-2* allele, *e1370* (Figures IA and IB; Table I). However, in regulating lifespan, the effect of the *daf-28(sa191)* mutation is weaker than that of the *daf-2(e1368)* mutation (Figures IIIC and IIIH and Table IV). Thus, *daf-28(sa191)* seems to affect the ILPs involved in the regulation of dauer entry more than the ILPs involved in lifespan regulation.

daf-16 largely suppresses the dauer entry phenotypes caused by _ilp_ mutations

The *C. elegans* ILPs have been predicted to act as ligands of the DAF-2 insulin/IGF receptor, because of their sequence and structural similarities to ILPs in other animals (Pierce et al., 2001; Li et al., 2003). However, there is no direct biochemical evidence that they actually bind DAF-2 and modulate its activity. Moreover, a group of mammalian ILPs, called relaxins, has been shown to bind to GPCRs, rather than insulin-like receptors (Liu and Lovenberg, 2008; Svendsen et al., 2008; Meyts et al., 2009).

To determine whether the different ILPs that are analyzed in this study act with the DAF-2 receptor, I tested how loss of *daf-16*, a downstream effector of *daf-2* (reviewed by Kenyon, 2005), affects the dauer entry phenotypes of the different *ilp* mutations. The canonical pathway downstream of the DAF-2 receptor targets the FOXO transcription factor DAF-16, which is negatively regulated by DAF-2 activity (reviewed by Kenyon, 2005). Hence, activation of DAF-16 promotes the expression of genes required for the induction of dauer arrest (Kenyon, 2005). At 25°C, the *daf-16(mu86)* deletion mutation completely suppresses the dauer entry phenotype of the *ins-6*; *daf-28(tm2308)* double mutants (Figure 1E), and almost completely suppresses that of the

daf-28(sa191) and other insulin deletion mutant combinations (Figure IC; Table I). At 27°C, removal of *daf-16* still causes a fraction of partial dauers to form in the *daf-28(sa191)* or *daf-28(tm2308)* mutant background, but more so in the *daf-28(sa191)* mutants (Figures ID and IE; Table II). Indeed, lack of *daf-16* activity in *daf-28(sa191)* at 25°C already leads to the formation of a small fraction of partial dauers (Figures IC). The small fraction of partial dauers that form in the *daf-16; daf-28(sa191)* double mutant, or other *ilp* mutant combinations with the *daf-16* deletion, do not have all the dauer characteristics. Although these partial dauers have a dauer-like shape and show nictation behavior [wherein dauers stand on their tails and sway their bodies in the air (Riddle and Albert, 1997)], their pharynges can still pump and are only slightly remodeled (data not shown). These partial dauers also do not accumulate fat in storage vesicles; and their cuticles and gonads are again only somewhat remodeled (data not shown). In addition, they sometimes show growth and reproductive defects, if and when they do develop to the adult stage. Thus, these findings suggest that the dauer arrest phenotypes of the *ilp* mutants are largely *daf-16*-dependent, which is consistent with these ILPs acting as DAF-2 ligands to regulate dauer arrest. Moreover, since the DAF-2 pathway has a DAF-16-independent component (Inoue and Thomas, 2000a; Paradis et al., 1999; Paradis and Ruvkun, 1998; Nanji et al., 2005), this component might contribute to the partial dauer formation in the *daf-16* deletion background. However, the formal possibility remains that at least some of these ILPs act in a DAF-2-independent manner, for example, by binding another receptor, which might directly or indirectly also target DAF-16.

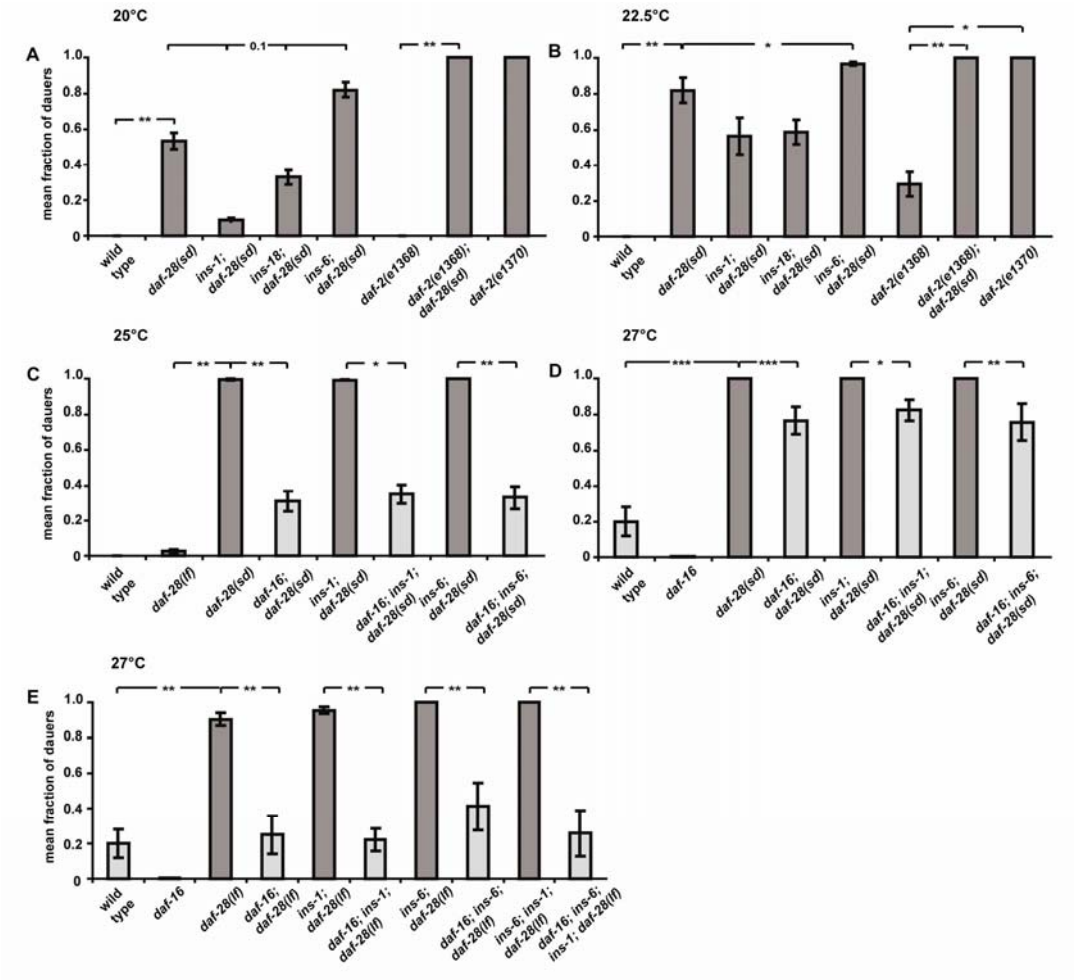

Figure I. *daf-28* acts with *ins-6* to inhibit dauer entry, whereas *ins-1* and *ins-18* promote it. (A-E) The mean fractions of wild-type and insulin mutant worms that form dauers are shown at the indicated temperature (dark grey bars). Each mean ± SEM includes at least three independent trials of approximately 100 worms, except for panel B, which includes at least two independent trials of approximately 100 worms. The detailed statistical comparisons between the dauer entry phenotypes of different genotypes under different conditions in these and subsequent analyses can be found in Tables I and II. * indicates $P \leq 0.05$; **, $P \leq 0.01$; and ***, $P \leq 0.001$. (A-B) The effect of different insulin deletions and the *daf-2(e1368)* mutation on dauer entry in the *daf-28(sa191)* mutant background at 20°C (A) and 22.5°C (B). (C-E) Light grey bars: Partial dauers. (C-D) The dauer entry phenotype of *daf-28(sa191)* and of different insulin deletions in the *daf-28(sa191)* mutant background is suppressed by the *daf-16(mu86)* mutation at 25°C (C) and 27°C (D). (E) The dauer entry phenotype of insulin deletion mutants is suppressed by the *daf-16(mu86)* mutation at 27°C. The *daf-28(tm2308)* deletion mutant is noted as *daf-28(lf)*, while the *daf-28(sa191)* gain-of-function mutant is noted as *daf-28(sd)*.

Strain/Treatment	Mean Fraction of Dauers ±SEM (%)	Total No. of Animals Observed (No. of Trials)	*P* Value Against Control*	*P* Value Against Specified Groups
20°C				
Wild type	0.00 ± 0.00	608 (6)	n.s.	
*daf-2(e1368)**	0.00 ± 0.00	595 (6)		
daf-2(e1368); ins-1	0.00 ± 0.00	430 (4)	0.07	
ins-6; daf-2(e1368)	0.05 ± 0.03	615 (6)	0.06	
ins-6; daf-2(e1368); ins-1	0.01 ± 0.01	431 (4)	0.2	
daf-2(e1368); daf-28(tm2308)	0.76 ± 0.08	621 (6)	0.002	0.1 [a]
daf-2(e1368); ins-1; daf-28(tm2308)	0.44 ± 0.09	438 (4)	0.004	0.06 [b]
ins-6; daf-2(e1368); daf-28(tm2308)	0.92 ± 0.05	437 (4)	0.004	
ins-6; daf-2(e1368); ins-1; daf-28(tm2308)	0.93 ± 0.04	419 (4)	0.004	1 [a]
20°C				
Wild type*	0.00 ± 0.00	608 (6)		
daf-28(sa191)	0.54 ± 0.05	207 (2)	0.009	
ins-1; daf-28(sa191)	0.09 ± 0.01	191 (2)	0.009	0.1 [c]
ins-18; daf-28(sa191)	0.33 ± 0.04	206 (2)	0.009	0.1 [c]
ins-6; daf-28(sa191)	0.82 ± 0.04	204 (2)	0.009	0.1 [c]
daf-2(e1368)	0.00 ± 0.00	595 (6)	n.s.	0.009 [c]
daf-2(e1368); daf-28(sa191)	1.00 ± 0.00	199 (2)	0.009	0.009 [d]
daf-2(e1370)	1.00 ± 0.00	205 (2)	0.009	0.009 [d]
22.5°C				
Wild type*	0.00 ± 0.00	488 (5)		
daf-28(sa191)	0.82 ± 0.07	394 (4)	0.008	
ins-1; daf-28(sa191)	0.57 ± 0.10	394 (4)	0.008	0.08 [c]
ins-18; daf-28(sa191)	0.59 ± 0.07	376 (4)	0.008	0.1 [c]
ins-6; daf-28(sa191)	0.97 ± 0.01	399 (4)	0.008	0.02 [c]
daf-2(e1368)	0.29 ± 0.07	487 (5)	0.005	0.01 [c]
daf-2(e1368); daf-28(sa191)	1.00 ± 0.00	502 (5)	0.003	0.005 [d]
daf-2(e1370)	1.00 ± 0.00	403 (4)	0.005	0.01 [d]
22.5°C				
Wild type*	0.00 ± 0.00	488 (5)		
ins-1(nr2091)	0.00	92 (1)		
ins-18(tm339)	0.00	98 (1)		
ins-6(tm2416)	0.00	98 (1)		
ins-6; ins-1	0.00	98 (1)		
daf-28(tm2308)	0.00 ± 0.00	292 (3)	n.s.	0.03 [e]
ins-1; daf-28(tm2308)	0.00 ± 0.00	391 (4)	n.s.	
ins-6; daf-28(tm2308)	0.31 ± 0.04	398 (4)	0.008	
ins-6; ins-1; daf-28(tm2308)	0.20 ± 0.03	393 (4)	0.008	0.04 [e]

Strain/Treatment	Mean Fraction of Dauers ±SEM (%)	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-7(tm1907)	0.00 ± 0.00	96 (1)	n.s.	
ins-18	0.02 ± 0.01	271 (3)	n.s.	
ins-18; ins-6	0.01 ± 0.01	192 (2)	n.s.	
ins-18; daf-28(tm2308)	0.00 ± 0.00	92 (1)	n.s.	
25°C				
Wild type*	0.00 ± 0.00	1005 (10)		
daf-28(tm2308)	0.02 ± 0.01	705 (7)	0.004	0.001 [c]
daf-28(sa191)	0.99 ± 0.00	673 (7)	0.0002	
daf-16; daf-28(sa191)	0.31 ± 0.06	488 (5)	0.001	0.006 [c]
ins-1; daf-28(sa191)	0.99 ± 0.00	402 (4)	0.001	0.5 [c]
daf-16; ins-1; daf-28(sa191)	0.35 ± 0.05	363 (4)	0.001	0.02 [f]
ins-6; daf-28(sa191)	1.00 ± 0.00	400 (4)	0.0004	0.1 [c]
daf-16; ins-6; daf-28(sa191)	0.33 ± 0.06	394 (4)	0.001	0.007 [g]

Table I. The dauer entry phenotypes of wild type and insulin-deficient worms. We assayed wild-type and mutant worms in parallel in independent trials at different temperatures and we 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 following indicate: *****, the control to which the different worms were compared in each trial; [a], compared to *ins-6(tm2416)*; *daf-2(e1368)*; *daf-28(tm2308)* mutants; [b], compared to *daf-2(e1368)*; *daf-28(tm2308)* mutants; [c], compared to *daf-28(sa191)* mutants; [d], compared to *daf-2(e1368)* mutants; [e], compared to *ins-6(tm2416); daf-28(tm2308)* mutants; [f], compared to *ins-1(nr2091)*; *daf-28(sa191)* mutants; [g], compared to *ins-6(tm2416); daf-28(sa191)* mutants; and n.s., not significant since $P > 0.1$. Some of the dauer entry data from the preceding section 3.1 are included here for comparison.

Table II. Partial dauers in wild-type, *daf-16* and/or insulin-deficient worms at 27°C

Strain/Treatment	Mean Fraction of Partial Dauers / Dauers ± SEM (%)	Total No. of Animals Observed (No. of Trials)	*P* Value Against Control*	*P* Value Against Specified Groups
27°C				
Wild type*	0.20 ± 0.08 / 0.00 ± 0.00	552 (6)		
daf-16(mu86)	0.00 ± 0.00 / 0.00 ± 0.00	538 (6)	0.01	
daf-28(tm2308)	0.00 ± 0.00 / 0.90 ± 0.04	585 (6)	0.004	
ins-16; daf-28(tm2308)	0.25 ± 0.11 / 0.00 ± 0.00	478 (5)	0.6	0.006 [a]
ins-1; daf-28(tm2308)	0.00 ± 0.00 / 0.96 ± 0.02	483 (5)	0.006	0.4 [a]
daf-16; ins-1; daf-28(tm2308)	0.22 ± 0.06 / 0.00 ± 0.00	483 (5)	0.9	0.009 [b]
ins-6; daf-28(tm2308)	0.00 ± 0.00 / 1.00 ± 0.00	490 (5)	0.004	0.01 [a]
daf-16; ins-6; daf-28(tm2308)	0.41 ± 0.13 / 0.00 ± 0.00	460 (5)	0.1	0.005 [c]
ins-6; ins-1; daf-28(tm2308)	0.00 ± 0.00 / 1.00 ± 0.00	477 (5)	0.004	n.s. [c]
daf-16; ins-6; ins-1; daf-28(tm2308)	0.26 ± 0.13 / 0.00 ± 0.00	490 (5)	1.0	0.005 [d]
27°C				
Wild type*	0.20 ± 0.08 / 0.00 ± 0.00	552 (6)		
daf-16(mu86)	0.00 ± 0.00 / 0.00 ± 0.00	538 (6)	0.01	
daf-28(sa191)	0.00 ± 0.00 / 1.00 ± 0.00	941 (10)	0.0003	
daf-16; daf-28(sa191)	0.77 ± 0.08 / 0.00 ± 0.00	467 (5)	0.006	0.0006 [e]
ins-1; daf-28(sa191)	0.00 ± 0.00 / 1.00 ± 0.00	295 (3)	0.02	0.6 [e]
daf-16; ins-1; daf-28(sa191)	0.82 ± 0.06 / 0.00 ± 0.00	284 (3)	0.02	0.04 [f]
ins-6; daf-28(sa191)	0.00 ± 0.00 / 1.00 ± 0.00	572 (6)	0.002	0.4 [e]
daf-16; ins-6; daf-28(sa191)	0.76 ± 0.10 / 0.00 ± 0.00	282 (3)	0.04	0.006 [g]

Table II. See also the Table I legend. The following indicate: *, the control to which the different worms were compared in each trial; [a], compared to *daf-28(tm2308)* mutants; [b], compared to *ins-1(nr2091); daf-28(tm2308)* mutants; [c], compared to *ins-6(tm2416)*; *daf-28(tm2308)* mutants; [d], compared to *ins-6(tm2416)*; *ins-1(nr2091); daf-28(tm2308)* mutants; [e], compared to *daf-28(sa191)* mutants; [f], compared to *ins-1(nr2091)*; *daf-28(sa191)* mutants; [g], compared to *ins-6(tm2416); daf-28(sa191)* mutants; and n.s., not significant since $P > 0.1$.

ins-6 can function in ASI or ASJ to promote dauer exit

By laser ablation studies, the ASJ neurons have been shown to be crucial for the induction of dauer exit (Bargmann and Horvitz, 1991a), which is also regulated by DAF-2 signaling (Gems et al., 1998). I have shown that the ILP *ins-6* functions to promote dauer exit (Figures 2 and 4; Table S2). In addition, I find that *ins-6* is expressed in the ASI neurons of reproductively growing worms (Figure 5; Table 1). However, it is downregulated in ASI in response to dauer-inducing pheromones and is upregulated in ASJ in dauers that are exposed to high levels of pheromones (Figure 5; Table 1). This suggests that *ins-6* can act from ASJ to promote dauer exit.

On the other hand, the dauer exit phenotype of *ins-6; daf-2(e1368)* double mutants can be rescued by high levels of *ins-6* expression from either the ASI or the ASJ neurons (Figure S2; Table S2). To clarify whether *ins-6* acts in ASJ to promote dauer exit, I analyzed worms in which the ASJ neurons are genetically ablated through the ASJ-specific expression of human caspase-1 (Zheng et al., 1999). As expected, the genetic ablation of ASJ causes a delay in dauer exit of *daf-2(e1368)* single mutants (Figure IIA; Table III). In addition, the deletion of *ins-6* has a similar effect as ablation of ASJ on the exit of *daf-2(e1368)* mutant dauers (Figure IIA; Table III). Surprisingly, however, ablation of ASJ can still enhance the dauer exit defect of *ins-6*; *daf-2(e1368)* double mutants (compare Figures IIA and IIB). This suggests that (i) *ins-6* also acts from a cell other than ASJ to promote dauer exit; and (ii) there are additional dauer-exit promoting signals from ASJ besides *ins-6*, *e.g.*, the ILP *daf-28,* which is also expressed in this neuron (Li et al., 2003).

At the same time, it should be noted that these ASJ ablation experiments were carried out in *daf-2(e1368)* mutant dauers that were exposed to low pheromone levels. Moreover, as shown in section 3.1, I found that this particular treatment, induction of *daf-2(e1368)* mutant dauers at 25°C under low pheromone exposure, is insufficient to downregulate *ins-6* in ASI or induce the switch in expression to ASJ (Table 1). Thus, these data suggest that *ins-6* can act in ASI to promote dauer exit under certain conditions, like a weak dauer arrest, since *daf-2(e1368)* dauers still exit in this environment. Indeed, this idea is consistent with the observation that the dauer exit phenotype of *ins-6*; *daf-2(e1368)* double mutants is rescued by *ins-6* expression from ASI either in the presence or absence of ASJ neurons (Figures S2 and IID; Tables S2 and III). Not surprisingly, higher levels of *ins-6* expressed in ASI also increases the rate of dauer exit of *daf-2(e1368*) mutants in which ASJ has been ablated (Figure IIC; Table III).

However, I have also found that the combined activities of high pheromone levels and the dauer program do shift *ins-6* expression from ASI to ASJ (Figure 5; Table 1). To determine whether *ins-6* acts in ASJ to promote dauer exit under a different context, future studies would be needed to test the effect of ASJ ablation on exit of *daf-2(e1368)* single mutant versus *ins-6*; *daf-2(e1368)* double mutant dauers that were induced by high levels of pheromone. Under these conditions, which represent strong dauer arrest because these dauers are less likely to exit, it is possible that loss of *ins-6* will not further enhance the dauer exit phenotype of *daf-2(e1368)* mutants in which the ASJ neurons have been genetically ablated.

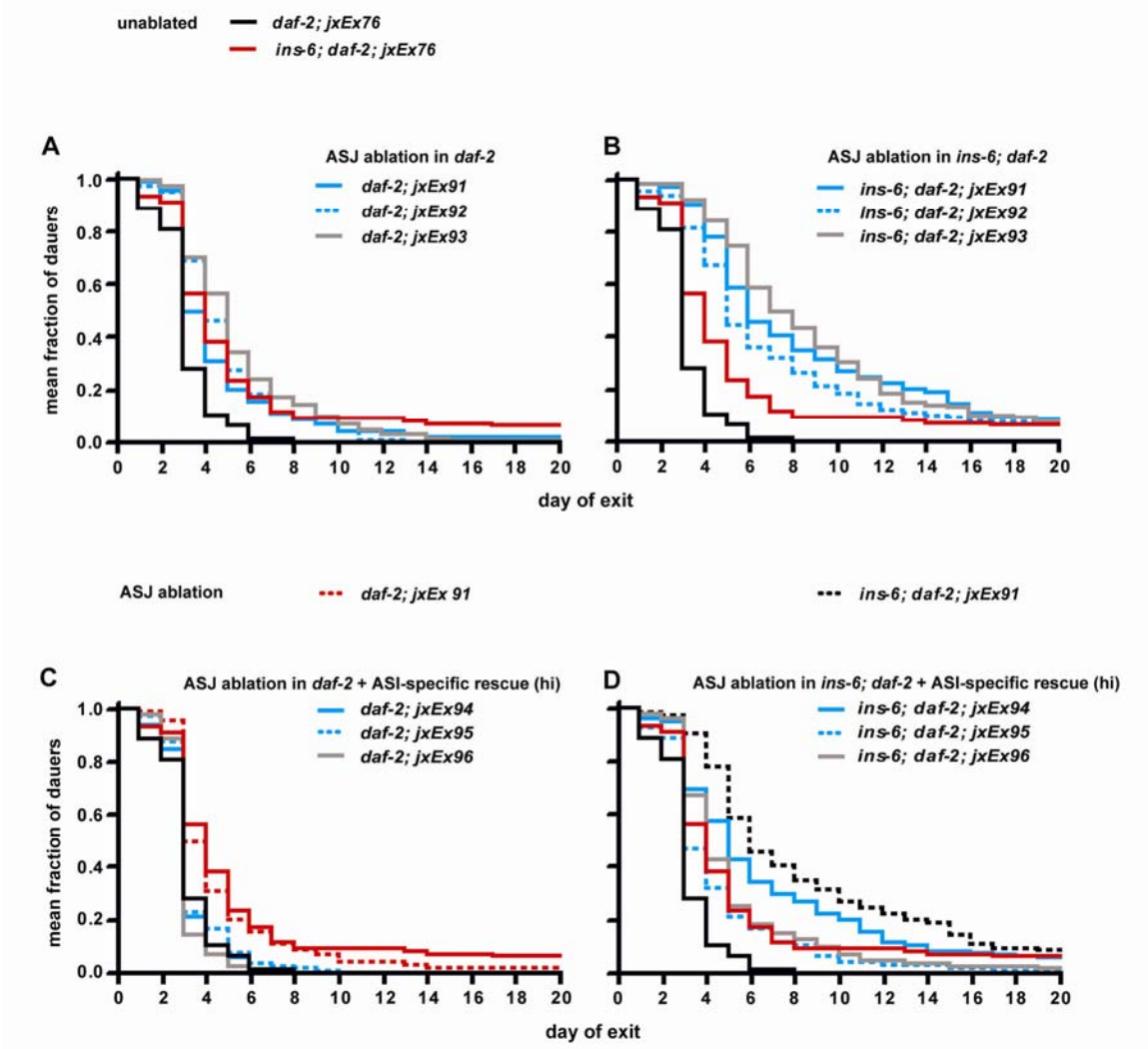

Figure II. **ASJ and *ins-6* function together to promote dauer exit.** (A-B) The rates of dauer exit at 25°C of *daf-2(e1368)* single (A) and *ins-6; daf-2(e1368)* double (B) mutant animals, in which ASJ is genetically ablated (*jxEx91*, *jxEx92* and *jxEx93)*. (C-D) The rates of dauer exit of *daf-2(e1368)* single (C) and *ins-6*; *daf-2(e1368)* double (D) mutants, in which ASJ is genetically ablated and in which *ins-6* expression is induced from an ASI-specific promoter at a high level (*jxEx94*, *jxEx95* and *jxEx96)*. (A-D) Control lines are *daf-2(e1368)* and *ins-6*; *daf-2(e1368)* mutants that carry the *trx-1p::gfp* ASJ marker and the *myo-3p::rfp* coinjection marker alone (*jxEx76*). Statistical analyses were performed according to the logrank test. See Table III for the statistical analyses of these experiments, and a comparison with two additional control lines.

Table III. Effect of genetic ablation of ASJ on dauer exit

Strain/Treatment	No. of Animals Observed/ Total Animals	No. of Trials	*P* Value Against Control (Logrank)	Rescue Effect	*P* Value Against Specified Groups (Logrank)	*P* Value Against Specified Groups (Logrank)
25°C						
trx-1p::gfp (25ng);						
myo-3p::rfp (30ng)						
daf-2; jxEx76	161/162	2				
daf-2; jxEx77	169/174	2				
daf-2; jxEx78	182/189	2				
ins-6; daf-2; jxEx76	135/153	2	< 0.0001[a]			
ins-6; daf-2; jxEx77	134/149	2	< 0.0001[b]			
ins-6; daf-2; jxEx78	158/218	2	< 0.0001[c]			
genetic ASJ ablation						
trx-1p::ICE (50ng);						
trx-1p::gfp (25ng);						
myo-3p::rfp (30ng)						
daf-2; jxEx91	142/149	2	< 0.0001[a]			
daf-2; jxEx92	209/218	2	< 0.0001[b]			
daf-2; jxEx93	221/228	2	< 0.0001[c]			
ins-6; daf-2; jxEx91	167/197	2	< 0.0001[a]			< 0.0001[ghi]
ins-6; daf-2; jxEx92	157/177	2	< 0.0001[b]			< 0.0001[ghi]
ins-6; daf-2; jxEx93	184/219	2	< 0.0001[c]			< 0.0001[ghi]
genetic ASJ ablation + ASI-specific rescue (high)						
trx-1p::ICE (50ng);						
trx-1p::gfp (25ng);						
myo-3p::rfp (30ng);						
str-3p::ins-6 (25ng)						
daf-2; jxEx94	133/133	1	n.s.[a]	++[a] +++[bc]		< 0.0001[def]
daf-2; jxEx95	142/143	1	n.s.[a]	++[a] +++[bc]		< 0.0001[def]
daf-2; jxEx96	124/124	1	n.s.[a]	++[a] +++[bc]		< 0.0001[def]
ins-6; daf-2; jxEx94	185/204	2	< 0.0001[a]	+[df] –[e]	< 0.0001[jl] n.s.[k]	< 0.0001[def]
ins-6; daf-2; jxEx95	169/180	2	< 0.0001[b]	++[de] +++[f]	< 0.0001[jkl]	n.s.[d] 0.06[e] 0.0005[f]
ins-6; daf-2; jxEx96	228/238	2	< 0.0001[c]	++[def]	< 0.0001[jkl]	0.04[d] n.s.[ef]
ASI-specific rescue (high)						
trx-1p::gfp (25ng);						
myo-3p::rfp (30ng);						
str-3p::ins-6(25ng)						
daf-2; jxEx84	128/129	1		+++[abc]		
daf-2; jxEx85	110/110	1		+++[abc]		
daf-2; jxEx86	119/119	1		+++[abc]		
ins-6; daf-2; jxEx84	114/114	1		+++[ghi]		
ins-6; daf-2; jxEx85	117/120	1		+++[ghi]		
ins-6; daf-2; jxEx86	119/122	1		+++[ghi]		

Table III. The combinatorial effect of genetic ablation of the ASJ neurons and *ins-6* deletion on dauer exit. The rates of dauer exit of *daf-2(e1368)* and *ins-6(tm2416); daf-2(e1368)* mutants with and without ASJ ablation at 25°C are shown together with the statistics from the cumulative experiments. We used the logrank test to determine the statistical significance of the differences among the groups. The following indicate: [a], compared to *daf-2*; *jxEx76* animals; [b], compared to *daf-2*; *jxEx77* animals; [c], compared to *daf-2*; *jxEx78* animals; [d], compared to *daf-2*; *jxEx91* animals; [e], compared to *daf-2*; *jxEx92* animals; [f], compared to *daf-2*; *jxEx93* animals; [g], compared to *ins-6*; *daf-2*; *jxEx76* animals; [h], compared to *ins-6*; *daf-2*; *jxEx77* animals; [i], compared to *ins-6*; *daf-2*; *jxEx78* animals; [j], compared to *ins-6*; *daf-2*; *jxEx91* animals; [k], compared to *ins-6*; *daf-2*; *jxEx92* animals; [l], compared to *ins-6*; *daf-2*; *jxEx93* animals; –, no rescue; +, partial rescue; ++, full rescue; +++, over rescue; and n.s., not significant since $P > 0.05$.

ASJ is the only the sensory neuron that has been previously identified to regulate dauer exit (Bargmann and Horvitz, 1991a). In this study, I identified a second sensory neuron that can regulate this step of the switch, ASI. This second role for ASI in the regulation of dauer arrest suggests that ASI not only inhibits dauer entry, but it also promotes dauer exit. However, considering that ASI might only promote exit after a weak dauer arrest, whereas ASJ might promote exit after a stronger dauer arrest, this also suggests that ASI, in contrast to ASJ, plays only a minor role in dauer exit.

Different ILPs have distinct and non-redundant effects on lifespan

The DAF-2/insulin-like pathway is a major signaling pathway that regulates C. *elegans* adult longevity (reviewed by Kenyon, 2005). Active DAF-2 signaling inhibits longevity, while downregulation of the pathway can increase lifespan by more than 100% (Kenyon et al., 1993). While mutations in the *daf-2* receptor gene and downstream components of the pathway have been studied in detail regarding this process, little is known about which ILPs might activate DAF-2 to inhibit longevity.

daf-28 has been implicated in lifespan regulation by the gain-of-function mutation *sa191,* which causes not only transient dauer arrest but also a slight increase in lifespan (Malone et al., 1996). In addition, RNAi of *ins-7* slightly increases lifespan in an RNAi-sensitive background (Murphy et al., 2003). In contrast, overexpression of *ins-1* slightly extends lifespan, which suggests that INS-1 might act to promote longevity, rather than inhibit it, although the *ins-1(nr2091)* deletion does not lead to a shorter lifespan (Pierce et al., 2001). Yet, all of these observations are based on methods that could interfere with endogenous ILPs that regulate lifespan.

Thus, I tested loss-of-function mutants of several *ilp* genes for a possible role in regulating longevity. At 25°C, I found that deletion of the *ilp* gene *ins-7*, *ins-18* or *daf-28* has no effect on lifespan, unlike those of *ins-6* and *ins-1*, which have significant but opposing effects (Figure III; Table IV). *ins-6* deletion mutants have a slightly, but significantly longer lifespan than wild type (11%; Figure IIIA; Table IV). In addition, deletion of *ins-6* further extends lifespan in both the *daf-28(tm2308)* and *daf-28(sa191)* mutant backgrounds (Figures IIIC and IIID; Table IV), and even more so in the *daf-2(e1368)* background (Figure IIIH; Table IV).

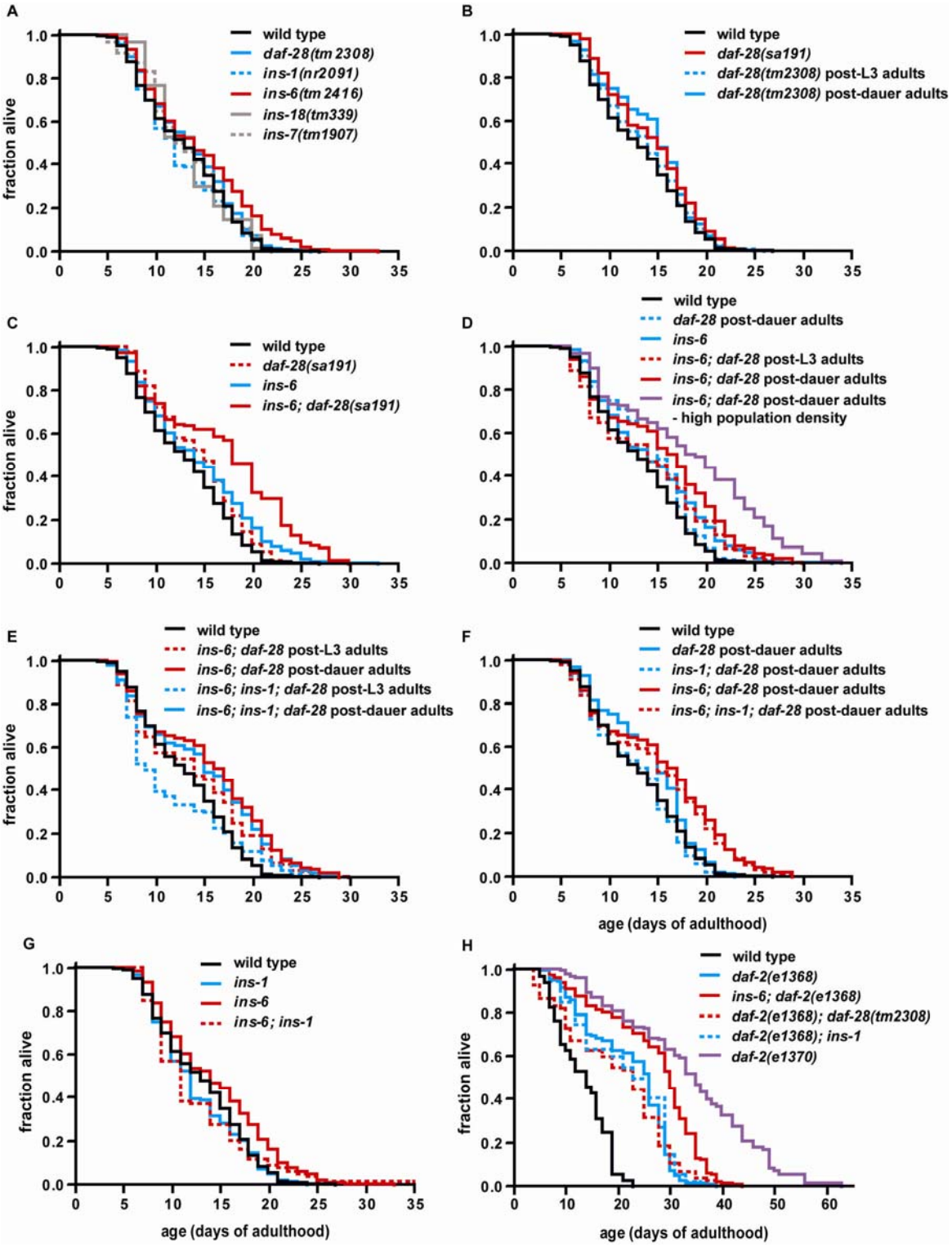
A
wild type
daf-28(tm2308)
ins-1(nr2091)
ins-6(tm2416)
ins-18(tm339)
ins-7(tm1907)
fraction alive
B
wild type
daf-28(sa191)
daf-28(tm2308) post-L3 adults
daf-28(tm2308) post-dauer adults
C
wild type
daf-28(sa191)
ins-6
ins-6; daf-28(sa191)
fraction alive
D
wild type
daf-28 post-dauer adults
ins-6
ins-6; daf-28 post-L3 adults
ins-6; daf-28 post-dauer adults
ins-6; daf-28 post-dauer adults - high population density
E
wild type
ins-6; daf-28 post-L3 adults
ins-6; daf-28 post-dauer adults
ins-6; ins-1; daf-28 post-L3 adults
ins-6; ins-1; daf-28 post-dauer adults
fraction alive
F
wild type
daf-28 post-dauer adults
ins-1; daf-28 post-dauer adults
ins-6; daf-28 post-dauer adults
ins-6; ins-1; daf-28 post-dauer adults
age (days of adulthood)
G
wild type
ins-1
ins-6
ins-6; ins-1
fraction alive
age (days of adulthood)
H
wild type
daf-2(e1368)
ins-6; daf-2(e1368)
daf-2(e1368); daf-28(tm2308)
daf-2(e1368); ins-1
daf-2(e1370)
fraction alive
age (days of adulthood)

Figure III. Insulin-like genes have distinct effects on lifespan. (A-G) All lifespan analyses were performed at 25°C. (A) The lifespan of *daf-28(tm2308), ins-1(nr2091), ins-6(tm2416), ins-7(tm1907)* and *ins-18(tm339)* deletion mutants. The *daf-28(tm2308)* mutants consist of continuously well-fed (post-L3) adults. (B) The lifespan of the two different *daf-28* mutant alleles: the semi-dominant allele *sa(191)*, which causes transient dauer arrest in all worms: and the loss-of-function allele *tm2308*, which leads to two different populations, post-L3 and post-dauer. The *daf-28(tm2308)* post-dauer population was induced by high population density at 25^{o}C. These postdauers are transient dauers that exit within 24 hours. (C) The combined effect on lifespan of the *ins-6* loss-of-function and *daf-28(sa191)* mutations. (D) The lifespan-extending effect of *ins-6* in the *daf-28(tm2308)* background. (E-G) *ins-1* loss-of-function shortens the lifespan of *ins-6* single mutants (G), *ins-6; daf-28(tm2308)* double mutant post-L3 adults (E), and *daf-28(tm2308)* single mutant post-dauer adults (F). However, *ins-1* loss-of-function does not suppress the lifespan extension seen in *ins-6; daf-28(tm2308)* double mutant post-dauer adults (E-F). (H) The lifespan of *daf-2(e1368)* in the absence of specific *ilps* and of *daf-2(e1370)*. Since *daf-2* mutants undergo dauer arrest at 25°C, these worms were grown at 20°C and then shifted to 25°C at the L4 stage to initiate lifespan analyses.

Similar to what has been previously reported (Malone et al., 1996), I also observed that the *daf-28(sa191)* mutation, which induces 100% transient dauer arrest at 25°C, extends lifespan by about 11% (Figures IIIB and IIIC; Table IV). At the same time, I found that the two different adult populations of *daf-28(tm2308)* exhibit different lifespan: the *daf-28(tm2308)* post-dauer adults show a lifespan increase similar to *daf-28(sa191)* mutants, whereas *daf-28(tm2308)* post-L3 adults, which did not go through the dauer stage, have a lifespan that is more similar to wild type (Figure IIIB; Table IV). Morover, because the *daf-28(tm2308)* mutation increases lifespan only of *ins-6* or *ins-6*; *ins-1* post-dauer, but not post-L3, adults (Figures IIID and IIIE; Table IV), it seems likely that it is the induction of dauer arrest, and not the loss of *daf-28*, that affects longevity. In support of this hypothesis, the induction of dauer arrest by high pheromone and low food levels in wild-type worms also slightly extends their adult lifespan (Hall et al., 2010).

Interestingly, the lifespan of the *ins-6; daf-28(tm2308)* post-dauer adults is further increased after exposure to an even higher pheromone to food ratio, as is the case in high population densities (Figure IIIC; Table IV), which suggests that dauer pheromones may also influence lifespan. Since exposure to pheromone levels alone has been reported to have no effect on lifespan (Alcedo and Kenyon, 2004), these observations together also suggest that dauer pheromones only affect lifespan in combination with the dauer program and low food availability.

On the other hand, unlike *ins-6* deletion or the dauer program or high pheromone to food ratios, the lack of *ins-1* activity shortens lifespan, but only in some of the *ilp* mutant backgrounds. I found that deletion of *ins-1* does not have an effect on lifespan in backgrounds that do not show increased longevity*, i.e.*, wild type and *daf-28(tm2308)*

post-L3 adults (Figure IIIA; Table IV). In contrast, loss of *ins-1* shortens lifespan in *ins-6* single mutants and *ins-6; daf-28* post-L3 adults, as well as *daf-28(tm2308)* post-dauer adults (Figures IIIE to IIIG; Table IV). Interestingly, *ins-1* does not shorten the longer lifespan of *ins-6*; *daf-28(tm2308)* post-dauer adults or *daf-2(e1368)* adults (Figures IIIE, IIIF and IIIH; Table IV). Since lack of *ins-1* function can only suppress small, but not larger, increases in lifespan, this suggests that *ins-1* only weakly antagonizes *daf-2* activity in regulating lifespan. This is again similar to the weak activity of *ins-1* in antagonizing *daf-2* signaling in the regulation of dauer entry (see Section 3.1).

Thus, in this study, I show that the wild-type function of *ins-6* is to shorten lifespan, while the wild-type function of *ins-1* is to lengthen lifespan. This suggests that ILPs have distinct and non-redundant functions in regulating longevity, like they do in the regulation of dauer arrest (see Section 3.1). The lifespan effect of *ins-1* is consistent with the previous observation that its overexpression promotes longevity (Pierce et al., 2001). However, my study shows that *ins-1* only plays a minor role in regulating this process.

I also show that the effects of these *ilps* are small compared to the effects of other insulin-like signaling mutants, such as *daf-2(e1368)* and *daf-2(e1370)* mutants (Figure IIIH; Table IV). Thus, there are likely other longevity-influencing ILPs that remain to be identified, although it is unclear if *ins-7* is one of these peptides. *ins-7* has previously been shown to affect lifespan (Murphy et al., 2007), but I find that loss of this gene has no effect under the conditions of my study. Nevertheless, future work could show that *ins-6* and *ins-1* function combinatorially with a group of ILPs to regulate longevity.

Table IV. Cumulative adult lifespan of ilp mutants at 25°C

Strain	Mean Lifespan ± SEM (Days)	75th %ile (Days)	No. Animals Observed / Total Initial Animals	% Wild Type	*P* Against Wild Type (Logrank)	*P* Against Specified Groups (Logrank)
25°C						
Wild Type	13.0 ± 0.1	17	1721/2049 (17)			
ins-1(nr2091)	12.6 ± 0.3	16	279/397(4)	-3	n.s.	
ins-18(tm339)	13.3 ± 0.4	16	84/100 (1)	+2	n.s.	
ins-7(tm1907)	13.3 ± 0.4	17	146/170 (2)	+2	n.s.	
ins-6(tm2416)	14.4 ± 0.2	19	552/697 (7)	+11	< 0.0001	< 0.0001 [a] 0.07 [b] n.s. [c]
daf-28(sa191)	14.4 ± 0.2	18	373/500 (5)	+11	< 0.0001	0.005 [a] n.s. [b]
daf-28(tm2308)						
post-L3 adults	13.6 ± 0.2	17	863/1100 (11)	+4	0.004	0.2 [b]
post-dauer adults	14.4 ± 0.3	18	182/300 (3)	+11	0.003	
ins-6(tm2416); daf-28(sa191)	17.0 ± 0.5	23	173/288 (3)	+31	< 0.0001	< 0.0001 [cd]
ins-6(tm2416); ins-1(nr2091)	12.5 ± 0.4	16	176/201 (2)	-4	n.s.	0.0001 [d] n.s. [e]
ins-18(tm339); ins-6(tm2416)	14.4 ± 0.7	21	70/100 (1)	+11	< 0.0001	n.s. [d]
ins-18(tm339); daf-28(tm2308)						
post-dauer adults	13.5 ± 0.6	16	58/100 (1)	+4	n.s.	n.s. [b]
ins-1(nr2091); daf-28(tm2308)						
post-L3 adults	12.7 ± 0.2	16	370/500 (5)	-2	n.s.	0.005 [a]
post-dauer adults	12.7 ± 0.3	17	199/300 (3)	-2	n.s.	0.0003 [b] n.s. [f]
ins-6(tm2416); daf-28(tm2308)						
post-L3 adults	13.9 ± 0.4	18	208/299 (3)	+7	< 0.0001	0.04 [g] n.s. [d]
mixed adult populations	14.7 ± 0.3	20	365/500 (5)	+13	< 0.0001	0.004 [a] 0.1 [d] < 0.0001 [a]
post-dauer adults	15.4 ± 0.4	21	207/300 (3)	+19	< 0.0001	0.01 [h] n.s. [g]
post-dauer adults – hi population density	18.3 ± 0.5	24	195/300 (3)	+41	< 0.0001	< 0.0001 [b] < 0.0001 [i]
ins-6(tm2416); ins-1(nr2091); daf-28(tm2308)						
post-L3 adults	11.6 ± 0.4	16	211/300 (3)	-10	n.s.	< 0.0001 [l] n.s. [fj]
mixed adult populations	13.2 ± 0.6	18	63/100 (1)	+2	n.s.	0.0005 [h] < 0.0001 [l] n.s. [k]
post-dauer adults	14.9 ± 0.5	20	184/301 (3)	+14	< 0.0001	< 0.0001 [m] n.s. [i]

Strain	Mean Lifespan ± SEM (Days)	75th %ile (Days)	No. Animals Observed / Total Initial Animals	% Wild Type	*P* Against Wild Type (Logrank)	*P* Against Specified Groups (Logrank)
shifted from 20°C to 25°C at L4 stage						
Wild Type	13.26 ± 0.38	17	170/200 (2)			
ins-1(nr2091)	12.17 ± 0.52	16	71/100 (1)	-8	0.03	
ins-6(tm2416)	13.62 ± 0.41	19	150/200 (2)	+3	n.s.	
daf-28(tm2308)	12.75 ± 0.49	17	82/100 (1)	-4	n.s.	
daf-2(e1368)	22.06 ± 0.65	29	152/200 (2)	+66	< 0.0001	
daf-2(e1368); ins-1	21.51 ± 1.12	29	58/100 (1)	+62	< 0.0001	
ins-6; daf-2(e1368)	27.08 ± 0.73	33	152/200 (2)	+104	< 0.0001	< 0.0001[n]
daf-2(e1368); daf-28(tm2308)	19.87 ± 1.07	28	82/100 (1)	+50	< 0.0001	n.s.[n]
daf-2(e1370)	33.72 ± 1.19	44	100/200 (2)	+154	< 0.0001	< 0.0001[n]

Table IV. The lifespan phenotypes of wild type and insulin-deficient worms. Lifespan was measured at 25°C, using worms that were grown at the same temperature, unless stated otherwise. All assays were initiated on the first day of adulthood and the worms were transferred daily to new plates during their reproductive period. The JMP 5.1 software was used to determine mean lifespan of cumulative trials and to perform statistical comparisons across the different genotypes. *P* values were determined by the logrank test and shown in the sixth and seventh columns. The following indicate: [a], compared to *daf-28(tm2308)* post-L3 adult mutants; [b], compared to *daf-28(tm2308)* post-dauer adults; [c], compared to *daf-28(sa191)* mutants; [d], compared to *ins-6(tm2416)* mutants; [e], compared to *ins-1(nr2091)* mutants; [f], compared to *ins-1(nr2091); daf-28(tm2308)* double mutants; [g], compared to *ins-6(tm2416); daf-28(tm2308)* mixed adult populations; [h], compared to *ins-6(tm2416); daf-28(tm2308)* double mutant post-L3 adults; [i], compared to *ins-6(tm2416); daf-28(tm2308)* double mutant post-dauer adults; [j], compared to *ins-6(tm2416); ins-1(nr2091); daf-28(tm2308)* triple mutant mixed adult populations; [k], compared to *ins-6(tm2416); ins-1(nr2091); daf-28(tm2308)* post-L3 adults; [l], compared to *ins-6(tm2416); ins-1(nr2091); daf-28(tm2308)* triple mutant post-dauer adults; [m], compared to *ins-1(nr2091); daf-28(tm2308)* post-dauer adults; [n], compared to *daf-2(e1368)* single mutants; and n.s., not significant since $P > 0.1$. The post-L3 adults are continuously well-fed adults, while post-dauer adults have undergone a transient dauer stage. The mixed population of adults contain both post-L3 and post-dauer adults. Some of the lifespan data from the previous chapter are included here for comparison.

3.2.4 **Materials and Methods**

All worm mutants used in this study were backcrossed six times to our lab wild-type (N2) strain before any phenotypic analysis was performed. However, a further outcross of *ins-7(tm1907)* failed to yield viable, homozygous *ins-7* mutant adults.

The worms were also continuously fed *E. coli* OP50 for at least two generations before each assay. The dauer entry and exit assays, lifespan assays and statistical analyses were as described in Section 3.1. For a complete list of all strains, transgenes and constructs analyzed in sections 3.1 and 3.2, see Tables V-VII.

Generation of ASJ-Genetically Ablated Worms

We generated independent transgenic lines using standard methods and a *myo-3p::rfp* coinjection marker (injected at 30 ng/µl) together with a *trx-1p::gfp* marker for the ASJ neurons. For controls, we generated *daf-2(e1368)* and *ins-6(tm2416); daf-2(e1368)* mutant worms that carry the *myo-3::rfp* coinjection marker together with the *trx-1p::gfp* marker.

To genetically ablate the ASJ neurons, we drove human caspase-1 (ICE; Zheng et al., 1999) transcription from the *trx-1* promoter (gift of P. Swoboda), *trx-1p::ICE*, which is specifically expressed from the ASJ neurons (Miranda-Vizuete et al., 2006). We generated a plasmid construct (pQZ37) in the pPD95.77 vector backbone (gift of A. Fire), in which the ICE cDNA is flanked by the 1-kb *trx-1* promoter and by the 0.7–kb 3' UTR of *unc-54*. We introduced the *trx-1p::ICE* construct at 50 ng/µl into *ins-6(tm2416)*; *daf-2(e1368)* mutants, which were subsequently crossed to *daf-2(e1368)* males to obtain the ablation arrays in the *daf-2(e1368)* background alone. In addition, the *str-3p::ins-6*

construct described in the Methods of section 3.1 was injected alone or together with *trx-1p::ICE* construct, to drive expression of *ins-6* from the ASI neurons.

The coinjected *trx-1p::gfp* marker (25 ng/ul) was still visible in about half of the *trx-1p::ICE*-expressing worms that were analyzed for GFP expression (data not shown). Thus, it is possible that the expression of *trx-1p::ICE* used in these experiments is insufficient for the complete ablation of the ASJ neurons, which suggests that some dauer-exit inducing signals could still be secreted from ASJ. This might explain the stronger dauer-exit defect of worms, in which the ASJ neurons were ablated with a laser (Bargmann and Horvitz, 1991a).

Table V Plasmid list

plasmid name	parent plasmid	construct	details
pQZ2	pPD117.01 (A. Fire)	*ins-6p::mCherry* (*ins-6* 5'cis only)	*mCherry* ORF flanked by 1.7-kb sequence upstream of the *ins-6* coding sequence
pQZ6	pDEST-R4-R3 (Gateway)	*ins-1p::CFP*	CFP ORF flanked by 4.3-kb sequence upstream and 1.1-kb sequence downstream of the *ins-1* coding sequence plus 0.8-kb intron region
pQZ10	pDEST-R4-R3 (Gateway)	*ins-6p::mCherry*	*mCherry* ORF flanked by 1.7-kb sequence upstream and 2.0-kb sequence downstream of the *ins-6* coding sequence
pQZ11	pCR-BluntII-Topo (Invitrogen)	*ins-6p::ins-6*	genomic *ins-6* locus with 1.7-kb upstream and 2.1-kb downstream sequence
pQZ33	pPD95.77 (A. Fire)	*str-3p::ins-6*	*ins-6* cDNA flanked by 3.1-kb *str-3* promoter[a] and 0.7-kb 3' UTR of *unc-54* (vector sequence)
pQZ34	pPD95.77 (A. Fire)	*trx-1p::gfp*	GFP ORF flanked by 1-kb *trx-1* promoter[b] and 0.7-kb 3' UTR of *unc-54* (vector sequence)
pQZ35	pPD95.77 (A. Fire)	*trx-1p::ins-6*	*ins-6* cDNA flanked by 1-kb *trx-1* promoter and 0.7-kb 3' UTR of *unc-54* (vector sequence)
pQZ36	pPD95.77 (A. Fire)	*str-3p::gfp*	GFP ORF flanked by 3.1-kb *str-3* promoter and 0.7-kb 3' UTR of *unc-54* (vector sequence)
pQZ37	pPD95.77 (A. Fire)	*trx-1p::ICE*	ICE coding sequence[c] flanked by 1-kb *trx-1* promoter and 0.7-kb 3' UTR of *unc-54* (vector sequence)

[a]str-3 promoter gift of C. Bargmann
[b]trx-1 promoter gift of P. Swoboda
[c]ICE DNA gift of V. Maricq

Table VI Extrachromosomal arrays by microinjection

array	injected constructs	concentrations
jxEx18[a]	*[ofm-1::gfp]*	25 ng/µl *ofm-1p::gfp*
jxEx20[a]	*[pQZ2(ins-6::mCherry) ofm-1::gfp]*	100 ng/µl *ins-6p::mCherry,* 25 ng/µl *ofm-1p::gfp*
jxEx21[a]	*[ofm-1::gfp]*	25 ng/µl *ofm-1p::gfp*
jxEx22[a]	*[ofm-1::gfp]*	25 ng/µl *ofm-1p::gfp*
jxEx24[a]	*[pQZ6(ins-1::cfp) ofm-1::gfp]*	100 ng/µl *ins-1p::cfp,* 25 ng/µl *ofm-1p::gfp*
jxEx25[a]	*[pQZ6(ins-1::cfp) ofm-1::gfp]*	100 ng/µl *ins-1p::cfp,* 25 ng/µl *ofm-1p::gfp*
jxEx26[a]	*[pQZ6(ins-1::cfp) ofm-1::gfp]*	100 ng/µl *ins-1p::cfp,* 25 ng/µl *ofm-1p::gfp*
jxEx27[a]	*[pQZ11(ins-6::ins-6) ofm-1::gfp]*	2 ng/µl *ins-6p::ins-6,* 25 ng/µl *ofm-1p::gfp*
jxEx28[a]	*[pQZ11(ins-6::ins-6) ofm-1::gfp]*	2 ng/µl *ins-6p::ins-6,* 25 ng/µl *ofm-1p::gfp*
jxEx29[a]	*[pQZ11(ins-6::ins-6) ofm-1::gfp]*	2 ng/µl *ins-6p::ins-6,* 25 ng/µl *ofm-1p::gfp*
jxEx30[a]	*[pQZ10(ins-6::mCherry) ofm-1::gfp]*	100 ng/µl *ins-6p::mCherry,* 25 ng/µl *ofm-1p::gfp*
jxEx31[a]	*[pQZ10(ins-6::mCherry) ofm-1::gfp]*	100 ng/µl *ins-6p::mCherry,* 25 ng/µl *ofm-1p::gfp*
jxEx32[a]	*[pQZ10(ins-6::mCherry) ofm-1::gfp]*	100 ng/µl *ins-6p::mCherry,* 25 ng/µl *ofm-1p::gfp*
jxEx50	*[pQZ33(str-3::ins-6) ofm-1::gfp]*	25 ng/µl *str-3p::ins-6,* 25 ng/µl *ofm-1p::gfp*
jxEx51	*[pQZ33(str-3::ins-6) ofm-1::gfp]*	25 ng/µl *str-3p::ins-6,* 25 ng/µl *ofm-1p::gfp*
jxEx52	*[pQZ33(str-3::ins-6) ofm-1::gfp]*	25 ng/µl *str-3p::ins-6,* 25 ng/µl *ofm-1p::gfp*
jxEx53	*[pQZ33(str-3::ins-6) ofm-1::gfp]*	2 ng/µl *str-3p::ins-6,* 25 ng/µl *ofm-1p::gfp*
jxEx54	*[pQZ33(str-3::ins-6) ofm-1::gfp]*	2 ng/µl *str-3p::ins-6,* 25 ng/µl *ofm-1p::gfp*
jxEx55	*[pQZ34(trx-1::gfp) myo-3::rfp]*	100 ng/µl *trx-1p::gfp,* 30 ng/µl *myo-3p::rfp*
jxEx56	*[pQZ34(trx-1::gfp) myo-3::rfp]*	100 ng/µl *trx-1p::gfp,* 30 ng/µl *myo-3p::rfp*
jxEx57	*[pQZ34(trx-1::gfp) myo-3::rfp]*	100 ng/µl *trx-1p::gfp,* 30 ng/µl *myo-3p::rfp*
jxEx58	*[pQZ35(trx-1::ins-6) ofm-1::gfp]*	25 ng/µl *trx-1p::ins-6,* 25 ng/µl *ofm-1p::gfp*
jxEx59	*[pQZ35(trx-1::ins-6) ofm-1::gfp]*	25 ng/µl *trx-1p::ins-6,* 25 ng/µl *ofm-1p::gfp*
jxEx60	*[pQZ35(trx-1::ins-6) ofm-1::gfp]*	25 ng/µl *trx-1p::ins-6,* 25 ng/µl *ofm-1p::gfp*
jxEx61	*[pQZ35(trx-1::ins-6) ofm-1::gfp]*	2 ng/µl *trx-1p::ins-6,* 25 ng/µl *ofm-1p::gfp*
jxEx62	*[pQZ35(trx-1::ins-6) ofm-1::gfp]*	2 ng/µl *trx-1p::ins-6,* 25 ng/µl *ofm-1p::gfp*
jxEx63	*[pQZ35(trx-1::ins-6) ofm-1::gfp]*	2 ng/µl *trx-1p::ins-6,* 25 ng/µl *ofm-1p::gfp*
jxEx64	*[pQZ33(str-3::ins-6) ofm-1::gfp]*	2 ng/µl *str-3p::ins-6,* 25 ng/µl *ofm-1p::gfp*
jxEx65	*[pQZ33(str-3::ins-6) ofm-1::gfp]*	2 ng/µl *str-3p::ins-6,* 25 ng/µl*ofm-1p::gfp*
jxEx66	*[pQZ33(str-3::ins-6) ofm-1::gfp]*	2 ng/µl *str-3p::ins-6,* 25 ng/µl *ofm-1p::gfp*
jxEx67	*[pQZ36(str-3::gfp); myo-3::rfp]*	2 ng/µl *str-3p::gfp,* 30 ng/µl *myo-3p::rfp*
jxEx68	*[pQZ36(str-3::gfp); myo-3::rfp]*	2 ng/µl *str-3p::gfp,* 30 ng/µl *myo-3p::rfp*
jxEx69	*[pQZ36(str-3::gfp); myo-3::rfp]*	2 ng/µl *str-3p::gfp,* 30 ng/µl *myo-3p::rfp*
jxEx70	*[pQZ34(trx-1::gfp) myo-3::rfp]*	2 ng/µl *trx-1p::gfp,* 30 ng/µl *myo-3p::rfp*
jxEx71	*[pQZ34(trx-1::gfp) myo-3::rfp]*	2 ng/µl *trx-1p::gfp,* 30 ng/µl *myo-3p::rfp*
jxEx72	*[pQZ34(trx-1::gfp) myo-3::rfp]*	2 ng/µl *trx-1p::gfp,* 30 ng/µl *myo-3p::rfp*
jxEx73	*[pQZ36(str-3::gfp); myo-3::rfp]*	25 ng/µl *str-3p::gfp,* 30 ng/µl *myo-3p::rfp*
jxEx74	*[pQZ36(str-3::gfp); myo-3::rfp]*	25 ng/µl *str-3p::gfp,* 30 ng/µl *myo-3p::rfp*
jxEx75	*[pQZ36(str-3::gfp); myo-3::rfp]*	25 ng/µl *str-3p::gfp,* 30 ng/µl *myo-3p::rfp*
jxEx76	*[pQZ34(trx-1::gfp) myo-3::rfp]*	25 ng/µl *trx-1p::gfp,* 30 ng/µl *myo-3p::rfp*
jxEx77	*[pQZ34(trx-1::gfp) myo-3::rfp]*	25 ng/µl *trx-1p::gfp,* 30 ng/µl*myo-3p::rfp*
jxEx78	*[pQZ34(trx-1::gfp) myo-3::rfp]*	25 ng/µl *trx-1p::gfp,* 30 ng/µl *myo-3p::rfp*
jxEx84	*[pQZ33(str-3::ins-6) pQZ34(trx-1::gfp) myo-3::rfp]*	25 ng/µl *str-3p::ins-6,* 25 ng/µl *trx-1p::gfp,* 30 ng/µl *myo-3p::rfp*
jxEx85	*[pQZ33(str-3::ins-6) pQZ34(trx-1::gfp) myo-3::rfp]*	25 ng/µl *str-3p::ins-6,* 25 ng/µl *trx-1p::gfp,* 30 ng/µl *myo-3p::rfp*
jxEx86	*[pQZ33(str-3::ins-6) pQZ34(trx-1::gfp) myo-3::rfp]*	25 ng/µl *str-3p::ins-6,* 25 ng/µl *trx-1p::gfp,* 30 ng/µl *myo-3p::rfp*
jxEx91	*[pQZ37(trx-1::ICE) pQZ34(trx-1::gfp) myo-3::rfp]*	50 ng/µl *trx-1p::ICE,* 25 ng/µl *trx-1p::gfp,* 30 ng/µl *myo-3p::rfp*
jxEx92	*[pQZ37(trx-1::ICE) pQZ34(trx-1::gfp) myo-3::rfp]*	50 ng/µl *trx-1p::ICE,* 25 ng/µl *trx-1p::gfp,* 30 ng/µl *myo-3p::rfp*

array	injected constructs	concentrations
jxEx93	*[pQZ37(trx-1::ICE) pQZ34(trx-1::gfp) myo-3::rfp]*	50 ng/µl *trx-1p::ICE,* 25 ng/µl *trx-1p::gfp,* 30 ng/µl *myo-3p::rfp*
jxEx94	*[pQZ37(trx-1::ICE) pQZ33(str-3::ins-6) pQZ34(trx-1::gfp) myo-3::rfp]*	50 ng/µl *trx-1p::ICE,* 25 ng/µl *str-3p::ins-6,* 25 ng/µl *trx-1p::gfp,* 30 ng/µl *myo-3p::rfp*
jxEx95	*[pQZ37(trx-1::ICE) pQZ33(str-3::ins-6) pQZ34(trx-1::gfp) myo-3::rfp]*	50 ng/µl *trx-1p::ICE,* 25 ng/µl *str-3p::ins-6,* 25 ng/µl *trx-1p::gfp,* 30 ng/µl *myo-3p::rfp*
jxEx96	*[pQZ37(trx-1::ICE) pQZ33(str-3::ins-6) pQZ34(trx-1::gfp) myo-3::rfp]*	50 ng/µl *trx-1p::ICE,* 25 ng/µl *str-3p::ins-6,* 25 ng/µl *trx-1p::gfp,* 30 ng/µl *myo-3p::rfp*
yxEx163[b]	*[pQZ11(ins-6::ins-6) ofm-1::gfp]*	25 ng/µl *ins-6p::ins-6,* 25 ng/µl *ofm-1p::gfp*
yxEx174[b]	*[pQZ11(ins-6::ins-6) ofm-1::gfp]*	25 ng/µl *ins-6p::ins-6,* 25 ng/µl *ofm-1p::gfp*
yxEx175[b]	*[pQZ11(ins-6::ins-6) ofm-1::gfp]*	25 ng/µl *ins-6p::ins-6,* 25 ng/µl *ofm-1p::gfp*

[a]injected by M. Gloeck
[b]injected by Yun Zhang lab

Table VII Strain list

strain[a]	genotype
QZ60[b]	*daf-16(mu86)I*
QZ61	*ins-7(tm1907)IV*
QZ78	*daf-28(sa191)V*
QZ80	*ins-1(nr2091)IV*
QZ81	*ins-6(tm2416)II*
QZ83	*daf-28(tm2308)V*
QZ91[b]	*daf-2(e1370)III*
QZ102	*ins-6(tm2416)II; daf-28(sa191)V*
QZ103	*ins-6(tm2416)II; daf-28(tm2308)V*
QZ104	*ins-18(tm339)I*
QZ106	*ins-6(tm2416)II; ins-1(nr2091)IV*
QZ107	*ins-1(nr2091)IV; daf-28(tm2308)V*
QZ118	*ins-1(nr2091)IV; daf-28(sa191)V*
QZ120	*daf-2(e1368)III*
QZ127	*ins-18(tm339)I; ins-6(tm2416)II*
QZ128	*ins-6(tm2416)II; daf-2(e1368)III*
QZ129	*daf-2(e1368)III; daf-28(tm2308)V*
QZ134	*ins-18(tm339)I; daf-28(tm2308)V*
QZ135	*daf-2(e1368)III; ins-1(nr2091)IV*
QZ136	*daf-16(mu86)I; ins-6(tm2416)II*
QZ143	*daf-16(mu86)I; daf-28(tm2308)V*
QZ151	*ins-6(tm2416)II; daf-2(e1368) III; daf-28(tm2308)V*
QZ152	*ins-6(tm2416)II; daf-2(e1368)III; ins-1(nr2091)IV*
QZ153	*daf-16(mu86)I; ins-6(tm2416)II; daf-28(tm2308)V*
QZ156	*daf-16(mu86)I; ins-1(nr2091)IV*
QZ158	*ins-6(tm2416)II; ins-1(nr2091)IV; daf-28(tm2308)V*
QZ159	*ins-18(tm339)I; daf-28(sa191)V*
QZ162	*daf-2(e1368)III; daf-28(sa191)V*
QZ174	*daf-2(e1368)III; ins-1(nr2091)IV; daf-28(tm2308)V*
QZ175	*daf-16(mu86)I; daf-28(sa191)V*
QZ176	*daf-16(mu86)I; ins-1(nr2091)IV; daf-28(tm2308)V*
QZ177	*daf-16(mu86)I; ins-6(tm2416)II; ins-1(nr2091)IV*
QZ183	*ins-18(tm339)I; ins-1(nr2091)IV*
QZ186[e]	*mgIs40[daf-28::gfp]*
QZ188	*ins-6(tm2416)II; daf-2(e1368)III; ins-1(nr2091)IV; daf-28(tm2308)V*
QZ191	*daf-16(mu86)I; ins-6(tm2416)II; daf-28(sa191)V*

strain[a]	genotype
QZ192	*daf-16(mu86)I; ins-1(nr2091)IV; daf-28(sa191)V*
QZ195	*daf-16(mu86)I; ins-6(tm2416)II; ins-1(nr2091)IV; daf-28(tm2308)V*
QZ206[c]	*jxEx18[ofm-1::gfp]*
QZ208[c]	*jxEx20[pQZ2(ins-6p::mCherry) ofm-1::gfp]*
QZ212[c]	*jxEx21[ofm-1::gfp]*
QZ213[c]	*jxEx22[ofm-1::gfp]*
QZ217	*daf-16(mu86)I; daf-2(e1368)III*
QZ226[c]	*jxEx24[pQZ6(ins-1p::cfp) ofm-1::gfp]*
QZ227[c]	*jxEx25[pQZ6(ins-1p::cfp) ofm-1::gfp]*
QZ228[c]	*jxEx26[pQZ6(ins-1p::cfp) ofm-1::gfp]*
QZ232[c]	*jxEx27[pQZ11(ins-6p::ins-6) ofm-1::gfp]*
QZ233[c]	*jxEx28[pQZ11(ins-6p::ins-6) ofm-1::gfp]*
QZ234[c]	*jxEx29[pQZ11(ins-6p::ins-6) ofm-1::gfp]*
QZ235[c]	*jxEx30[pQZ10(ins-6p::mCherry) ofm-1::gfp]*
QZ236[c]	*jxEx31[pQZ10(ins-6p::mCherry) ofm-1::gfp]*
QZ237[c]	*jxEx32[pQZ10(ins-6p::mCherry) ofm-1::gfp]*
QZ250	*ins-6(tm2416)II; daf-28(tm2308)V; jxEx18[ofm-1::gfp]*
QZ252	*ins-6(tm2416)II; daf-28(tm2308)V; jxEx21[ofm-1::gfp]*
QZ254	*ins-6(tm2416)II; daf-28(tm2308)V; jxEx22[ofm-1::gfp]*
QZ257	*ins-6(tm2416)II; jxEx18[ofm-1::gfp]*
QZ258	*ins-6(tm2416)II; jxEx21[ofm-1::gfp]*
QZ259	*ins-6(tm2416)II; jxEx22[ofm-1::gfp]*
QZ260	*ins-6(tm2416)II; jxEx27[pQZ11(ins-6p::ins-6) ofm-1::gfp]*
QZ261	*ins-6(tm2416)II; jxEx28[pQZ11(ins-6p::ins-6) ofm-1::gfp]*
QZ262	*ins-6(tm2416)II; jxEx29[pQZ11(ins-6p::ins-6) ofm-1::gfp]*
QZ263	*ins-6(tm2416)II; daf-2(e1368)III; jxEx18[ofm-1::gfp]*
QZ264	*ins-6(tm2416)II; daf-2(e1368)III; jxEx21[ofm-1::gfp]*
QZ265	*ins-6(tm2416)II; daf-2(e1368)III; jxEx22[ofm-1::gfp]*
QZ266	*ins-6(tm2416)II; daf-2(e1368)III; jxEx27[pQZ11(ins-6p::ins-6) ofm-1::gfp]*
QZ267	*ins-6(tm2416)II; daf-2(e1368)III; jxEx28[pQZ11(ins-6p::ins-6) ofm-1::gfp]*
QZ268	*ins-6(tm2416)II; daf-2(e1368)III; jxEx29[pQZ11(ins-6p::ins-6) ofm-1::gfp]*
QZ274	*ins-6(tm2416)II; daf-28(tm2308)V; jxEx27[pQZ11(ins-6p::ins-6) ofm-1::gfp]*
QZ275	*ins-6(tm2416)II; daf-28(tm2308)V; jxEx28[pQZ11(ins-6p::ins-6) ofm-1::gfp]*
QZ276	*ins-6(tm2416)II; daf-28(tm2308)V; jxEx29[pQZ11(ins-6p::ins-6) ofm-1::gfp]*
QZ287	*ins-6(tm2416)II; daf-28(tm2308)V; yxEx163[ins-6p::ins6(pQZ11) ofm-1::gfp]*
QZ288	*ins-6(tm2416)II; daf-28(tm2308)V; yxEx174[ins-6p::ins6(pQZ11) ofm-1::gfp]*
QZ289	*ins-6(tm2416)II; daf-28(tm2308)V; yxEx175[ins-6p::ins6(pQZ11) ofm-1::gfp]*
QZ290	*ins-6(tm2416)II; daf-2(e1368)III; yxEx163[ins-6p::ins6(pQZ11) ofm-1::gfp]*
QZ291	*ins-6(tm2416)II; daf-2(e1368)III; yxEx174[ins-6p::ins6(pQZ11) ofm-1::gfp]*
QZ292	*ins-6(tm2416)II; daf-2(e1368)III; yxEx175[ins-6p::ins6(pQZ11) ofm-1::gfp]*
QZ293	*daf-2(e1368)III; jxEx18[ofm-1::gfp]*
QZ294	*daf-2(e1368)III; jxEx21[ofm-1::gfp]*
QZ295	*daf-2(e1368)III; jxEx22[ofm-1::gfp]*
QZ304[d]	*daf-2(e1368)III; jxEx30[pQZ10(ins-6p::mCherry) ofm-1::gfp]*
QZ305[d]	*daf-2(e1368)III; jxEx31[pQZ10(ins-6p::mCherry) ofm-1::gfp]*
QZ309	*ins-6(tm2416)II; daf-2(e1368)III; jxEx50[pQZ33(str-3::ins-6) ofm-1::gfp]*
QZ310	*ins-6(tm2416)II; daf-2(e1368)III; jxEx51[pQZ33(str-3::ins-6) ofm-1::gfp]*
QZ311	*ins-6(tm2416)II; daf-2(e1368)III; jxEx52[pQZ33(str-3::ins-6) ofm-1::gfp]*
QZ312	*ins-6(tm2416)II; daf-2(e1368)III; jxEx53[pQZ33(str-3::ins-6) ofm-1::gfp]*
QZ313	*ins-6(tm2416)II; daf-2(e1368)III; jxEx54[pQZ33(str-3::ins-6) ofm-1::gfp]*
QZ314	*jxEx55[pQZ34(trx-1::gfp) myo-3::rfp]*
QZ315	*jxEx56[pQZ34(trx-1::gfp) myo-3::rfp]*
QZ316	*jxEx57[pQZ34(trx-1::gfp) myo-3::rfp]*
QZ317	*ins-6(tm2416)II; daf-2(e1368)III; jxEx58[pQZ35(trx-1::ins-6) ofm-1::gfp]*
QZ318	*ins-6(tm2416)II; daf-2(e1368)III; jxEx59[pQZ35(trx-1::ins-6) ofm-1::gfp]*

strain[a]	genotype
QZ319	*ins-6(tm2416)II; daf-2(e1368)III; jxEx60[pQZ35(trx-1::ins-6) ofm-1::gfp]*
QZ320	*ins-6(tm2416)II; daf-28(tm2308)V; jxEx61[pQZ35(trx-1::ins-6) ofm-1::gfp]*
QZ321	*ins-6(tm2416)II; daf-28(tm2308)V; jxEx62[pQZ35(trx-1::ins-6) ofm-1::gfp]*
QZ322	*ins-6(tm2416)II; daf-28(tm2308)V; jxEx63[pQZ35(trx-1::ins-6) ofm-1::gfp]*
QZ323	*daf-16(mu86)I; jxEx30[pQZ10(ins-6p::mCherry) ofm-1::gfp]*
QZ324	*daf-16(mu86)I; jxEx31[pQZ10(ins-6p::mCherry) ofm-1::gfp]*
QZ327	*ins-6(tm2416)II; daf-2(e1368)III; jxEx64[pQZ33(str-3::ins-6) ofm-1::gfp]*
QZ328	*ins-6(tm2416)II; daf-2(e1368)III; jxEx65[pQZ33(str-3::ins-6) ofm-1::gfp]*
QZ329	*ins-6(tm2416)II; daf-2(e1368)III; jxEx66[pQZ33(str-3::ins-6) ofm-1::gfp]*
QZ330	*ins-6(tm2416)II; daf-28(tm2308)V; jxEx50[pQZ33(str-3::ins-6) ofm-1::gfp]*
QZ331	*ins-6(tm2416)II; daf-28(tm2308)V; jxEx51[pQZ33(str-3::ins-6) ofm-1::gfp]*
QZ332	*ins-6(tm2416)II; daf-28(tm2308)V; jxEx52[pQZ33(str-3::ins-6) ofm-1::gfp]*
QZ333	*ins-6(tm2416)II; daf-28(tm2308)V; jxEx53[pQZ33(str-3::ins-6) ofm-1::gfp]*
QZ334	*ins-6(tm2416)II; daf-28(tm2308)V; jxEx54[pQZ33(str-3::ins-6) ofm-1::gfp]*
QZ335	*ins-6(tm2416)II; daf-28(tm2308)V; jxEx58[pQZ35(trx-1::ins-6) ofm-1::gfp]*
QZ336	*ins-6(tm2416)II; daf-28(tm2308)V; jxEx59[pQZ35(trx-1::ins-6) ofm-1::gfp]*
QZ337	*ins-6(tm2416)II; daf-28(tm2308)V; jxEx60[pQZ35(trx-1::ins-6) ofm-1::gfp]*
QZ338	*ins-6(tm2416)II; daf-2(e1368)III; jxEx61[pQZ35(trx-1::ins-6) ofm-1::gfp]*
QZ339	*ins-6(tm2416)II; daf-2(e1368)III; jxEx62[pQZ35(trx-1::ins-6) ofm-1::gfp]*
QZ340	*ins-6(tm2416)II; daf-2(e1368)III; jxEx63[pQZ35(trx-1::ins-6) ofm-1::gfp]*
QZ342	*ins-6(tm2416)II; daf-28(tm2308)V; jxEx64[pQZ33(str-3::ins-6) ofm-1::gfp]*
QZ343	*ins-6(tm2416)II; daf-28(tm2308)V; jxEx65[pQZ33(str-3::ins-6) ofm-1::gfp]*
QZ344	*ins-6(tm2416)II; daf-28(tm2308)V; jxEx66[pQZ33(str-3::ins-6) ofm-1::gfp]*
QZ345	*daf-28(tm2308)V; jxEx27 [pQZ11(ins-6p::ins-6) ofm-1::gfp]*
QZ346	*daf-28(tm2308)V; jxEx28 [pQZ11(ins-6p::ins-6) ofm-1::gfp]*
QZ347	*daf-28(tm2308)V; jxEx29 [pQZ11(ins-6p::ins-6) ofm-1::gfp]*
QZ348	*daf-28(tm2308)V; yxEx163[ins-6p::ins6(pQZ11) ofm-1::gfp]*
QZ349	*daf-28(tm2308)V; yxEx174[ins-6p::ins6(pQZ11) ofm-1::gfp]*
QZ350	*daf-28(tm2308)V; yxEx175[ins-6p::ins6(pQZ11) ofm-1::gfp]*
QZ355	*jxEx67[pQZ36 (str-3::gfp) myo-3::rfp]*
QZ356	*jxEx68[pQZ36 (str-3::gfp) myo-3::rfp]*
QZ357	*jxEx69[pQZ36 (str-3::gfp) myo-3::rfp]*
QZ358	*jxEx70[pQZ34 (trx-1::gfp) myo-3::rfp]*
QZ359	*jxEx71[pQZ34 (trx-1::gfp) myo-3::rfp]*
QZ360	*jxEx72[pQZ34 (trx-1::gfp) myo-3::rfp]*
QZ361	*jxEx73[pQZ36 (str-3::gfp) myo-3::rfp]*
QZ362	*jxEx74[pQZ36 (str-3::gfp) myo-3::rfp]*
QZ363	*jxEx75[pQZ36 (str-3::gfp) myo-3::rfp]*
QZ364	*jxEx76[pQZ34 (trx-1::gfp) myo-3::rfp]*
QZ365	*jxEx77[pQZ34 (trx-1::gfp) myo-3::rfp]*
QZ366	*jxEx78[pQZ34 (trx-1::gfp) myo-3::rfp]*
QZ378	*ins-6(tm2416)II; daf-2(e1368)III; jxEx84[pQZ33(str-3::ins-6) pQZ34(trx-1::gfp) myo-3::rfp]*
QZ379	*ins-6(tm2416)II; daf-2(e1368)III; jxEx85[pQZ33(str-3::ins-6) pQZ34(trx-1::gfp) myo-3::rfp]*
QZ380	*ins-6(tm2416)II; daf-2(e1368)III; jxEx86[pQZ33(str-3::ins-6) pQZ34(trx-1::gfp) myo-3::rfp]*
QZ381	*daf-2(e1368)III; jxEx76 [pQZ34 (trx-1::gfp) myo-3::rfp]*
QZ382	*daf-2(e1368)III; jxEx77 [pQZ34 (trx-1::gfp) myo-3::rfp]*
QZ383	*daf-2(e1368)III; jxEx78 [pQZ34 (trx-1::gfp) myo-3::rfp]*
QZ384	*ins-6(tm2416)II; daf-2(e1368)III; jxEx76 [pQZ34 (trx-1::gfp) myo-3::rfp]*
QZ385	*ins-6(tm2416)II; daf-2(e1368)III; jxEx77 [pQZ34 (trx-1::gfp) myo-3::rfp]*
QZ386	*ins-6(tm2416)II; daf-2(e1368)III; jxEx78 [pQZ34 (trx-1::gfp) myo-3::rfp]*
QZ396	*ins-6(tm2416)II; daf-2(e1368)III; jxEx91[pQZ37(trx-1::ICE) pQZ34 (trx-1::gfp) myo-3::rfp]*

strain[a]	genotype
QZ397	*ins-6(tm2416)II; daf-2(e1368)III; jxEx92[pQZ37(trx-1::ICE) pQZ34 (trx-1::gfp) myo-3::rfp]*
QZ398	*ins-6(tm2416)II; daf-2(e1368)III; jxEx93[pQZ37(trx-1::ICE) pQZ34 (trx-1::gfp) myo-3::rfp]*
QZ399	*ins-6(tm2416)II; daf-2(e1368)III; jxEx94[pQZ37(trx-1::ICE) pQZ33(str-3::ins-6) pQZ34 (trx-1::gfp) myo-3::rfp]*
QZ400	*ins-6(tm2416)II; daf-2(e1368)III; jxEx95[pQZ37(trx-1::ICE) pQZ33(str-3::ins-6) pQZ34 (trx-1::gfp) myo-3::rfp]*
QZ401	*ins-6(tm2416)II; daf-2(e1368)III; jxEx96[pQZ37(trx-1::ICE) pQZ33(str-3::ins-6) pQZ34 (trx-1::gfp) myo-3::rfp]*
QZ402	*daf-2(e1368)III; jxEx84[pQZ33(str-3::ins-6) pQZ34(trx-1::gfp) myo-3::rfp]*
QZ403	*daf-2(e1368)III; jxEx85[pQZ33(str-3::ins-6) pQZ34(trx-1::gfp) myo-3::rfp]*
QZ404	*daf-2(e1368)III; jxEx86[pQZ33(str-3::ins-6) pQZ34(trx-1::gfp) myo-3::rfp]*
QZ405	*daf-2(e1368)III; jxEx91[pQZ37(trx-1::ICE) pQZ34 (trx-1::gfp) myo-3::rfp]*
QZ406	*daf-2(e1368)III; jxEx92[pQZ37(trx-1::ICE) pQZ34 (trx-1::gfp) myo-3::rfp]*
QZ407	*daf-2(e1368)III; jxEx93[pQZ37(trx-1::ICE) pQZ34 (trx-1::gfp) myo-3::rfp]*
QZ408	*daf-2(e1368)III; jxEx94[pQZ37(trx-1::ICE) pQZ33(str-3::ins-6) pQZ34 (trx-1::gfp) myo-3::rfp]*
QZ409	*daf-2(e1368)III; jxEx95[pQZ37(trx-1::ICE) pQZ33(str-3::ins-6) pQZ34 (trx-1::gfp) myo-3::rfp]*
QZ410	*daf-2(e1368)III; jxEx96[pQZ37(trx-1::ICE) pQZ33(str-3::ins-6) pQZ34 (trx-1::gfp) myo-3::rfp]*
ZC208	*yxEx163[pQZ11(ins-6p::ins-6) ofm-1::gfp]*
ZC238	*yxEx174[pQZ11(ins-6p::ins-6) ofm-1::gfp]*
ZC239	*yxEx175[pQZ11(ins-6p::ins-6) ofm-1::gfp]*

[a]by injection and by crossing
[b]outcrossed by Bakhthiyor Adilov
[c]generated by Mario Gloeck;
[d]generated by Joy Alcedo
[e]gift of G. Ruvkun

4. Discussion

4. 1 Insulin-like peptides function coordinately to regulate dauer arrest and lifespan

4.1.1 Insulin-like peptides in dauer regulation

The *C. elegans* ILPs, which are predicted ligands of the DAF-2 insulin-like receptor, are thought to control dauer arrest and longevity by modulating DAF-2 signaling (Li et al., 2003; Pierce et al., 2001). Some of the numerous insulin-like genes have been implicated in dauer and lifespan regulation through gain-of-function and RNA interference studies (Li et al., 2003; Malone et al., 1996; Murphy et al., 2003; Pierce et al., 2001). For example, *daf-28* has previously been implicated in the inhibition of dauer formation (Li et al., 2003; Malone et al., 1996), whereas *ins-1* and *ins-18* have been proposed to antagonize *daf-2* signaling to promote dauer arrest (Pierce et al., 2001).

In this study, I find that ILPs that promote reproductive development and inhibit dauer arrest include not only *daf-28* but also *ins-6* (Figures 1, 2 and 4.1; Tables.S1 and S2). However, I also show that *daf-28* mainly functions to inhibit entry into the dauer program, while *ins-6* primarily functions to promote exit from the program (Figure 4.1). At the same time, I find that *ins-1* and, to a minor extent, *ins-18* antagonize DAF-2 signaling to promote dauer entry (Figures 1, I and 4.1; Tables S1 and II). In addition, I identified a second role for *ins-1*, which is to inhibit dauer exit (Figures 2 and 4.1; Table S2). Thus, the different ILPs have distinct and non-redundant functions to regulate dauer arrest.

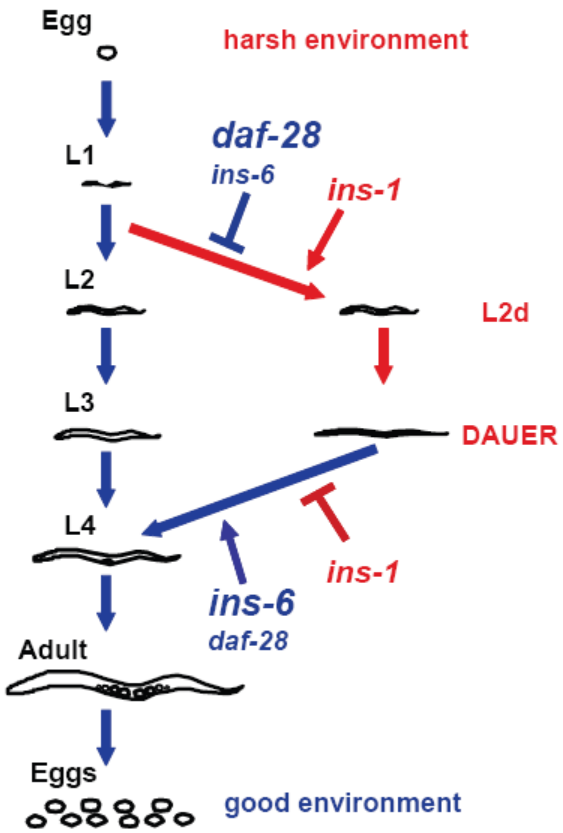

Figure 4.1. The functions of *daf-28*, *ins-6* and *ins-1* in dauer regulation. *daf-28* and *ins-6* inhibit dauer entry and promote dauer exit, whereas *ins-1* promotes dauer entry and inhibits dauer exit. While *daf-28* plays a more prominent role in inhibiting dauer entry (depicted with *daf-28* in larger font than *ins-6*), *ins-6* has a more primary role in promoting dauer exit (depicted with *ins-6* in larger font than *daf-28*).

4.1.2 Insulin-like peptides in lifespan regulation

ILPs also have distinct and non-redundant functions in regulating lifespan (Figures 6 and III; Table IV): While one group of ILPs, *ins-7*, *ins-18* and *daf-28*, does not have any effect on longevity under the conditions of my study, I find that *ins-6* does have a minor role in inhibiting longevity. Consistent with a previous observation (Pierce et al., 2001), I also show that *ins-1* lengthens lifespan, but that this role of *ins-1*, like that of *ins-6*, is

again minor compared to the effects of reduced DAF-2 signaling (Figure III; Table IV). Thus, these observations suggest that additional *ilp* genes modulate DAF-2 activity to regulate longevity.

The fact that these ILPs act non-redundantly but combinatorially to regulate lifespan, as well as dauer arrest (Table 4.1), raises the possibility that different combinations of ILPs are involved in different processes that are regulated by DAF-2 signaling. Indeed, *daf-28* has a major function in dauer entry, while it has only a minor function in dauer exit and no effect on longevity. On the other hand, *ins-6* has a minor function in dauer entry and lifespan regulation, but a major function in dauer exit. In comparison, *ins-1*, *ins-7* and *ins-18* plays either only a minor role or no role in all three processes. Yet, *ins-1* has been shown to have a major role in *C. elegans* salt-associated chemotaxis learning behavior (Tomioka et al., 2006) and food-associated thermotaxis (Kodama et al., 2006). Thus, these data are consistent with specific subsets of ILPs acting coordinately to modulate DAF-2 activity in diverse processes. Moreover, since *ins-1* can act not only as an antagonist of the pathway (in dauer arrest and thermotactic behavior) but also as an agonist (salt-dependent chemotactic learning), it is possible that other ILPs may do the same, depending on the process involved.

Table 4.1. Functions of DAF-2 and ILPs in the regulation of diverse biological processes

insulin-like receptor	**dauer entry inhibition**	**dauer exit induction**	**longevity inhibition**	**other processes**
daf-2	promote	promote	promote	embryonic and larval development, morphology, reproduction, fat storage, metabolic rate, stress response, locomotion, salt chemotaxis learning, food-associated thermotaxis
insulin-like peptide				
daf-28	major, agonist (refs. 1-4)	minor, agonist (ref. 5)	none (ref. 6);	NA
ins-6	minor, agonist (refs. 1, 3, 4)	major, agonist (ref. 5)	minor, agonist (ref. 6)	NA
ins-1	minor, antagonist (refs. 3, 4, 7)	minor, antagonist (ref. 5)	minor, antagonist (refs. 6, 7)	antagonist in food-associated thermotaxis (ref. 8); major agonist in salt chemotaxis learning (ref. 9)
ins-7	none (refs. 1, 4); minor (ref. 10)	NA	none (ref. 6); slight effect (ref. 10)	NA
ins-18	minor, antagonist (refs. 4, 7)	minor, antagonist (ref. 11)	none (ref. 6); slight effect (ref. 12)	NA
ins-4	minor, agonist (ref. 1; P. Fardel, data not shown)	NA	NA	NA
ins-9, ins-17, ins-19, ins-21, ins-22, ins-23, ins-31	none (refs. 1, 7)	NA	NA	NA

1. Li et al., 2003; 2. Malone et al. 1996; 3. Figure 1 and Table S1; 4. Figure I and Tables I and II; 5. Figure 2 and Table S2; 6. Figure III and Table IV; 7. Pierce et al., 2001; 8. Kodama et al., 2006; 9. Tomioka et al., 2006; 10. Murphy et al., 2003; 11. Ouellet et al., 2008; 12. Kawano et al., 2000.

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4.1.3 Possible mechanisms of DAF-2 activation

By what mechanism might the ILPs coordinately modulate DAF-2 activity to control different physiological outputs of the same pathway? The data on INS-1, which seems to act agonistically or antagonistically on the DAF-2 receptor, depending on the process, suggest the possible mechanism of competitive inhibition. In this scenario, different ILPs would have different binding affinities for DAF-2, and the presence or absence of specific ILPs, with different activating potential, within the local environment of the target cell would determine that cell's level of DAF-2 signaling. The resulting level of insulin-like signaling within the cell might then lead to different levels of active DAF-16 in the nucleus and at the regulatory sites of target genes. In turn, this could promote different transcriptional outputs, which would result in different physiological responses. INS-1 might thus be a weak agonist of DAF-2, and high concentrations of *ins-1* within the local environment might prevent the binding of stronger ILP agonists of the pathway. Consequently, loss of INS-1 would allow the strong agonists in the environment to activate the pathway at very high levels.

It is also possible that the ILPs might bind and activate the receptor as hetero- or homooligomers, which could again have varying affinities for DAF-2. In humans, ILP molecules are known to form dimers and multimers, which have also been shown to bind one receptor with different affinities (De Meyts, 1995). Another possibility is that some of the ILPs also act to stabilize or even sequester other ILPs away from the receptor. *C. elegans* has few predicted ILP-binding proteins (*e.g.*, T23G11.6; and a DAF-2 isoform

Table 4.2. Expression patterns and transcriptional regulation of *ilp* genes

***ilp* gene**	**identified neurons and tissues**	**unidentified neurons**	**other tissues**	**specific regulation**
*daf-28**	ASI and ASJ amphid sensory neurons (at all well-fed larval stages and adult worms, strong expression), PQR tail neuron, hindgut (ref. 1; confirmed by my analyses)	several head neurons (late embryogenesis and again with age; ref. 1)	pharyngeal muscle, hypodermis (late embryogenesis), somatic gonad (with age; ref. 1)	downregulated in ASI and ASJ by dauer program (in *daf-1*, *daf-7*, *daf-11*, or *daf-22* mutant dauers) and pheromones; upregulated in *daf-6*, *osm-1*, or *tax-4* sensory mutants (ref. 1)
*ins-1**	ASI, ASJ, ASE amphid sensory neurons and AIA interneurons (ref. 2, ASI and ASJ confirmed by our analyses); ADF, AIA, AIM, ASE, ASG, ASH, ASI, ASJ, AWA, BAG, and NSM (ref. 3)	amphid and several different types of head neurons and tail neurons in embryos to adult worms (refs. 2 and 4; confirmed by my analyses)	vulval muscle, intestine in embryos to adult worms (ref. 4)	none identified (ref. 5)
*ins-18**		in amphid and several other neurons in embryos to adult worms (ref. 4)	intestine in embryos to adult worms (ref. 4)	
*ins-6**	ASI (during reproductive development and in post-L3 adults); ASJ (during dauer arrest and in post-dauer adults) (ref. 5)	in amphid and several other neurons in embryos to adult worms (ref. 4); none (ref. 5)	none (refs. 4 and 5)	specifically downregulated in ASI by dauer pheromones; upregulated in ASJ by the combined effect of pheromones and dauer program (ref. 5)

* Expression during embryogenesis (refs. 1, 4 and 6).

1. Li et al., 2003; 2. Tomioka et al., 2006; 3. Kodama et al., 2006; 4. Pierce et al., 2001; 5. Figure 5 and Table 1; 6. Gregoire et al., 1998.

that only has the extracellular ligand binding domain, Y55D5A.5b; see www.wormbase.org) that could stabilize or sequester the different ILPs.

Although it has never been shown directly that a *C. elegans* ILP actually binds the DAF-2 receptor, INS-6, which has an insulin-like fold according to NMR analyses, was shown to have a weak binding affinity for the human insulin receptor (Hua et al., 2003). Other ILPs have also been predicted to adopt an IGF-I-like fold (Duret et al., 1998). However, the possibility remains that at least some of the ILPs bind receptors other than DAF-2, for example, GPCRs. Indeed, members of the mammalian relaxin family, which are related to ILPs, have been shown to bind such GPCRs (Liu and Lovenberg, 2008; Meyts et al., 2009; Svendsen et al., 2008).

4.1.4 Tissue-specific functions of ILP signaling

Since the numerous *ilp* genes have partially overlapping expression patterns [Table 4.2; (Li et al., 2003; Pierce et al., 2001)], different combinations of ILPs might be present at the local environments of different target tissues. At the same time, the expression levels of *ilp* genes could be modulated differently in different cells. Although ILPs are believed to act like hormones and spread through the worm's interstitial fluid after secretion (Li et al., 2003; Pierce et al., 2001), it is possible that they also act in a locally restricted manner. Thus, different DAF-2 regulated processes might be controlled independently, depending on which cells secrete the ILPs and in which target cells DAF-2 signaling is activated.

Genetic evidence suggests that dauer arrest and lifespan regulation by DAF-2 signaling are decoupled in space and time. Mosaic analyses indicate that *daf-2* functions cell nonautonomously in both processes (Apfeld and Kenyon, 1998). These and other data also suggest that DAF-2 functions primarily in the nervous system to control these processes, while it functions in muscle tissue to control metabolism (Apfeld and Kenyon, 1998; Wolkow et al., 2000). In addition, the downstream FOXO transcription factor DAF-16 has been found to regulate dauer arrest and lifespan in distinct tissues. DAF-16 activity in the nervous system is sufficient to induce dauer arrest and intestinal DAF-16 activity seems to control longevity more than dauer arrest (Libina et al., 2003). Besides the tissue specificity of its functions, different DAF-2 regulated processes also depend on different temporal activities of DAF-2 signaling. For example, DAF-2 signaling is required during larval development to regulate dauer arrest, while larval DAF-2 activity has little effect on longevity (Dillin et al., 2002). On the other hand, DAF-2 activity in adult worms is sufficient to inhibit longevity (Dillin et al., 2002).

Since many ILPs are largely expressed in neurons (Li et al., 2003; Pierce et al., 2001), it is possible that DAF-2 signaling in specific neurons starts a signaling cascade that again activates DAF-2, as well as other signaling pathways, in specific downstream cells or tissues. The transcriptional outputs in the downstream tissues could specify the tissue-specific responses. These responses would include dauer morphology or physiological changes that contribute to longevity, *e.g.*, the induction of stress-response mechanisms, a change in metabolic rate and/or reduced reproductive activity. Moreover, the transcriptional outputs from the downstream cells or tissues could feed back onto upstream cells to amplify the original signal.

4.2 Insulin-like peptides regulate physiology in response to environmental cues

As noted, the *C. elegans ilp* genes are primarily expressed in partly overlapping subsets of neurons, and some of them in amphid sensory neurons [Table 4.2; (Li et al., 2003; Pierce et al., 2001)]. The dauer-regulating ILPs *daf-28*, *ins-1* and *ins-6* are expressed in amphid sensory neurons (Kodama et al., 2006; Li et al., 2003; Tomioka et al., 2006; Figure 5; Tables 1 and 4.2), which have been shown to control dauer arrest by ablation studies (Bargmann and Horvitz 1991a; Schackwitz et al., 1996). Moreover, *ins-1* has also been shown to act in interneurons, which receive direct inputs from sensory neurons, to control behavioral and learning responses (Kodama et al., 2006; Tomioka et al., 2006). These findings raise an intriguing possibility that ILPs encode sensory inputs to induce specific physiological outputs, and thus coordinate the physiological and behavioral responses to the worm's environment. For example, low food levels not only induce dauer arrest at the dauer decision stage L1, which is accompanied by changes in metabolism and stress responses, but also induce adult worms to have altered behaviors and metabolism, reduced and delayed reproduction and increased longevity (Houthoofd et al., 2005).

4.2.1 The dauer-regulating neurons ASI and ASJ secrete insulin-like peptides to control dauer arrest

How are specific environmental inputs, such as starvation or crowding, transmitted into physiological outputs? First, specific cues, like the dauer pheromone mixture, are sensed by sensory amphid neurons (Bargmann and Horvitz 1991a; Schackwitz et al., 1996) that contain the cognate receptors within their cilia (Kim et al., 2009). In response to these cues, the sensory neurons regulate *ilps,* as well as other signals, at the transcriptional level (Li et al., 2003; Murakami et al., 2001; Ren et al., 1996; Schackwitz et al., 1996) and also likely at the post-transcriptional level. These signaling molecules, in turn, regulate the different physiological outputs.

The entry into and exit from the dauer program are known to be regulated by the amphid neurons, ASI, ADF, ASG, ASJ and ASK (Bargmann and Horvitz 1991a; Kim et al., 2009; Schackwitz et al., 1996). These neurons likely sense the food and pheromone cues that control dauer arrest. The neurons ASI, ADF and, to a minor extent, ASG inhibit dauer entry, whereas ASJ and, to a minor extent, ASK are required for the pheromone-induced dauer entry (Bargmann and Horvitz, 1991a; Schackwitz et al., 1996). At the same time, ASJ is required for dauer exit in response to lowered pheromone levels (Bargmann and Horvitz, 1991a). Recently, two receptors, the GPCRs SRBC-64 and SRBC-66, for a subset of the dauer pheromone mixture have been identified in the ASK neurons (Kim et al., 2009). However, no pheromone receptor has been found so far in ASJ. Although it is possible that additional pheromone receptors are expressed in the ASJ

neurons, the function of ASJ in this process might also be indirectly activated by the ASK neurons.

In the ASI neurons, different dauer-inhibiting signals are coordinately regulated in response to environmental cues (Ren et al., 1996; Schackwitz et al., 1996; Murakami et al., 2001; Li et al., 2003). The TGF-*β*-like ligand *daf-7*, which is exclusively expressed in ASI, is downregulated in response to high pheromone and low food levels, which induces dauer arrest (Ren et al., 1996; Schackwitz et al., 1996). In contrast, *daf-7* becomes upregulated in response to a low pheromone to food ratio, which induces dauer exit (Ren et al., 1996; Schackwitz et al., 1996). The *ilp* gene *daf-28* is also downregulated in ASI, as well as in ASJ, upon treatment with the pheromone mixture and in response to starvation (Figure 4.2) and the induction of the dauer program through genetic manipulations (Li et al., 2003). In addition, *daf-28* expression is upregulated by *daf-7,* which also stimulates its own expression, in an autocrine feedback loop (Li et al., 2003). On the other hand, transcription of *ins-1*, which promotes dauer entry and inhibits dauer exit and is expressed in both ASI and ASJ (Kodama et al., 2006; Tomioka et al., 2006), does not change in response to starvation, high temperature or pheromones (Table 1). However, *ins-1* might be regulated by these same sensory cues at a posttranscriptional level.

Interestingly, the *ilp* gene *ins-6*, whose expression is restricted to ASI in reproductively growing worms, shifts its expression to ASJ after a strong dauer arrest (Figures 5 and 4.2; Table 1). The dauer pheromone mixture downregulates *ins-6* in ASI, so that *ins-6* is largely depleted in the ASI neurons of dauers and post-dauer adults (Figure 5; Table 1). Yet, the pheromone cue alone does not induce the switch to the ASJ

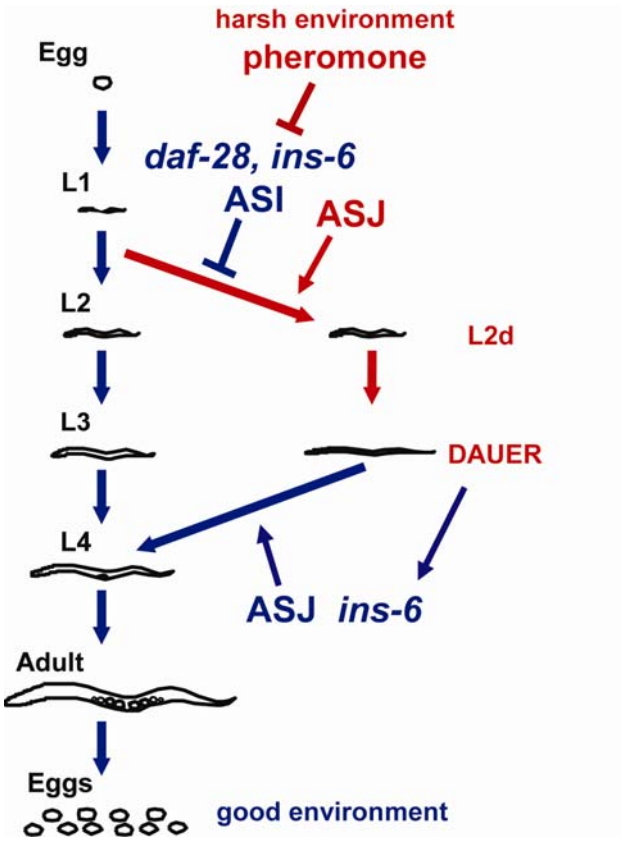

Figure 4.2. *ins-6* and *daf-28* act from ASI and ASJ to regulate dauer arrest. The pheromone cue downregulates *daf-28* and *ins-6* in ASI to allow for dauer entry, and, together with the dauer program, upregulates *ins-6* in ASJ, to prepare for dauer exit.

neurons in non-dauers (Table 1). Surprisingly, dauer arrest that is triggered by genetically reduced levels of *daf-2* activity under conditions of low pheromone to food ratio is also insufficient to induce *ins-6* expression fully in ASJ (Table 1). Indeed, *ins-6* expression is only upregulated in the ASJ neurons by the coordinated activities of the pheromone cues and the dauer program (Table 1). Thus, these observations show that specific cues determine the *ilp* genes that are transcribed, their level of expression, and presumably, the peptides secreted from specific neurons.

4.2.2 Insulin-like peptides might function hormonally or locally within neural circuits

Following secretion, the ILPs might either act as hormones on their distant target tissues or signal locally within neuronal circuits to specific target neurons, which might in turn secrete other ILPs or other signals to act on downstream tissues. An example of an ILP that can act as part of a neural circuit is *ins-1* (Tomioka et al., 2006). *ins-1* regulates salt chemotaxis learning from an interneuron, which receives direct synaptic inputs from sensory neurons and sends synaptic outputs to other neurons that are part of a circuit (Tomioka et al., 2006). On the other hand, other processes might require ILPs to act like hormones. Thus, depending on the stability and range of the specific ILP, it might induce local or wide-spread activation of the DAF-2 pathway.

The switch in *ins-6* expression to ASJ (Figures 5 and 4.2; Table 1), together with the function of *ins-6* and the ASJ neurons in dauer exit [Figure 2; Table S2; (Bargmann and Horvitz 1991a)], suggest that *ins-6* is secreted from ASJ to induce dauer exit. This might also suggest that *ins-6* acts locally as part of one circuit that induces dauer exit versus another that inhibits dauer entry. However, *ins-6* expression in either ASI or ASJ can induce dauer exit (Figure S2; Table S2), although this might be due to the fact that under the conditions of these particular experiments *ins-6* is expressed at higher than endogenous levels. Nonetheless, dauers that are induced through reduced DAF-2 activity at low pheromone levels, which reflect a weaker form of arrest, are still capable of exiting from the dauer stage (Figures 2 and II), when *ins-6* remains primarily expressed in ASI and does not completely switch to ASJ (Table 1). Not surprisingly, *ins-6* can still

induce exit after ablation of the ASJ neurons in these dauers (Figure II, Table III), which would suggest that *ins-6* can act from ASI to promote exit after a weak dauer arrest.

In contrast, dauers that are induced at high pheromone levels, which reflect a stronger form of arrest as these dauers do not easily exit, have almost no *ins-6* expression in ASI and a very strong *ins-6* expression in ASJ (Table 1). Thus, under these conditions *ins-6* might act from ASJ to promote exit, a hypothesis that remains to be tested. Such a hypothesis also raises the possibility that the circuit that induces exit after a weak arrest is different from the circuit that induces exit after a stronger arrest and that *ins-6* acts as part of these distinct circuits.

It is possible that *ins-6* becomes specifically upregulated in ASJ, because ASJ has the receptors that receive not only the inputs that indicate a strong dauer arrest but also the inputs that induce exit after such an arrest. Moreover, because the upregulation of *ins-6* expression in the ASJ neurons of arrested dauers does not immediately induce exit, this suggests that *ins-6* is likely further regulated posttranscriptionally in response to the actual dauer-exit inducing signals. This posttranscriptional regulation of *ins-6* might be at the level of translation and/or secretion.

Thus, the neurons that regulate dauer entry and exit might act combinatorially, whether by integrating outputs into a common downstream circuit, or by producing local or more organism-wide gradients of specific dauer-regulating signals, which would include ILPs like *ins-6*.

4.3 The impact of dauer arrest on adult physiology

Dauer arrest can be induced by different means (Cassada and Russell, 1975; Golden and Riddle, 1982): through overcrowding, which leads to high levels of pheromones in the presence of low food availability; by the direct addition of high concentrations of pheromone, while there is plenty of food; or by genetic manipulations, such as using temperature-sensitive *daf-2* mutations, in which dauer arrest can be induced even on high food and low pheromone levels. Hence, this suggests that the pheromone cues can overcome the food signals. On the other hand this also shows that lowered DAF-2 signaling mimics the perception of dauer-inducing cues, because decreased DAF-2 activity is likely a consequence of sensing increased pheromone and decreased food in the environment.

The pheromone cues induce specific expression changes in particular neurons: *daf-7* and *ins-6* are specifically downregulated in ASI; and *daf-28*, in ASI and ASJ; (Li et al., 2003; Ren et al., 1996; Figure 5 and Table 1). Interestingly, the pheromones and the dauer program together upregulate *ins-6* in ASJ, a change in expression that persists in adults (Figure 5; Tables 1 and 4.2). This suggests that post-dauer adults are different from post-L3 adults that never went through the dauer stage. Since the switch in *ins-6* expression perdures up to five-day-old adults, *ins-6* might also have a different function in adults from ASJ compared to its function from ASI. Thus, *ins-6* in ASJ might lead not only to dauer exit but also to changes in adult behavior and physiology.

The dauer arrest that is induced by a high pheromone to food ratio differs from the dauer arrest that is induced in the *daf-2(e1368)* mutant at a low pheromone to food

ratio. *ins-6* expression in the latter dauers is not downregulated in ASI and is only incompletely upregulated in ASJ (Table 1). This observation suggests that the transcriptional programs of dauers induced by different means might also differ, and that these differences might also perdure into adulthood.

Transient dauer arrest causes a slight increase in adult lifespan, as demonstrated by the lifespan of *daf-28* or *ins-6*; *daf-28* mutant post-dauers (Figures 6 and III; Table IV) and of adults that exited from dauer arrest induced by a high pheromone to food ratio [(Hall et al., 2010); P. Fardel, data not shown]. Because there are different classes of dauers, *e.g.*, a weak versus a strong form of arrest, the dauer program might also have different effects on lifespan or even other aspects of adult phsyiology. A weak arrest that does not induce the full dauer program might have little or no effect on lifespan, whereas a stronger arrest that induces the full program might have a more significant effect. Another factor one might consider is the duration of the dauer arrest. For instance, a longer time period spent in the dauer stage leads to elevated levels of reproductive defects and decreased brood sizes in post-dauer adults (Kim and Paik, 2008). However, it remains to be investigated whether the duration of dauer arrest positively correlates with longevity. These differences between post-dauer adults and adults that developed under a replete environment might be part of a system that remembers developmental conditions, which consequently leads to adaptive changes in adult behaviors and physiology.

4.4 Insulin-like signaling in the nervous system of other species

The insulin-like signaling module downstream of the DAF-2 receptor is conserved between species, and the processes controlled by insulin-like signaling in different species are also largely overlapping. In *Drosophila* and higher organisms, insulin-like signaling controls metabolism, growth and development, reproduction, stress resistance and longevity (reviewed by Kenyon, 2005). ILPs have also been shown to act from the nervous system of *Drosophila* and mammals (Ayer-le Lievre et al., 1991; Brogiolo et al., 2001; Bathgate et al., 2002; Ikeya et al., 2002; Rulifson et al.,2002; Sherwood, 2004; Liu and Lovenberg, 2008; Yang et al., 2008; Meyts et al., 2009).

In *Drosophila*, there are seven known ILPs, *dilp1* to *dilp7*, which function in the regulation of body size, growth, stress response, metabolism and longevity (Tatar et al. 2001; Garofalo 2002; Rulifson et al., 2002; Rhodes and White, 2002; Oldham and Hafen 2003; Broughton et al., 2005). The *Drosophila ilp* genes are expressed in different neuronal and non-neuronal cells (Ikeya et al., 2002; Rulifson et al., 2002; Yang et al., 2008; Slaidina et al., 2009). Four of the *ilp* genes, *dilp1* to *dilp3* and *dilp5*, are expressed in the head in a group of neurosecretory cells, which are termed insulin-producing cells (Brogiolo et al., 2001; Bathgate et al., 2002; Ikeya et al., 2002; Rulifson et al.,2002). The activities of these neurons might be regulated indirectly by gustatory inputs (Scott et al., 2001; Rulifson et al., 2002; Melcher and Pankratz, 2005; Yang et al., 2008). *dilp2*, *dilp3* and *dilp5* are thought to regulate insulin-like signaling in response to nutrient availability (Ikeya et al., 2002; Broughton et al., 2008; Zhang et al., 2009), whereas *dilp7* regulates

the fly's egg-laying decision in response to food cues (Yang et al., 2008). Thus, *Drosophila* ILPs may also have distinct functions in the sensory regulation of specific processes.

The mammalian ILP superfamily is comprised of at least seven members (Nef and Parada, 2000). Besides insulin and the insulin-like growth factors IGF-I and IGF-II, mammals have several so-called relaxins, which belong to the insulin-like family based on their tertiary structures (reviewed by Sherwood, 2004). However, the sequence identities of relaxins to the human insulin are low. All mammalian ILPs are derived of prohormones with a signal peptide, B- and A-chains, and an interjacent C-chain, which is sometimes removed. The seven human relaxins are relaxin 1-3, the Leydig cell insulin-like peptide (INSL3), the early placenta insulin-like peptide (INSL4), and the insulin-like factors INSL5 and INSL6 (Sherwood 2004). Four of these have been shown to bind specific GPCRs rather than insulin-like receptors (De Meyts et al., 2009). The mammalian *ilp* genes are expressed in neuronal and non-neuronal tissues (Ayer-le Lievre et al., 1991; Liu and Lovenberg, 2008; De Meyts et al., 2009). While the functions of insulin, IGF-I and IGF-II have been studied in detail (Nakae et al., 2001; Sherwood, 2004; Kenyon, 2005), less is known about the mammalian relaxins. So far, they have been mainly implicated in reproductive processes, but they may also function in the nervous system based on their expression patterns (Sherwood, 2004).

Since insulin-like signaling has been discovered to inhibit *C. elegans* longevity, several studies showed similar effects of insulin-like signaling on mammalian lifespan (Kenyon et al., 1993; Gems and Partridge, 2001; Wolkow, 2002; Bluher et al., 2003; Taguchi et al., 2007). For example, female mice that are heterozygous for the IGF-I

deletion live significantly longer than wild-type females, and have little or no change in their metabolism and fertility (Holzenberger et al., 2003). In addition, the growth-hormone receptor knockout mice (Laron dwarf mice) live longer than wild type, likely also because of their reduced IGF-1 levels (Coschigano et al., 2000). However, although insulin-like signaling is important for the growth and survival of neurons (reviewed by Aleman and Torres-Aleman, 2009; Broughton and Partridge, 2009; Mattson and Wan, 2008) the possibility of a sensory component to the ILP regulation of lifespan and/or other processes in mammals remains to be investigated. Thus, further investigations might reveal additional functions for ILPs, which might also play a role in encoding sensory information to regulate physiology in higher organisms.

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03/2008	**Talk – European Worm Meeting 2008,** Carmona, Spain
06/2007	**Poster – 16th International *C. elegans* Meeting,** Los Angeles, USA
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8. Appendix

The thioredoxin TRX-1 modulates the activity of the insulin signaling pathway to regulate dauer formation in *C. elegans*.

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Running title: Involvement of TRX-1 on dauer formation in *C. elegans*.

Key words: *C. elegans*, thioredoxin, dauer, insulin, stress-resistance, aging.

ABSTRACT

Thioredoxins comprise a conserved family of redox regulators involved in many biological processes, such as stress-resistance and aging. We report that the *C. elegans* thioredoxin TRX-1 acts in a pair of head sensory neurons called ASJ, as a novel negative regulator of the insulin signaling pathway in a redox-independent manner. We show that increased formation of the stress-resistant, long-lived dauer larva in mutants for the gene encoding the insulin-like peptide DAF-28 requires TRX-1 acting upstream of the insulin receptor DAF-2, in the ASJ neurons. During dauer development, transcriptional green fluorescent reporters of *trx-1* and *daf-28* showed an opposing expression pattern, not reproduced in normally growing L2/L3 larvae. These findings, together with the requirement of *trx-1(+)* for *daf-28* down-regulation during dauer development, demonstrate that TRX-1 antagonizes DAF-28 activity and contributes to the regulation of *daf-28* production during the dauer stage. We propose that TRX-1 acts as a neuronal signaling mediator that fluctuates within the ASJ neurons to monitor the adjustment of DAF-28 production during adverse conditions at early stages of development and thus promote stress-resistance and long-life.