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COMMENTARY

L1 arrest, *daf-16*/FoxO and nonautonomous control of post-embryonic development

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ABSTRACT

Post-embryonic development is governed by nutrient availability. L1 arrest, dauer formation and aging illustrate how starvation, anticipation of starvation and caloric restriction have profound influence on *C. elegans* development, respectively. Insulin-like signaling through the Forkhead box O transcription factor *daf-16*/FoxO regulates each of these processes. We recently reported that *ins-4*, *ins-6* and *daf-28* promote L1 development from the intestine and chemosensory neurons, similar to their role in dauer development. *daf-16* functions cell-nonautonomously in regulation of L1 arrest, dauer development and aging. Discrepancies in *daf-16* sites of action have been reported in each context, but the consensus implicates epidermis, intestine and nervous system. We suggest technical limitations of the experimental approach responsible for discrepant results. Steroid hormone signaling through *daf-12*/NHR is known to function downstream of *daf-16* in control of dauer development, but signaling pathways mediating cell-nonautonomous effects of *daf-16* in aging and L1 arrest had not been identified. We recently showed that *daf-16* promotes L1 arrest by inhibiting *daf-12*/NHR and *dbl-1*/TGF-β Sma/Mab signaling, two pathways that promote L1 development in fed larvae. We will review these results on L1 arrest and speculate on why there are so many signals and signaling centers regulating post-embryonic development.

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Introduction

Post-embryonic development is sensitive to nutrient availability. Upon hatching, L1-stage larvae of the nematode *C. elegans* delay development in a state called L1 arrest (or L1 diapause) until food is available (reviewed in[4]). L1 arrest ensures that post-embryonic development is not initiated without adequate nutrition, and arrested larvae adapt physiologically in order to prolong survival of starvation. Larvae that hatch with limited food available develop relatively slowly, and if they experience sufficient population density they enter a true developmental diapause in the third larval stage known as dauer arrest.[1,43] Larvae assess population density via a pheromone cocktail,[23] which is used as a cue along with dwindling food supply in anticipation of starvation. In addition to affecting the rate of post-embryonic development and dauer arrest, nutrient availability influences adult size, fecundity and aging,[14,15,18] with the increase in lifespan caused by caloric restriction being most renowned. These effects of nutrient availability on post-embryonic development raise the question of how the worm senses and responds to environmental conditions in order to alter developmental physiology. This commentary addresses this question with an emphasis on recent work investigating L1 arrest.

Insulin-like signaling is sensitive to nutrient availability, and it regulates L1 arrest, dauer formation and aging.[32] The sole insulin-like receptor *daf-2*/InsR is activated in response to food, promoting larval development, bypass of dauer formation and aging. Mutations disrupting *daf-2*/InsR function cause constitutive L1 arrest in fed larvae and increased survival in starved larvae.[6,12,31] *daf-2* signals through a conserved phosphoinositide 3-kinase (PI3K) pathway to antagonize activity of the transcription factor *daf-16*/FoxO.[21,35] Mutations disrupting *daf-16* function cause an L1 arrest-defective phenotype and decreased survival in starved larvae.[6,31] Each of the somatic developmental events that occur during normal L1 development in fed

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larvae (i.e., cell divisions, migrations and fusions) occurs in starved *daf-16* mutants in the same chronological order but at a slower rate[6] *daf-16* is also required for starvation-induced developmental arrest in L3 and L4 larvae, affecting somatic development of the entire animal as in L1 arrest.[40] These global phenotypic effects indicate that *daf-16* provides extensive permissive control of larval development.

ins-4, ins-6 *and* daf-28 *regulate L1 arrest*

Despite *daf-2* being the only known insulin-like receptor in the worm, the genome encodes 40 insulin-like peptides (ILPs).[36] Humans are thought to have nine ILPs, eight have been discovered in Drosophila, and the silkmoth B. mori has 39.[8,22,30] Although the worm is not alone in having a large ILP family, it is unclear why there are so many ILPs and how specificity is achieved with a single receptor. Despite clear examples of overlapping function, a variety of studies have provided evidence for functional specificity as well. Disruption of specific peptides affects aging, dauer formation and germline proliferation.[9,16,19,26,27,29,34] Functional specificity was confirmed in a comprehensive analysis of ILPs.[10] We showed that ILPs have dynamic, nutrient-dependent expression throughout the life cycle and during the L1 stage[5,7] and reporter gene analysis revealed distinct anatomical expression patterns.[38] Genetic analysis suggests that ILPs act as agonists or antagonists of *daf-2*/InsR.[9,26,27,36] Expression analysis reveals significant correlation between function as an agonist or antagonist and positive or negative transcriptional regulation in response to feeding.[7] Such correlation suggests that candidate agonists and antagonists can be identified by relative expression in fed and starved worms.

We identified ten candidate agonists in L1 larvae based on expression in an effort to identify the ILPs that promote L1 development in response to feeding.[7] Deletion of candidate agonists is expected to weakly phenocopy disruption of *daf-2*/InsR, delaying L1 development and increasing starvation survival. Simultaneous deletion of the candidate agonists *ins-4* and *daf-28* increased starvation survival, but development was not detectably delayed.[7] Deletion of either gene alone did not cause an observable phenotype, reflecting functional redundancy. Overexpression of candidate agonists *ins-4*, *ins-6* or *daf-28* alone phenocopied loss of *daf-16*/FoxO, causing a decrease in starvation survival and an arrest-defective phenotype. Together these results suggest that *ins-4*, *ins-6* and *daf-28* function in concert to promote L1 development in response to feeding.[7] These ILPs have not been implicated in regulation of aging, but they do contribute to regulation of dauer formation and recovery along with additional ILPs.[9] Furthermore, *ins-4*, *ins-6* and *daf-28* are expressed in ASI and ASJ chemosensory neurons, as well as other neurons, during L1 arrest, and they are transcriptionally up-regulated in the intestine in response to feeding (Fig. 1). These ILPs are expressed in the same sites in late L2 and L3 larvae, when they function to regulate dauer development.[9,16,19] Expression in these sites suggests that sensory neurons and the intestine directly sense nutrient availability, comprising critical signaling centers in developmental control of the entire animal. We speculate that sensory neurons, where agonistic ILPs are expressed even in starved L1 larvae, sense external nutrient status and respond by controlling secretion (and possibly transcription and translation) of agonistic ILPs, providing a rapid regulatory input on short time scales. In contrast, we speculate that the intestine, the entire endoderm of the animal and a site of fat storage, senses internal nutrient status and responds by controlling transcription (and likely translation and secretion) of agonistic ILPs, providing a slower regulatory input on longer time scales (Fig. 1).

Cell-nonautonomy of daf-16/*FoxO*

ILPs are secreted into the body cavity where they are thought to function as hormones, but they do not directly affect all of the tissues under their regulation. That is, the insulin-like signaling pathway, comprised by *daf-2*/InsR, the PI3K pathway and *daf-16*/FoxO, functions cell-nonautonomously to regulate L1 arrest, dauer development and aging.[3,11,16,17,20,42,43] Using transgenes with tissue-specific heterologous promoters to complement mutations affecting *daf-16*, we found that *daf-16* functions in the nervous system, epidermis and intestine to regulate L1 arrest.[17] Using a similar approach to complement mutations affecting *daf-16*, *daf-2* or *age-1*/PI3K, the intestine and nervous system were identified as key sites of action for aging and dauer formation.[16,17,20,42] (Table 1). The epidermis is also a key site of action for aging,[44] as well as L1 arrest and starvation-induced arrest in L3 and L4 larvae,[11,17,40] but the role of the epidermis has not been reported for dauer formation. Taken together, these

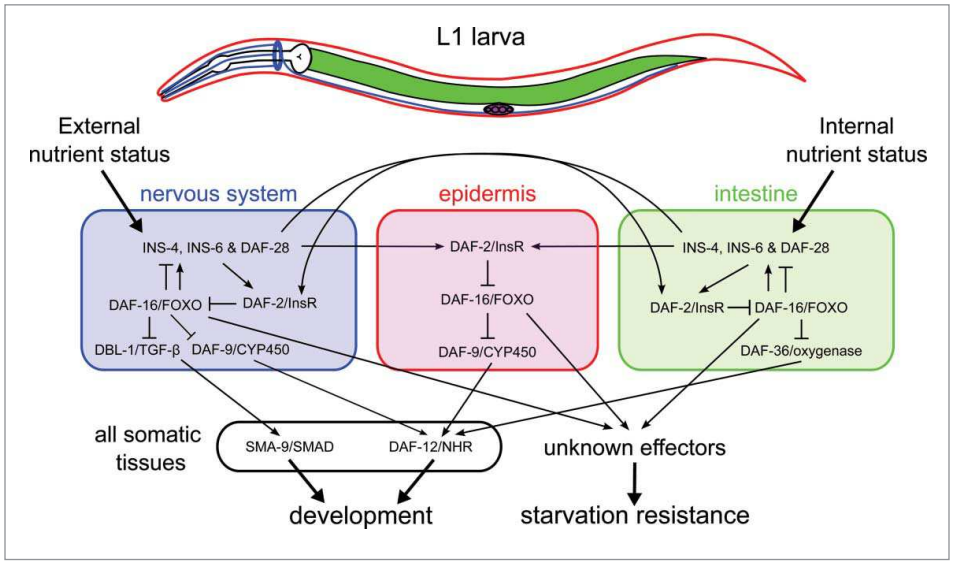

Figure 1. A model of the regulatory network governing L1 development in response to nutrient availability. The gross anatomy of an L1 larva is depicted, with the nervous system, epidermis and intestine presented in different colors. Chemosensory and other neurons sense external nutrient status and regulate secretion of ILPs *ins-4, ins-6* and *daf-28*. Intestinal cells autonomously sense internal nutrient status and regulate expression and secretion of *ins-4, ins-6* and *daf-28*. These three ILPs, and possibly others, signal to *daf-2*/InsR in the nervous system, intestine and epidermis. *daf-2* signals through the PI3K pathway to antagonize *daf-16*/FoxO in each of those tissues. *daf-16* inhibits *daf-12*/NHR and *dbl-1*/TGF-β signaling by repressing, directly or indirectly, transcription of different components of each pathway in different tissues. Signaling activity may also be regulated post-transcriptionally. *daf-12*/NHR and *dbl-1*/TGF-β signaling promote L1 development in fed larvae, and their inhibition by *daf-16* promotes L1 arrest. *daf-16* also promotes starvation resistance, but it does so through other, unidentified effector genes. *daf-16* also activates and represses transcription of different ILPs, producing positive and negative feedback. This feedback, or FOXO-to-FOXO signaling, couples the state of the insulin-like signaling pathway within and between tissues and potentially contributes to phenotypic variation among individuals.

results implicate the nervous system, intestine and epidermis in regulation of post-embryonic development.

The relative importance ascribed to different anatomical sites has varied between publications for aging, dauer formation and L1 arrest. The differences are mostly quantitative, but some tissues have no effect in one study and a prominent effect in another (see Table 1 and the Discussion of ref.[17] for a more complete review). We believe these discrepancies reflect shortcomings of the transgene-based approach used in these studies. Complementation of positive and negative regulators of the same pathway (e.g., *daf-2* or *age-1* as opposed to *daf-18* or *daf-16*) need not give the same results, as there may be differences in where pathway activity is necessary as

Table 1. A summary of studies investigating sites of action of insulin-like signaling in regulation of post-embryonic development reveals discrepancies but collectively implicates the nervous system, intestine and epidermis. The phenotype assayed is indicated along with the sites of action identified, the protein expressed, the genetic background and the citation. Epidermal function has not been addressed for dauer formation. Abbreviations: gf, gain of function; lf, loss of function; -, null mutant.

Phenotype	Site of Action	Protein Expressed	Promoters	Background	Citation
Aging	neuronal	AGE-1	*unc-14, ges-1*	*age-1(-)*	42
	strong neuronal, weak intestinal	DAF-2	*unc-14, unc-119, ges-1*	*daf-2(lf)*	42
	strong intestinal weak neuronal	GFP::DAF-16a	*unc-119, ges-1, myo-1*	*daf-16(-); daf-2(lf)*	20
	epidermal intestinal	GFP::DAF-16a	*lin-26, ges-1*	*daf-16(-); daf-2(lf)*	44
Dauer	neuronal	GFP::DAF-16a	*unc-119, ges-1, myo-1*	*daf-16(-); daf-2(lf)*	20
	intestinal	DAF-2	*rgef-1, ges-1, myo-3*	*daf-2(lf)*	16
	intestinal	GFP::DAF-16a	*rgef-1, ges-1, myo-3*	*daf-16(-); daf-2(lf)*	16
L1 arrest	strong epidermal weak neuronal, weak intestinal	DAF-18	*dpy-7, rgef-1, myo-3, ges-1*	*daf-18(-)*	11
	epidermal	AKT-1(gf)::GFP	*dpy-7, rgef-1, pgp-1*	wild type	11
	epidermal intestinal neuronal	GFP::DAF-16a	*unc-119, myo-3, ges-1, col-12*	*daf-16(-)*	17
L3/L4 arrest	epidermal	GFP::DAF-16a	*unc-119, unc-115, myo-3, ges-1, col-12*	*daf-16(-)*	40
Starvation survival	epidermal intestinal neuronal	GFP::DAF-16a	*unc-119, myo-3, ges-1, col-12*	*daf-16(-)*	17

opposed to sufficient to alter phenotype.[20,42] Moreover, mutations are rescued by a high-copy transgene driven by a heterologous promoter. Thus, gene expression levels are not native or well controlled. In addition, the effects of site of expression and the promoter used to drive expression are confounded, and in some cases different promoters were used in different studies for the same tissue. Furthermore, there is substantial allelic variation among transgenic lines obtained from a single transformation, with some lines displaying highly penetrant rescue and others no rescue.[17,20] This variation raises the possibility of being misled by analyzing one or a small number of independent alleles, possibly explaining some discrepancies among published results. Taken together, we believe these caveats are sufficient to account for the discrepant results reported for sites of action for the insulin-like signaling pathway. Experimentalists and reviewers should generally be more critical of this approach to identifying sites of action and the conclusions drawn from it.

daf-16/*FoxO inhibits* daf-12/*NHR and* dbl-1/*TGF-β signaling*

daf-16/FoxO cell-nonautonomy suggests that the transcription factor regulates one or more signaling pathways to exert its systemic effects. Genetic epistasis analysis suggests that the nuclear hormone receptor *daf-12*/NHR functions downstream of insulin-like signaling in regulation of dauer development,[37] but downstream signaling pathways for regulation of aging have not been identified. We used mRNA-seq to identify genes whose expression is affected by mutation of *daf-16* in starved L1 larvae. We found that *daf-12*/NHR, an additional component of the steroid hormone signaling pathway (*daf-36*) and the Sma/Mab gene *dbl-1*/TGF-β were each expressed at higher levels in the mutant, suggesting that *daf-16* activity leads to their transcriptional repression. Genetic epistasis analysis suggests that *dbl-1*/TGF-β and *daf-12*/NHR steroid hormone signaling function downstream of *daf-16* in regulation of L1 arrest (Fig. 1).[17] We found that these two pathways promote development in fed L1 larvae, and we conclude that their inhibition by *daf-16* during starvation promotes developmental arrest. Notably, *dbl-1*/TGF-β and *daf-12*/NHR steroid hormone signaling are not epistatic to *daf-16* for starvation resistance, an additional aspect of L1 arrest affected by insulin-like signaling. This result implies that *daf-16* regulates starvation resistance through other, unidentified effectors, and it reveals that *daf-16* mutants do not die rapidly during starvation as a consequence of inappropriate development.

Expression patterns of key components of the *daf-12*/NHR and *dbl-1*/TGF-β pathways overlap with *daf-16*/FoxO sites of action, suggesting why *daf-16* function in any one of these sites is sufficient to promote developmental arrest. *dbl-1*/TGF-β is expressed in neurons while *daf-36*, a Rieske oxygenase in the *daf-12*/NHR steroid hormone pathway, is expressed in the intestine.[39,41] Another component of the *daf-12*/NHR steroid hormone pathway, *daf-9*/CYP450, is expressed in neurons and the epidermis.[13] It follows that *daf-16* activity can potentially repress *dbl-1* and *daf-9* in neurons, *daf-9* in the epidermis, and *daf-36* in the intestine. However, we have not determined if DAF-16 regulates these genes directly or indirectly, and it is unclear if they are the terminal regulators of cell division, migration and fusion downstream of insulin-like signaling. Nonetheless, we believe that such widely distributed regulation of these genes enables insulin-like signaling to robustly control *daf-12*/NHR steroid hormone and *dbl-1*/TGF-β signaling in response to nutrient availability.

Why so many signals and signaling centers?

Why are so many signals and signaling centers involved in regulation of L1 arrest and post-embryonic development in general? Before speculating on potentially adaptive features of this regulatory network, it is worth noting that various aspects of the network could be somewhat arbitrary, stemming from developmental systems drift or other neutral processes[24], b). Nevertheless, it is attractive to speculate that network complexity increases robustness of the system, allowing the rate of development to be coordinated among tissues and across the whole animal. Such complexity may also allow for integration of diverse regulatory inputs. Specifically, *daf-12*/NHR and *dbl-1*/TGF-β signaling are sensitive to more than just insulin-like signaling, and insulin-like signaling is sensitive to more than just nutrient availability. We suggest that chemosensory neurons assess external nutrient status to control insulin secretion on a short time scale and that the intestine assesses internal nutrient status to control secretion on a long time scale (Fig. 1). *daf-2*/InsR and the PI3K pathway can then integrate these different inputs. Indeed, insulin-like signaling has been

shown to mediate neuron-intestine communication in regulation of dauer development.[16] Along these lines, systemic and cell-autonomous signals must also be integrated to ensure appropriate coordination of physiological state across the animal.

Even with these tentative explanations for network complexity, it remains striking that there are so many ILPs in *C. elegans*. There clearly is a degree of functional overlap among various ILPs, and it has been suggested that gene dosage can evolve through duplication to affect regulation of ecologically relevant traits.[28] This could be true insofar as the ILPs are functionally redundant, but specific mutant phenotypes continue to be discovered for individual ILPs. We propose that the large number of ILPs actually facilitates integration of diverse regulatory inputs and coordination of responses across the animal. Insulin-like signaling actually regulates expression of ILPs, producing positive and negative feedback, or FOXO-to-FOXO signaling[10,26,33,34,38] The physiological significance of FOXO-to-FOXO signaling is unclear, but it has been suggested to contribute to metabolic homeostasis.[2] Feedback regulation may reflect paracrine function of the ILPs, rather than systemic function as hormones. Local signaling and feedback could be used to relay signaling between tissues, integrating signals from different cells and environmental cues in the process. Such a model suggests that specific perturbations to the insulin-signaling network will alter spatiotemporal response dynamics, disrupting coherence of the worm's physiological state. That is, disruption of individual feedback loops should uncouple tissues so that their response to fluctuations in environmental conditions is uncoordinated. In addition, feedback regulation could contribute to variation in physiological state among individual worms. The combination of positive and negative feedback could enable individuals to establish different set points in metabolism and growth rate in response to continuous differences in nutrient availability. Furthermore, feedback could promote or maintain distinct stable physiological states resulting from stochastic differences between individuals in the same conditions. Such inter-individual phenotypic variation could support an evolutionary bet-hedging strategy to optimize fitness in uncertain conditions. In this case, perturbations to the insulin-signaling network should alter phenotypic variation among individuals.

Conclusions

L1 arrest provides a powerful model for nutritional control of development that complements research on dauer development and aging. Investigating the role of *daf-16*/FoxO in each context enriches understanding of how these developmental processes relate to each other. As a more complete view of the regulatory network controlling post-embryonic development emerges, it is clear that it is comprised of many different signals and signaling centers. There is no reason to think that such complexity is worm-specific. To the contrary, the tractability of the worm is bringing the complexity to light, and we expect *C. elegans* research to continue at the forefront of the field.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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