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Should I stay or should I go? Identification of novel nutritionally regulated developmental checkpoints in *C. elegans*

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After embryogenesis, developing organisms typically secure their own nutrients to enable further growth. The fitness of an organism depends on developing when food is abundant and slowing or stopping development during periods of scarcity. Although several key pathways that link nutrition with development have been identified, a mechanistic understanding of how these pathways coordinate growth with nutritional conditions is lacking. We took advantage of the stereotyped development and experimental accessibility of *C. elegans* to study nutritional control of late larval development. We discovered that *C. elegans* larval development is punctuated by precisely time checkpoints that globally arrest growth when nutritional conditions are unfavorable. Arrest at the checkpoints is regulated by insulin- and insulin-like signaling and steroid hormone signaling. These pathways are conserved in mammals, suggesting that similar mechanisms could regulate growth and development in humans. We highlight several implications of our research, including quiescence of diverse cellular behaviors as an adaptive response to unfavorable growth conditions, the existence of oscillatory checkpoints that coordinate development across tissues, and the connections between systemic and cell-autonomous regulators of nutritional response. Together, our findings describe a fascinating developmental strategy in *C. elegans* that we expect will not only provide insight into nutritional regulation of development, but also into poorly understood cellular processes such as quiescence and aging.

Keywords: developmental checkpoints, insulin-like signaling, nutrition, oscillations, quiescence, starvation, steroid hormones

Abbreviations: IIS, insulin- and insulin-like signaling; VPC, vulval precursor cell; ILP, insulin-like peptide; dsRNA, double-stranded RNA; DA, dafachronic acid; NHR, nuclear hormone receptor.

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Introduction

Development is an energy-intensive process that requires a sufficient amount of nutrients to sustain growth. Developing organisms in the wild that obtain their nutrients through feeding often encounter a range of dietary conditions, including periods of starvation. Prolonged nutrient deprivation necessitates adaptive responses to ensure that limited resources are conserved for essential survival functions. One adaptation that organisms make in response to starvation is slowing or arresting development.[1,2] Any change in progression through development must occur in a synchronous manner so that an organism maintains the coordination of tissue development necessary for organ function.[3] The response to starvation therefore is controlled both at the level of the whole organism to orchestrate a systemic response, and at the level of individual cells to modulate cellular processes. Research conducted in *C. elegans*, insects, and mouse has uncovered key pathways that connect nutritional conditions with growth and development. These include insulin and insulin-like signaling (IIS) and steroid hormone signaling.[1,4,5] The conservation of these pathways in animals highlights the evolutionary importance of responding to changes in nutritional conditions with commensurate changes in development.

The rapid development of *C. elegans* makes it a particularly amenable model to study how nutrition affects development. Following hatching, *C. elegans* progresses through four larval stages (L1–L4) to adulthood over a period of about 2 d.

Growth arrest occurs when nutritional conditions are unfavorable. The best characterized arrests occur early in the L1 stage when larvae hatch in the absence of food,[1] and in the dauer diapause, an alternative developmental stage that initiates late in the L1 stage in response to adverse environmental factors, including low nutritional availability.[6] In contrast to early larval development, the effects of nutrient deprivation in late larval development are poorly understood. During the L3 and L4 stages there is rapid growth of the tissues that form the reproductive system. This growth encompasses a wide range of cellular processes, including division, migration, invasion, and cell-cell fusion.[7–9] Studying the effects of starvation in the L3 and L4 stages therefore offers a way to connect nutritional conditions to diverse aspects of larval growth and tissue morphogenesis.

We used the extensive tissue development that occurs in the L3 and L4 stages to assess the *C. elegans* response to starvation during late larval development.[10] We found that checkpoints are present early in each larval stage that arrest tissue development throughout the organism in response to adverse nutritional conditions. Arrest only occurs at these checkpoints, as bypassing one checkpoint causes progression through the larval stage the next checkpoint. Arrest at the checkpoints is regulated by the IIS pathway, which functions upstream of steroid hormone signaling. Taken together with L1 arrest and dauer formation, our studies reveal that *C. elegans* undergo adaptive arrest at specific, regularly timed checkpoints throughout larval development. Furthermore, the identification of these checkpoints provides an experimental model to understand several interesting aspects of nutritional response mechanisms that we expect are conserved in animals and important in human diseases and aging. These include the quiescence and activation of diverse cellular processes, the synchronization of growth rates throughout the organism, and the integration of systemic and cell-autonomous growth control pathways.

Identification of Developmental Checkpoints in the L3 and L4 Larval Stages

To assess the response to nutritional conditions during late larval development, we first examined the effects of starvation on the formation of the hermaphrodite vulva. The vulva derives from three vulval precursor cells (VPCs) that undergo three rounds of cell division during the L3 stage. During these divisions, the VPCs initiate a morphogenetic program that includes migration, invagination, cell-cell fusion, and lumen formation[8] (**Fig. 1A**). The steps of vulval formation are easily observed in live animals, providing a way to rapidly assess the developmental response to nutrient removal at the level of an individual tissue. To accomplish this, we grew a synchronized population of wild type animals to the late L2 stage, about 5 h prior to the first VPC divisions, and then completely removed these animals from food. We next examined progression through the stages of vulval development over an extended period of starvation. We found that all animals molted into the L3 stage and arrested prior to VPC divisions, with no further progression through the L3 stage observable even after 10 d starvation. This result suggested that developmental arrest occurred in a precise and invariant manner following removal from food. This hypothesis was validated in a second experiment in which animals were removed from food in the mid L3 stage, after the first VPC divisions. We found that animals continued development through the L3 stage, molted into the L4 stage, and then arrested early in L4 after completion of all 3 rounds of VPC divisions. In a final experiment, animals were removed from food in the L4 stage, and were found to continue development through L4 and arrest in young adulthood. Arrest occurred only at these specific times in vulval development (**Fig. 1A**). Our work shows that arrest in vulval formation is not variable, but instead involves precisely timed checkpoints that stop tissue development when nutritional conditions are inadequate.

The timing of arrest early in the L3 and L4 stages suggested a relationship between nutritional conditions and the molting cycle, the oscillatory pattern of cuticle synthesis and shedding that occurs in each larval stage. By examining a destabilized GFP reporter for *mlt-10*, a gene required for proper execution of the molt,[11] we determined that arrest occurs after ecdysis (cuticle shedding, the final step of molting), and prior to the onset of cuticle synthesis in the subsequent larval stage (**Fig. 1B**). Therefore, similar to vulval development, the molting cycle also arrests in a precise manner early in the larval stage.

Multiple Cell Types Enter Quiescence in Response to Nutrient Removal

In addition to vulval development and the molting cycle, we examined other developmental events that take place in the L3 and L4 stage, and found that the timing of arrest was systemic in nature. Similar to the VPCs, myoblasts and germ cell precursors also arrested cell divisions in the early L3 stage. In addition to cell divisions, other cellular processes including migration, invasion, and cell-cell fusion also stopped at the checkpoints. All of these arrests were reversible, as 98% of animals that were recovered on food after 8 d of starvation resumed development to adulthood without obvious defects in egg-laying or movement.

The ability of cells to reversibly arrest in response to external conditions is termed quiescence. Quiescence occurs in stem cells and tumor cells, both of which can survive in a non-dividing state for years.[12,13] In the case of tumor cells, reactivation of cell divisions is a major cause of cancer recurrence after chemotherapy.[12] There is increasing evidence that quiescence is an actively maintained cellular state that encompasses extensive transcriptional changes.[14] The genes involved in entry into and exit from quiescence are not well defined, in part due to the lack of *in vivo* models.[15] The larval arrest checkpoints described here offer a tractable model to identify the genes that regulate quiescence. Furthermore, studying late larval arrest can provide insight into the largely unexplored question of how

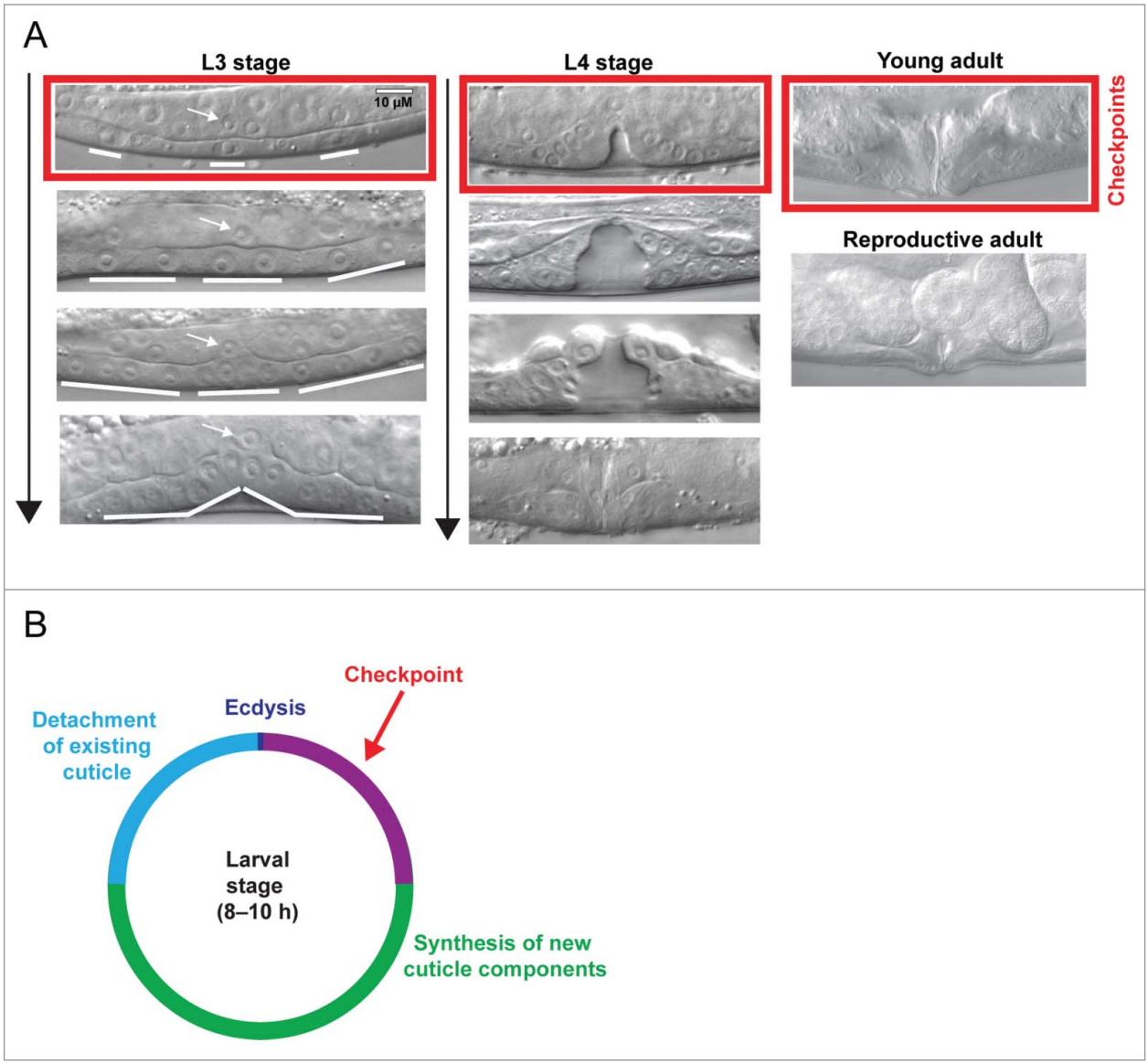

Figure 1. Starvation checkpoints in *C. elegans* larval development. (**A**) Vulval development in the L3 and L4 stages. In the L3 stage, 3 vulval precursor cells (VPCs), underlined with white bars, divide 3 times. In the L3 and L4 stages, these VPCs are invaded by the uterine anchor cell (white arrow), migrate toward the vulval midline of the animal, undergo cell-cell fusion, and form a lumen. In the young adult the vulva has adopted its final shape, but reproduction has not initiated. Red boxes highlight the times when vulval development arrests in the absence of food. (**B**) Schematic of the larval stage at 20°C. The starvation checkpoints occur after the existing cuticle has been shed during ecdysis and prior to the synthesis of new cuticle components.

non-mitotic cellular processes are also able to reversibly arrest in response to adverse environmental conditions.

The Role of Cellular Oscillations in Timing Development

Our work shows that *C. elegans* late larval development progresses from one checkpoint to the next, with an opportunity to arrest at each checkpoint should conditions become unfavorable. Because these arrest points occur at specific times and repeat in the L3, L4, and young adult stages, it appears that some kind of oscillatory mechanism exists that places nutritionally sensitive control points early in each larval stage. A prime example of a known biological oscillator in *C. elegans* is the molting cycle, which similarly occurs regularly during the larval stages. It is intriguing to speculate that the nutritionally sensitive control points in the L3, L4 and early adult, which occur shortly after the completion of the L2/L3, L3/L4 and L4/adult molts, respectively, might be linked to the oscillator that drives the molting cycle. A key component of the oscillator that drives the molting cycle is the gene *lin-42*, which is related to the mammalian and Drosophila Period genes.[16] LIN-42 protein is expressed in many tissues and oscillates with similar periodicity in those tissues.[17] *lin-42* mutants have delayed molts, precocious

divisions of VPCs and myoblasts, and gonadal migration abnormalities.[16,17] The expression of LIN-42 and the range of defects in *lin-42* mutants is consistent with the possibility that LIN-42 could regulate the timing windows in which worms arrest in response to starvation. We suspect that LIN-42 or another oscillating regulatory system may act in all cells of the animal to coordinately sensitize cells to arrest, or periodically functions in a specific tissue or group of cells that non-autonomously control the timing of arrest. It will clearly be interesting in the future to explore the possible links between LIN-42 oscillations and the timing of the developmental arrest checkpoints.

The IIS Pathway Regulates Arrest in the L3 and L4 Stages

Having established that checkpoints exist in the L3 and L4 larval stages, we sought to identify the molecular regulators that determine whether animals arrest at the checkpoints or continue development. We first focused on the insulin and insulin-like signaling (IIS) pathway, which regulates entry into dauer and cell division arrest in starved L1s.[18,19] Insulin-like peptides are secreted when food is consumed and serve as agonists for DAF-2, the sole *C. elegans* insulin-like receptor.[20] Ligand-bound DAF-2 acts through several intermediate proteins to phosphorylate a FoxO transcription factor, DAF-16. Phosphorylated DAF-16 is sequestered in the cytoplasm, where it is inactive, promoting growth and development (**Fig. 2**). During starvation, the DAF-2–mediated phosphorylation of DAF-16 is lost, allowing DAF-16 to enter the nucleus and transcriptionally regulate genes implicated in developmental arrest.[18,21]

We first tested a role for the IIS pathway in the L3 and L4 stages by examining a partial loss-of-function mutant of daf-2. We found that mutant animals were delayed in advancing through the L3 and L4 stages compared to wild type. Significantly, *daf-2* mutants temporarily arrested at the L3 and L4 checkpoints even in the presence of ample food, indicating that reduced insulin-like signaling necessitates a longer duration of feeding to bypass the checkpoints.

We next asked whether loss of *daf-16* compromised the ability to arrest at the checkpoints by performing starvation experiments on *daf-16* null animals. When starved late in the L2 stage, *daf-16* null animals initially stopped development at the L3 checkpoint; however, unlike wild type animals that remained arrested, they bypassed L3 arrest over time and developed to the L4 checkpoint. These results suggest that DAF-16 is important for maintaining the arrested state, likely by shutting down pro-development pathways when resources are limited. The timing of arrest was the same in wild type and *daf-16* null animals, indicating that the IIS pathway does not control the location of the checkpoints in the larval stage, but instead acts to couple nutritional conditions with progression through these checkpoints.

Steroid Hormone Signaling Functions Downstream of the IIS Pathway During Late Larval Arrest

DAF-16 has been shown to function cell-nonautonomously in the neurons to regulate dauer formation and in the intestine to regulate lifespan and muscle maintenance.[22,23] We wanted to determine if DAF-16 similarly functioned cell-nonautonomously in regulation of arrest at the L3 and L4 checkpoints. Using tissue-specific rescue of the daf-16 null phenotype and tissue-specific RNAi against *daf-16*, we found that *daf-16* functioned cell-nonautonomously in the hypodermis to promote arrest at the checkpoints.

The fact that hypodermal expression of DAF-16 regulated arrest in other tissues (e.g., vulva, myoblasts) implicated a systemic signaling mechanism downstream of DAF-16. One strong candidate was steroid hormone signaling, which is downstream of the IIS pathway during the dauer decision.[24-26] An enzyme required for the production of *C. elegans* steroid hormones is DAF-9/cytochrome P450, which is expressed in the hypodermis during larval development.[27,28] We hypothesized that steroid hormones regulate progression through the L3 and L4 stages, and that the bypass of arrest caused by loss of *daf-16* was due to the continued expression of steroid hormones. We tested this hypothesis by reducing *daf-9* by dsRNA feeding in *daf-16* null animals, a treatment that would be predicted to inhibit the production of steroid hormones and thereby suppress the bypass defect of daf-16 nulls. We found that this was the case, as the number of *daf-16* null animals bypassing arrest at the L3 and L4 checkpoints was reduced greater than 2-fold following *daf-9* dsRNA feeding. These data suggest that under starvation conditions, DAF-16 normally functions to inhibit *daf-9* in the arrest checkpoints (**Fig. 2**).

Research has shown that levels of daf-9 mRNA are negatively regulated by DAF-16 when animals are starved of cholesterol, an adverse growth environment.[29] This finding suggests that DAF-16 inhibits growth during unfavorable conditions at least partly through reduced *daf-9* expression. The regulation of *daf-9* by DAF-16 may be indirect, as *daf-9* has not been identified in screens for transcriptional targets of DAF-16.[30-32] It will be important to determine how the IIS pathway regulates steroid hormone release, as the conservation of these pathways in animals suggests that similar signaling networks may have roles in human development and disease.

Since a reduction of steroid hormone production promoted arrest at the checkpoints, we hypothesized that an increase in steroid hormone production could cause continued development even in the absence of food. To test this, we examined a strain overexpressing functional DAF-9, which would be predicted to generate a higher level of steroid hormones. Strikingly, we found that daf-9 overexpressing animals removed from food late in the L2 stage developed into adulthood. Thus, overexpression of daf-9 bypassed not only the L3 checkpoint, the stage at which wild type animals arrested, but also the L4 checkpoint. These results indicate that the levels of DAF-9 are a critical determinant of whether animals stop or continue development through the checkpoints.

During the dauer decision, DAF-9 is required for the synthesis of dafachronic acids (DAs), steroid hormones that bind

to the DAF-12 nuclear hormone receptor (NHR) to promote development past dauer.[33] We tested a role for both DAs and DAF-12 in L3 and L4 arrest and were unable to find a comparable role to dauer formation. Treatment with exogenous DA had no effect on arrest timing of starved animals, and a null allele of daf-12 that is defective in dauer formation arrested at the late larval checkpoints similarly to wild type. Other steroid hormones have been identified in *C. elegans*,[34] and there are 284 *C. elegans* NHRs,[35] raising the possibility that a different ligand/receptor signaling pathway may function downstream of DAF-9 during the late larval stages.

Arrest at the Checkpoints Involves a Developmental Decision

Both *daf-16* null and *daf-9* overexpressing animals bypass the checkpoints and continue development for one or 2 larval stages. An implication of these observations is that the rapid arrest that occurs in wild type animals is not due to a shortage of available resources, since these other strains are capable of further development. Instead, it appears that wild type animals are making a developmental decision that nutritional conditions are unfavorable for growth past the checkpoints. This decision to arrest at the checkpoints is likely a means of prolonging survival during periods of nutritional scarcity by allocating resources away from developmental pathways toward survival pathways. This is borne out by the observation that wild type animals survive for an average of 12 d during starvation, whereas *daf-16* null and *daf-9* overexpressing animals survive only 8 d In contrast, *daf-2* mutants survived for 21 d (A. Schindler, unpublished observations), a survival increase also observed in L1-arrested *daf-2* mutants.[18] These findings demonstrate that an inverse correlation exists between bypassing arrest and survival during starvation. A delay in bypassing arrest, which occurs in *daf-2* mutants, correlates with extended survival; increased bypass, which occurs in *daf-16* null and *daf-9* overexpressing animals, correlates with shortened survival. Interestingly, both the IIS and steroid hormone signaling pathways regulate lifespan,[25,34,36,37] suggesting that the developmental decisions on

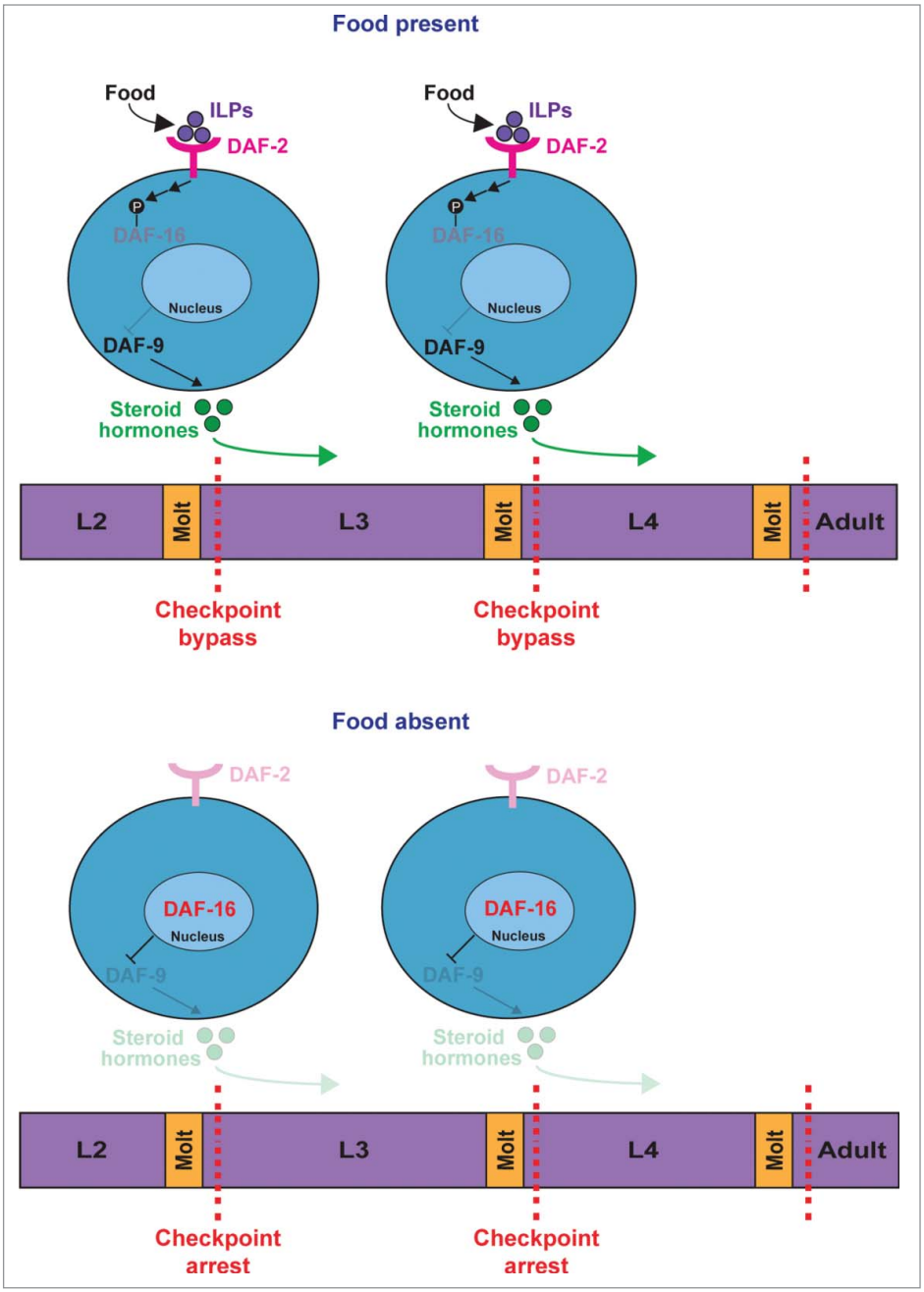

Figure 2. Pathways regulating arrest at the starvation checkpoints. Active pathways are darkened; inactive pathways are lightened. Top: Food leads to the production of insulin-like peptides (ILPs) that signal through DAF-2, the insulin-like receptor. Ligand-bound DAF-2 acts through several intermediate proteins to promote the phosphorylation of a FoxO transcription factor, DAF-16. Phosphorylated DAF-16 is localized to the cytoplasm where it is inactive, allowing DAF-9/cytochrome P450 to generate steroid hormones that promote development past the early larval stage checkpoints. Bottom: In the absence of food, DAF-16 enters the nucleus, where it inhibits the DAF-9–mediated production of steroid hormones, preventing animals from bypassing the checkpoints.

resource allocation that occur at the L3 and L4 checkpoints may also factor into organismal viability in adulthood.

The discovery that animals make a decision at the checkpoints to either arrest or continue development raises the question of what factor(s) is being sensed to dictate this decision. We offer 3 possible mechanisms that may connect nutritional conditions with the developmental decision at the checkpoints: 1) Components of food trigger a signaling pathway that stimulates development. Amino acids are likely to be one component, as work in *Drosophila* has shown that dietary amino acids act through the Tor pathway to induce the release of ILPs that stimulate growth.[38,39] 2) Chemosensation of food activates neural pathways that promote growth. This possibility is supported by recent work in *C. elegans* showing that the proliferation of germline progenitor cells is regulated by the TGFβ signaling pathway in chemosensory neurons, which relays information about nutritional availability.[40] 3) A size-sensing mechanism allows continued development only upon attainment of a threshold body size. This scenario occurs in insect development, in which metamorphosis initiates only after reaching a critical body weight.[41] These 3 scenarios are not mutually exclusive, as crosstalk could exist between different nutrition-sensing mechanisms. The genetic tools available in *C. elegans* and the easily observed readout of the L3 and L4 checkpoints should allow insight into this question.

Perspectives and Future Directions

We have identified checkpoints present in the L3 and L4 stages that arrest development in response to nutritional deprivation. This work adds to our understanding of *C. elegans* development by showing that adaptive arrest can occur at various times in the animal's life cycle. The precise timing of arrest early in the larval stage demonstrates that *C. elegans* development is punctuated by critical points at which nutritional conditions are sensed and dictate an all-or-none decision to either arrest or continue development.

We believe that the L3 and L4 checkpoints provide an experimental framework to study several important questions about developmental cell biology. These include how diverse cell types are able to enter into and exit from quiescence in response to external signals, the role of oscillations in developmental timing, and the interplay between systemic and cell-autonomous regulators of development. We have only begun to scratch the surface in understanding the complex mechanisms that animals have evolved to adapt to their environment. Elucidating these mechanisms will not only be fascinating in their own right, but also relevant to important aspects of human biology and disease, including aging and cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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