

To Grow or Not to Grow: Nutritional Control of Development During *Caenorhabditis elegans* L1 Arrest

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ABSTRACT It is widely appreciated that larvae of the nematode *Caenorhabditis elegans* arrest development by forming dauer larvae in response to multiple unfavorable environmental conditions. *C. elegans* larvae can also reversibly arrest development earlier, during the first larval stage (L1), in response to starvation. “L1 arrest” (also known as “L1 diapause”) occurs without morphological modification but is accompanied by increased stress resistance. Caloric restriction and periodic fasting can extend adult lifespan, and developmental models are critical to understanding how the animal is buffered from fluctuations in nutrient availability, impacting lifespan. L1 arrest provides an opportunity to study nutritional control of development. Given its relevance to aging, diabetes, obesity and cancer, interest in L1 arrest is increasing, and signaling pathways and gene regulatory mechanisms controlling arrest and recovery have been characterized. Insulin-like signaling is a critical regulator, and it is modified by and acts through microRNAs. DAF-18/PTEN, AMP-activated kinase and fatty acid biosynthesis are also involved. The nervous system, epidermis, and intestine contribute systemically to regulation of arrest, but cell-autonomous signaling likely contributes to regulation in the germline. A relatively small number of genes affecting starvation survival during L1 arrest are known, and many of them also affect adult lifespan, reflecting a common genetic basis ripe for exploration. mRNA expression is well characterized during arrest, recovery, and normal L1 development, providing a metazoan model for nutritional control of gene expression. In particular, post-recruitment regulation of RNA polymerase II is under nutritional control, potentially contributing to a rapid and coordinated response to feeding. The phenomenology of L1 arrest will be reviewed, as well as regulation of developmental arrest and starvation survival by various signaling pathways and gene regulatory mechanisms.

VIRTUALLY all organisms must cope with fluctuations in nutrient availability. A variety of mechanisms evolved to buffer animal development from these fluctuations. In particular, the evolution of multicellularity required animals to have coordinated responses to starvation and feeding, promoting the evolution of systemic regulation rather than relying on cell-autonomous mechanisms. Given the ancient and pervasive effects of nutrient availability, it impacts organismal biology broadly. For example, growth rate and stress resistance are influenced by caloric restriction, as well as adult size, behavior, and fertility. Caloric restriction can also extend lifespan, though the mechanistic basis is not well established. In humans, alterations in energy homeostasis can cause diseases such as obesity and diabetes, and cancer

often stems from inappropriate responses to lack of nutrition. Mechanistic understanding of these phenomena requires animal models to study the effects of nutrient availability on developmental physiology.

Life in the wild is characterized by feast or famine for *Caenorhabditis elegans*, and postembryonic development is governed by nutrient availability. First stage larvae (L1) that hatch in the absence of food (*Escherichia coli* in the lab) enter a state of developmental arrest known as “L1 arrest” (also known as “L1 diapause”) (Figure 1). L1 arrest has always been an important aspect of *C. elegans* husbandry, and most investigators are familiar with it as a convenient way to synchronize cultures. Bleaching gravid hermaphrodites yields a sterile preparation of eggs, which enter arrest when hatched without food and synchronously initiate larval development upon feeding. Because arrested L1s are stress resistant, they can also be frozen for long-term storage (Lewis 1995). Johnson and coworkers showed that arrested larvae are “ageless” in 1984 (i.e., time spent in arrest does

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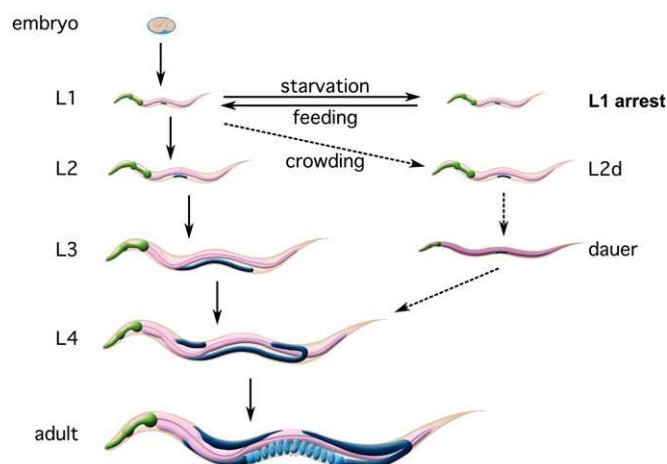


Figure 1 Feeding is required to initiate larval development, and hatching in the absence of food causes L1 arrest. Larvae can survive L1 arrest for weeks, and they are resistant to a variety of environmental stresses. Unlike L1 arrest, dauer formation involves an alternative developmental program (indicated by dashed arrows) producing a morphologically modified alternative to the third larval stage. Images were adapted from WormAtlas.org.

not reduce adult lifespan) (Johnson *et al.* 1984), but little more was published on the phenomenon for another 20 years. Interest in understanding the role of nutrition in development, stress resistance, and lifespan sparked reexamination of L1 arrest, and there has been a surge of interest in recent years.

L1 arrest is distinct from dauer arrest (Table 1). Dauer larvae form as an alternative to the third larval stage in response to high population density, limited food, and high temperature (Hu 2007). Population density is the primary determinant in dauer formation, and it is sensed via a cocktail of ascaroside-based pheromones (Ludewig and Schroeder 2013). It is unclear how multiple environmental signals are integrated in regulation of dauer formation. L1 arrest provides an opportunity to investigate nutritional control of development alone, independent of confounding environmental factors and distinctions arising from the alternative developmental program underlying dauer formation. Despite the lack of morphological modification, L1 arrest increases resistance to environmental stress. Notably, dauer formation is often inappropriately referred to as a starvation response—larvae must feed to form dauers, and hatching in the complete absence of food results in L1 arrest. As regulatory mechanisms come to light, it will continue to be valuable to compare L1 arrest and dauer formation.

Diapause in animal development has three defining features: arrest occurs at a specific stage in the lifecycle; arrest and recovery are triggered by specific environmental cues; and the animal prepares for arrest by altering metabolism in advance (Kostal 2006). Based on this definition, L1 arrest is technically not a diapause, though dauer arrest is. Starvation is not a specific cue, and arrest does not occur in advance of actual starvation nor is there any evidence that it is associated with preparation. *C. elegans* embryos do not grow, completing embryogenesis entirely on

Table 1 Critical distinctions between L1 arrest and dauer arrest

	L1 arrest	Dauer arrest
Larval stage	L1	L3
Causes	Starvation	High density (pheromone) Limited food High temperature
Morphology	Standard	Modified
Survival	Weeks	Months

Arrested larvae are stress resistant in both cases. L1 arrest results when eggs hatch in the absence of food, but starvation can cause arrest at other stages as well. Heat and other stresses can also cause arrest at the L1 stage, but the mechanistic basis of these types of arrest is unclear (see *Related Arrest Phenomena*).

maternal stores, and larvae must feed to initiate postembryonic development. As a consequence, embryos cultured without food arrest as L1 larvae. Rather than reflecting a stage-specific response, larvae starved at any stage will arrest development in similar fashion (Johnson *et al.* 1984; Van Gilst *et al.* 2005b; Ruaud and Bessereau 2006; Kang *et al.* 2007). Although L1 arrest is under systemic regulation and it includes metabolic adaptation and increased stress resistance, it is more appropriate to consider L1 arrest as a form of starvation-induced quiescence than a diapause (Kostal 2006).

L1 arrest raises a variety of mechanistic questions at the molecular, cellular, and organismal level, offering a powerful metazoan system for genetic analysis of integrative organismal biology. How is nutrient availability sensed, and how are the developmental and physiological responses implemented? What signaling pathways mediate regulation, what are their sites of action, and how are they integrated to provide coordinated responses? How does signaling affect gene expression to bring about developmental arrest, metabolic adaptation, and stress resistance? How is arrest maintained and robust recovery accomplished? How does regulation of L1 arrest compare to regulation of dauer formation and aging? Investigation of L1 arrest is an emerging area of *C. elegans* research, and this review seeks to address these questions while covering what is currently known.

Insulin-Like Signaling and Developmental Arrest

DAF-2/InsR

Insulin-like signaling contributes to developmental homeostasis by buffering multicellular animals from fluctuations in nutrient availability. This conserved function is manifested in regulation of dauer formation, stress resistance, and lifespan in *C. elegans*. Mutations affecting the sole insulin-like receptor DAF-2/InsR (Figure 2) form dauers constitutively (Riddle *et al.* 1981; Hu 2007), are stress resistant (Honda and Honda 1999; Scott *et al.* 2002; Lamitina and Strange 2005), and extend adult lifespan (Kenyon *et al.* 1993; Kimura *et al.* 1997). Strong loss-of-function alleles also display constitutive L1 arrest (Gems *et al.* 1998). However, this phenotype was originally interpreted as a lethal,

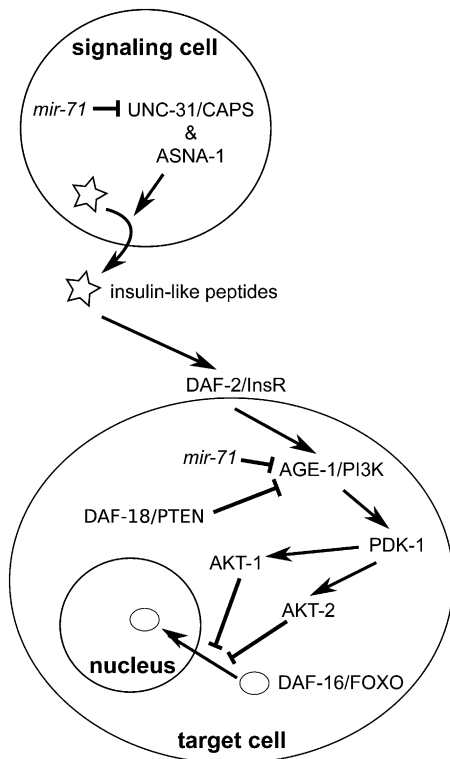


Figure 2 Model of the *C. elegans* insulin-like signaling pathway. There are 40 putative insulin-like peptides in *C. elegans*, and their secretion is mediated by UNC-31/CAPS and the conserved ATPase ASNA-1. DAF-2/InsR is the sole insulin/IGF receptor in *C. elegans*, and it signals through a conserved PI3K pathway to antagonize function of DAF-16/FOXO. DAF-16/FOXO activates transcription of genes that promote L1 arrest, dauer formation, stress resistance, and lifespan extension. DAF-16/FOXO is not the only effector of insulin-like signaling (Tullet *et al.* 2008). DAF-16/FOXO is also regulated by additional factors and signaling pathways (Landis and Murphy 2010). DAF-18/PTEN functions independently of DAF-16/FOXO in the germline (Fukuyama *et al.* 2006).

terminal arrest phenotype. Consistent with this interpretation, the null phenotype of *daf-2* includes embryonic lethality (Gems *et al.* 1998; Patel *et al.* 2008). However, *daf-2* mutants are temperature sensitive, and Baugh and Sternberg (2006) showed that constitutive L1 arrest of *daf-2* (*e979*) could be reversed at the permissive temperature producing reproductive adults. Furthermore, growth arrest is not due to an inability of *daf-2*(*e979*) to feed (Baugh and Sternberg 2006). *daf-2* mutants are also resistant to starvation and survive L1 arrest longer than wild type, consistent with insulin-like signaling regulating L1 arrest (Munoz and Riddle 2003; Baugh and Sternberg 2006). Disruption of insulin-like peptide secretion also results in constitutive L1 arrest and increased starvation survival (see *Insulin-like peptide secretion*), providing additional evidence that insulin-like signaling regulates L1 arrest (Kao *et al.* 2007).

DAF-16/FOXO

The FOXO transcription factor DAF-16 is a major effector of DAF-2/InsR signaling (Lin *et al.* 1997; Ogg *et al.* 1997), and DAF-16 is required for L1 arrest (Baugh and Sternberg

2006). *daf-16* null mutants have no obvious phenotype in standard culture conditions, but they are sensitive to starvation and die rapidly during L1 arrest (Munoz and Riddle 2003; Baugh and Sternberg 2006). Insulin-like signaling regulates localization and activity of DAF-16/FOXO (Lin *et al.* 1997; Ogg *et al.* 1997; Henderson and Johnson 2001; Lee *et al.* 2001), and the protein translocates to the nucleus during L1 arrest where it regulates gene expression (Weinkove *et al.* 2006). *daf-16* mutants are also L1 arrest defective and initiate postembryonic development in the absence of food, indicating that DAF-16/FOXO promotes developmental arrest in response to starvation (Baugh and Sternberg 2006). The *daf-16* L1 arrest-defective phenotype includes the normal pattern of cell divisions, migrations, and fusions that occur during L1 development, though much slower than normal development of fed larvae. DAF-18/PTEN regulates L1 arrest in the germline (see *Germline arrest*), and insulin-like signaling appears to be transduced by AGE-1/PI3K during L1 arrest (Weinkove *et al.* 2006; Zhang *et al.* 2011), but other components of the pathway have not been examined (Figure 2). Consistent with the pathway having the same structure as in regulation of dauer formation (Hu 2007), *daf-16*/FOXO is epistatic to *daf-2*/InsR (Baugh and Sternberg 2006). In summary, the L1 arrest phenotypes of *daf-2*/InsR and *daf-16*/FOXO suggest that insulin-like signaling provides permissive, nutrient-dependent regulation of L1 development (Table 2).

AMP-activated kinase

The AMP-activated kinase (AMPK) interacts with insulin-like signaling (Apfeld *et al.* 2004). Like mutations affecting *daf-16*/FOXO, mutation of the AMPK α -subunit encoded by *aak-2* results in an L1 arrest-defective phenotype (Baugh and Sternberg 2006). This phenotype also includes the normal L1 developmental pattern of cell divisions, migrations and fusions, though much slower than in fed larvae.

Cell cycle arrest and developmental timing

The cyclin-dependent kinase inhibitor *cki-1*/CIP/KIP/p27 and the microRNA (miRNA) *lin-4* function downstream of insulin-like signaling in L1 arrest and development. Ambros and coworkers showed that *cki-1* is required for blast cells to remain undivided during L1 arrest and that the cell cycle is arrested at the G1/S transition (Hong *et al.* 1998). *cki-1* reporter expression is up-regulated in blast cells during L1 arrest, and up-regulation requires *daf-16*/FOXO, providing a mechanistic link between insulin-like signaling and cell cycle arrest (Baugh and Sternberg 2006). *lin-4* is a key regulator of postembryonic developmental timing, being the most upstream component of the heterochronic pathway (Lee *et al.* 1993). *lin-4* is expressed during the mid-late L1 stage, and the timing of *lin-4* miRNA accumulation controls the timing of postembryonic development. Ambros and coworkers hypothesized that a signal that requires feeding leads to accumulation of *lin-4* miRNA (Feinbaum and Ambros 1999). Indeed, a *lin-4* reporter gene is normally not

Table 2 Genes with developmental arrest phenotypes

Gene	Mutant arrest phenotype	Somatic or germline	Function
<i>asna-1</i>	Constitutive	Both	Insulin-like peptide secretion
<i>daf-2/InsR</i>	Constitutive	Both	Insulin-like receptor
<i>daf-16/FOXO</i>	Defective	Somatic	Insulin-like signaling
<i>mir-235</i>	Defective	Somatic	Downstream of insulin-like signaling
<i>daf-18/PTEN</i>	Defective	Germline	Insulin-like signaling
<i>aak-2/AMPK</i>	Defective	Both	Energy homeostasis
<i>cki-1/CIP/KIP/p27</i>	Defective	Somatic	Cell cycle
<i>mdf-1/Mad1p</i>	Defective	Germline	Cell cycle

The loss-of-function mutant L1 developmental arrest phenotype is indicated. Arrest of somatic or germline cells may be affected, and with some genes both are affected.

expressed during L1 arrest, but in a *daf-16/FOXO* mutant it is expressed in some individuals, placing insulin-like signaling upstream of developmental timing (Baugh and Sternberg 2006). However, whether *cki-1/CIP/KIP/p27* or *lin-4* are direct targets of *DAF-16/FOXO* has not been addressed, and other pathways also contribute to their regulation (Kniazeva *et al.* 2008; Ow *et al.* 2008).

miRNAs and developmental arrest

In addition to regulating developmental timing in fed larvae, miRNAs also contribute to regulation of developmental arrest during starvation. Screening 85 deletion mutants affecting 105 miRNA genes identified *mir-235* as a critical regulator of L1 developmental arrest (Kasuga *et al.* 2013). Similar to a *daf-16* mutant, *mir-235(n504)* is L1 arrest defective, with all of the cell divisions, migrations, and fusions that occur during normal L1 development occurring during starvation in standard sequence. Consistent with *mir-235* being an effector of *DAF-16*, *mir-235* expression is up-regulated during L1 arrest by *DAF-16*. *mir-235* is expressed in the epidermis and glial-like socket cells surrounding the amphid chemosensory neurons, and expression in either of these sites can rescue the arrest-defective phenotype, revealing cell-nonautonomous function. *mir-235* directly regulates expression of *nhr-91/GCNF* via its 3' UTR, and *nhr-91* is epistatic to *mir-235* for the L1 arrest-defective phenotype (Kasuga *et al.* 2013). *mir-235* is conserved, and the extent to which its regulation and function are conserved remains to be determined.

Germline arrest

Primordial germ cells arrest at a different point in the cell cycle and by a different mechanism than somatic cells during L1 arrest. There are two primordial germ cells when the L1 larva hatches, Z2 and Z3 (Sulston and Horvitz 1977). These cells arrest in G2 after DNA synthesis with condensed chromosomes (Fukuyama *et al.* 2006). Arrest of Z2 and Z3 depends on *daf-18/PTEN*, a negative regulator of the insulin-like pathway composed of *daf-2/InsR*, *age-1/PI3K* and *akt-1/Akt/PKB* (Ogg and Ruvkun 1998; Fukuyama *et al.* 2006). The germ cell arrest defect of a *daf-18* mutant depends on *age-1* and *akt-1*; but, in contrast to somatic cell arrest, it does not require *daf-16/FOXO* function (Baugh and Sternberg 2006; Fukuyama *et al.* 2006). Germ cell arrest

does not depend on the cyclin-dependent kinase inhibitor *cki-1/CIP/KIP/p27*, also in contrast to somatic cell arrest (see below) (Hong *et al.* 1998; Fukuyama *et al.* 2003; Baugh and Sternberg 2006). Germ cell arrest does rely on the mitotic spindle checkpoint protein *MDF-1/Mad1p* acting downstream of *DAF-18/PTEN* and *AKT-1/Akt/PKB* (Watanabe *et al.* 2008). These results indicate that although the insulin-like/PI3K pathway regulates germ cell arrest, an effector other than *daf-16/FOXO* is utilized (Fukuyama *et al.* 2006). In addition to *daf-18/PTEN*, the α -subunits of AMPK encoded by *aak-1* and *aak-2* are redundantly required for germ cell arrest (Fukuyama *et al.* 2012; Lee *et al.* 2012). Furthermore, AMPK mutants suffer accelerated damage to the gonad during starvation and give rise to sterile adults following recovery from arrest, though sterility is not observed in continuously fed animals (Fukuyama *et al.* 2012; Lee *et al.* 2012). *DAF-18/PTEN* and AMPK function independently in regulation of germ cell arrest, but they converge on inhibition of TOR complex 1, which is required for ectopic germ cell proliferation during L1 arrest (Fukuyama *et al.* 2012). The site of action of *DAF-18/PTEN* and AMPK in regulation of germ cell proliferation is unclear, but there is evidence that it is cell autonomous in each case (Suzuki and Han 2006; Fukuyama *et al.* 2012).

Insulin-like signaling sites of action

Several lines of evidence suggest that the insulin-like signaling pathway does not function cell autonomously to regulate L1 arrest. Kenyon and coworkers used a transgene-based approach to characterize the site of action of insulin-like signaling in regulation of lifespan (Libina *et al.* 2003). They started with a *daf-2*; *daf-16* double mutant and used tissue-specific, heterologous promoters to drive *daf-16* expression in this background. With this approach, *DAF-16/FOXO* will be highly active in the tissue where it is expressed. Notably, transgenic lines expressing *daf-16* in the epidermis could not be recovered, possibly reflecting the embryonic lethal *daf-2* null phenotype (Gems *et al.* 1998; Libina *et al.* 2003; Patel *et al.* 2008). Furthermore, panneuronal expression of *daf-16* resulted in a constitutive L1 arrest phenotype with incomplete penetrance at 25.5° (Libina *et al.* 2003), similar to mutation of *daf-2* (Baugh and Sternberg 2006). This phenotype was not evident with forced *daf-16* expression in muscle or intestine except at

27°, suggesting that the nervous system is a more important site of action (Libina *et al.* 2003). Indeed, *DAF-2* expression is abundant in the nervous system, and it is regulated by nutrient availability (Kimura *et al.* 2011).

The nervous system does not appear to be the only site of action for insulin-like signaling. *mir-235* expression is regulated by *DAF-2* signaling via *DAF-16*, and the L1 arrest-defective phenotype of *mir-235* can be rescued by expression in the epidermis, nervous system, or glial cells (see *miRNAs and developmental arrest*), suggesting that insulin-like signaling to each of these tissues alone can affect developmental arrest nonautonomously (Kasuga *et al.* 2013). In addition, forced expression of miRNA-induced silencing complex (miRISC) component *AIN-2* in the intestine rescues the starvation-sensitive phenotype of its redundant homolog *ain-1* (see *miRNAs and starvation survival*), suggesting that the insulin-like signaling pathway can have appreciable function in the intestine during L1 arrest (Zhang *et al.* 2011). This result and others suggest that different organs contribute to regulation of developmental arrest and starvation survival (see *DAF-16/FOXO*, *DAF-18/PTEN*, *AMPK and starvation survival*). In summary, these results are consistent with the insulin-like signaling pathway functioning cell-nonautonomously to regulate developmental arrest, though signals functioning downstream of *DAF-16* to mediate such regulation have not been identified.

Insulin-like peptides

The insulin-like peptides that regulate L1 arrest have not been identified. The *C. elegans* genome encodes an unusually large family of putative insulin-like peptides comprising 40 genes (Pierce *et al.* 2001). In mixed stage cultures reporter gene expression is observed primarily in the nervous system but also in the intestine, epidermis, vulva, and pharynx (Pierce *et al.* 2001). The large size of the gene family suggests functional redundancy will preclude identification of loss-of-function phenotypes. Nevertheless, specific loss-of-function phenotypes affecting dauer formation (*daf-28*, *ins-1*, *ins-17*, and *ins-18*), dauer recovery (*ins-6*), lifespan (*ins-7* and *ins-18*), and germline proliferation (*ins-3* and *ins-33*) have been reported, revealing specificity of function (Li *et al.* 2003; Murphy *et al.* 2003; Michaelson *et al.* 2010; Cornils *et al.* 2011; Matsunaga *et al.* 2012). In addition, overexpression of *ins-4*, *daf-28*, or *ins-6* promotes recovery from dauer arrest (Kao *et al.* 2007; Cornils *et al.* 2011). Nevertheless, the *DAF-2*/InsR agonists that presumably promote L1 development in response to feeding have not been identified.

In contrast to their well-established role promoting growth and development, insulin-like peptides can also promote arrest in *C. elegans*. Overexpression of *ins-1*, *ins-18*, or *ins-17* actually promotes dauer arrest and to a lesser extent L1 arrest, suggesting that they antagonize *DAF-2*/InsR (Pierce *et al.* 2001; Matsunaga *et al.* 2012). The loss-of-function phenotype for each of these three genes confirms that they promote dauer arrest (Cornils *et al.* 2011; Matsunaga

et al. 2012). An insulin-like peptide in *Drosophila* has recently been reported to promote developmental arrest (Colombani *et al.* 2012), suggesting antagonistic function is conserved in other animals. *ins-17* reporter gene expression is up-regulated during dauer arrest (Matsunaga *et al.* 2012), suggesting that expression analysis can be used to predict *DAF-2*/InsR antagonists that function during L1 arrest. Consistent with this hypothesis, *ins-17* and *-18* are up-regulated during dauer and L1 arrest, as well as *ins-10*, *-16*, *-20*, and *-24* (Baugh *et al.* 2011), but their function has not been characterized during L1 arrest.

Chemosensation and systemic regulation

Chemosensory neurons likely provide systemic control of L1 arrest. Depending on temperature, mutations affecting the structure and function of amphid chemosensory neurons have either dauer-defective or dauer-constitutive phenotypes, suggesting that these neurons regulate dauer formation in response to sensory stimuli (Albert *et al.* 1981; Vowels and Thomas 1992; Starich *et al.* 1995; Ailion and Thomas 2000). Laser ablation of specific amphid chemosensory neurons also causes a dauer-constitutive phenotype, implicating ASI, ASJ, and ADF in particular (Bargmann and Horvitz 1991). An L1 arrest phenotype was not reported following laser ablation, but cells do not die immediately after ablation, so it is possible that these experiments would not necessarily reveal such a phenotype. Likewise, mutations affecting chemosensory neuron structure and function may not eliminate all sensory or signaling function. Nevertheless, such mutations enhance the L1 arrest-constitutive phenotype of *daf-2*/InsR mutants (Vowels and Thomas 1992). These genetic interactions suggest that chemosensory neurons promote L1 development in response to sensory stimuli. Insulin-like peptides are expressed in and secreted from these neurons (see *Insulin-like peptide secretion*), suggesting that they could directly sense the environment and autonomously respond by modulating peptide secretion providing systemic regulation.

Insulin-like peptide secretion

In humans, the immediate response of pancreatic β -cells to increased blood glucose levels is dense-core vesicle secretion of insulin (Saltiel and Pessin 2007), suggesting that secretion is acutely regulated by nutrient availability in *C. elegans*. Mutation of *unc-31*/CAPS, a calcium-activated regulator of dense-core vesicle release, extends starvation survival during L1 arrest in *daf-16*/FOXO-dependent fashion, similar to the *daf-2*/InsR mutant phenotype (Baugh and Sternberg 2006; Lee and Ashrafi 2008). Expression of *unc-31* in ciliated chemosensory neurons, ADL and ASH in particular, restores starvation sensitivity in an *unc-31* mutant (Lee and Ashrafi 2008). Moreover, mutation of *ocr-2*, which encodes a TRPV channel that localizes to the cilia of these sensory neurons, diminishes insulin-like peptide secretion, and increases starvation survival, implicating sensory perception in regulation of L1 arrest (Lee and Ashrafi 2008).

Secretion of insulin-like peptides from the intestine also regulates L1 arrest. *asna-1* encodes a conserved ATPase that is required for insulin-like peptide secretion and larval development (Kao *et al.* 2007). Mutation of *asna-1* disrupts secretion causing a constitutive-L1 arrest phenotype similar to the *daf-2*/InsR mutant phenotype, confirming that insulin-like signaling regulates L1 arrest (Baugh and Sternberg 2006; Kao *et al.* 2007). A directed RNAi screen of genes predicted to interact with *ASNA-1* suggests that the Golgi network and mitochondria influence insulin-like peptide secretion affecting larval development (Billing *et al.* 2012). An *asna-1* reporter is expressed in a subset of chemosensory neurons, intestine, and epidermis (Kao *et al.* 2007), each a site of insulin-like peptide reporter expression (Pierce *et al.* 2001). Overexpression of *asna-1* phenocopies overexpression of insulin-like peptides *ins-4* and *daf-28*, promoting dauer recovery (Kao *et al.* 2007). Expression of *asna-1* in ciliated sensory neurons or intestine alone is sufficient for rescue of the L1 arrest-constitutive phenotype (epidermal expression was not tested for rescue), though larval development is slower than wild-type in both cases (Kao *et al.* 2007). These results show that chemosensory neurons and intestine contribute in additive fashion to regulation of post-embryonic development via insulin-like peptide secretion.

Feeding Behavior and Metabolism

MAPK, autophagy, and feeding

The pharynx is *C. elegans*' feeding organ. The pharyngeal muscle rhythmically contracts and relaxes to pump food into the intestinal lumen (Avery and You 2012). The rate of pumping determines the rate of ingestion, and it is modulated in response to nutrient availability. Pumping decreases when food is removed, it increases when food is encountered, and it increases more when starved animals encounter food (Avery and Horvitz 1990). After the initial decrease in pumping, it increases over the first few hours of starvation, as if to prime starved animals for rapid recovery when food is encountered again (You *et al.* 2006). Avery and coworkers have shown that pumping is regulated by acetylcholine release from the MC motor neurons acting through nicotinic and muscarinic acetylcholine receptors in the pharynx (Raizen *et al.* 1995; McKay *et al.* 2004; Steger and Avery 2004). Starvation activates a muscarinic acetylcholine receptor, it signals through Gq α and PKC to phosphorylate MAPK in the pharynx, and MAPK activity promotes the increased pumping rate that occurs during the first few hours of starvation (You *et al.* 2006).

Pumping is energy intensive, and this increase in rate depends on autophagy within the pharynx (Kang *et al.* 2007). Autophagy is regulated by MAPK activity, and genetic analysis indicates that either too little or too much autophagy reduces starvation survival and recovery from L1 arrest. This dual role of autophagy reflects the fact that it is required to maintain viability and pumping, but too much

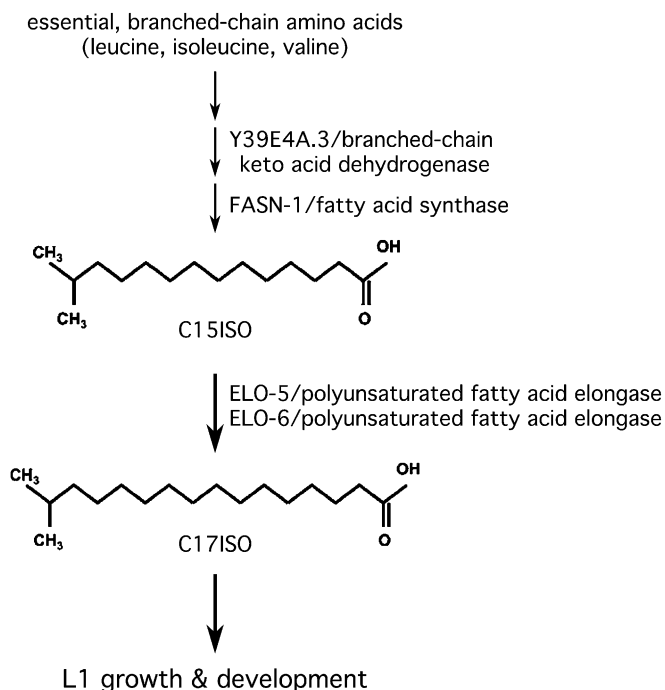


Figure 3 Synthesis of the monomethyl branched-chain fatty acid C17ISO is necessary to initiate L1 growth and development. Catabolism of essential, branched-chain amino acids produces the precursors of C15ISO, which is converted into C17ISO by the polyunsaturated fatty acid elongases ELO-5 and ELO-6. The pathway depicted for C15ISO synthesis is incomplete.

autophagy damages the organ causing it to malfunction, compromising recovery from L1 arrest (Kang *et al.* 2007). These results provide an integrative understanding of how nutrient availability influences physiology of the feeding organ.

Monomethyl branched-chain fatty acid metabolism

Monomethyl branched-chain fatty acids are common in many organisms, but their origin and physiological role is poorly understood. The monomethyl branched-chain fatty acid C17ISO (Figure 3) is a product of leucine catabolism, and synthesis of C17ISO is necessary to initiate postembryonic development in *C. elegans* (Kniazeva *et al.* 2004). Disruption of either of the genes encoding fatty acid elongation enzymes, *elo-5* or *elo-6*, causes an L1 arrest-constitutive phenotype. *elo-5* and *elo-6* are necessary for synthesis of C17ISO, and larval development can be rescued after their disruption with exogenous C17ISO (Kniazeva *et al.* 2004). The dose response for this rescue is ultrasensitive and all or none, and Han and coworkers suggested that C17ISO plays a signaling role as opposed to structural (e.g., membrane structure) (Kniazeva *et al.* 2008). They also showed that regulation of L1 development by C17ISO acts in parallel to insulin-like signaling. In particular, arrest of M lineage and seam cell divisions does not depend on *daf-16*/FOXO function, though starvation-induced arrest of these cell divisions does (Baugh and Sternberg 2006; Kniazeva *et al.* 2008). *daf-16* is also dispensible for C17ISO mediated

up-regulation of *cki-1*/CIP/KIP/p27 in seam cells, indicating that insulin-like signaling and C17ISO regulation converge on this arrest mechanism and probably others as well (Kniazeva *et al.* 2008). C17ISO homeostasis is maintained via feedback involving transcriptional regulation of *elo-5* and the acyl-CoA ligase *acs-1* by *sbp-1*/SREBP-1c. Consistent with it being a product of leucine catabolism, C17ISO synthesis is sensitive to absorption of digested oligopeptides by intestinal cells (Kniazeva *et al.* 2008). The L1 arrest-constitutive phenotype of C17ISO-deficient animals is suppressed by disruption of the P-type ATPase *TAT-2*, which functions in the intestine (Seamen *et al.* 2009). How fatty acid metabolism in the intestine leads to systemic control of development is unclear. However, this system comprises a mechanism for regulation of postembryonic development in response to the metabolic state of the organism, providing insight into the physiological function of monomethyl branched-chain fatty acids.

Intermediary metabolism

C. elegans provides a valuable metazoan model for how metabolism is regulated in response to nutrient availability. The genome encodes the vast majority of enzymes involved in intermediary metabolism in other animals (Braeckman *et al.* 2009), suggesting a conserved starvation response. Metabolic gene expression has been investigated in dauer larvae compared to developing larvae and over time in response to acute starvation (Van Gilst *et al.* 2005b; McElwee *et al.* 2006). Dauers do not feed, and general patterns of regulation are similar in dauer and starved larvae. In particular, enzymes mediating β -oxidation of fatty acids, the glyoxylate pathway, and gluconeogenesis are up-regulated, while the citric acid cycle and mitochondrial respiratory chain are down-regulated. The results reflect altered metabolic flux, with fat stores being mobilized to produce carbohydrate and provide energy during starvation. A similar pattern of gene expression was observed in response to starvation during each larval stage and in adults (Van Gilst *et al.* 2005b), suggesting that L1 arrest is not metabolically distinct from starvation during other stages. Analysis of fed *daf-2*/InsR mutant adults revealed patterns of metabolic gene expression overlapping with wild-type dauer larvae, showing that insulin-like signaling, which regulates dauer formation and L1 arrest, contributes to regulation of metabolic gene expression (McElwee *et al.* 2006). In addition, mutations affecting the nuclear hormone receptor *nhr-49*/HNF4 and its coactivator, the mediator subunit *mdt-15*/MED15, alter metabolic gene expression, indicating that *nhr-49* and *mdt-15* regulate fatty acid metabolism in response to nutrient availability (Van Gilst *et al.* 2005a,b; Taubert *et al.* 2006).

Starvation Survival and Stress Resistance

Environmental factors affecting starvation survival

A variety of environmental factors influence the amount of time arrested L1's survive starvation. Assays of starvation

survival can easily be confounded by such factors if not properly controlled. For example, the rate of metabolism affects starvation survival, such that survival is shorter at higher temperature (Lee *et al.* 2012). Worm density also affects starvation survival, as does the type of closure used on glass test tubes (L. Avery and A. Artyukhin, personal communication).

Presence of a carbon source in the buffer has a strong effect on starvation survival. Larvae arrested in the salt buffer M9 survive L1 arrest for ~10 days, but larvae arrested in buffer S-basal survive about three times longer. Lewis (1995) described the composition of these buffers. The critical difference between the two buffers is ethanol: cholesterol dissolved in ethanol is added to S-basal, resulting in a final concentration of 0.1% (20 mM) ethanol. As little as 1 mM ethanol extends starvation survival to a similar degree, as do n-propanol and n-butanol but not methanol or isopropanol (Castro *et al.* 2012). Clarke and colleagues showed that pharyngeal pumping is necessary for ethanol to extend survival and that it is used as a carbon source to synthesize fatty acids and amino acids (Castro *et al.* 2012). In this sense the larvae are not truly starved, but the carbon source alone is not sufficient to initiate larval development. However, initiation of postembryonic development prior to death in a *daf-16* mutant during starvation requires the addition of ethanol or an alternative carbon source (Baugh and Sternberg 2006). The effects of environmental factors are interesting but should encourage caution in experimental design and interpretation.

Amino acid sensation

The interneurons AIY and AIB provide systemic control of the starvation response during L1 arrest. Treatment of arrested L1's with leucine, an essential amino acid, reduces autophagy, stress resistance, and starvation survival (Kang and Avery 2009a). The effect of leucine on the starvation response was discovered via its suppression of the starvation-sensitive phenotype of *gpb-2*/G β 5, which results from this mutant being hypersensitive to excessive muscarinic signaling (see *MAPK, autophagy, and feeding*). Kang and Avery (2009a) showed that the metabotropic glutamate receptors *mgl-1* and *mgl-2* function in AIY and AIB, respectively, to provide systemic control of these starvation responses. AIY and AIB receive synaptic input from amphid chemosensory neurons (White *et al.* 1986). However, regulation of L1 arrest is intact in an *eat-4*/BNPI mutant, which has disrupted glutamate neurotransmission, suggesting that AIY and AIB autonomously sense and respond to internal amino acid levels (Kang and Avery 2009a). AIY and AIB also control food- and odor-evoked behavior in adults, providing additional evidence that this signaling system provides systemic control of the starvation response (Chalasani *et al.* 2007). The systemic signals produced by AIY and AIB in response to amino acid levels are unknown, but it has been suggested that they are neuropeptides (Kang and Avery 2009a).

Table 3 Genes that affect starvation survival during L1 arrest

Gene	Mutant starvation survival phenotype	Function
<i>ocr-2/TRPV</i>	Increased	Chemosensation
<i>osm-6/IFT52 et al.</i>	Increased	Chemosensation
<i>unc-31/CAPS</i>	Increased	Insulin-like peptide secretion
<i>daf-2/InsR</i>	Increased	Insulin-like receptor
<i>age-1/PI3K</i>	Increased	Insulin-like signaling
<i>daf-18/PTEN</i>	Decreased	Insulin-like signaling
<i>daf-16/FOXO</i>	Decreased	Insulin-like signaling
<i>aak-2/AMPK</i>	Decreased	Energy homeostasis
<i>ife-2/eIF4E</i>	Increased	Translation initiation
<i>ain-1</i>	Decreased	miRISC component
<i>mir-71</i>	Decreased	Targets insulin-like signaling
<i>eat-2</i>	Decreased	Pharyngeal pumping
<i>isp-1/ISP</i>	Decreased	Mitochondrial respiration
<i>clk-1/COQ7/CAT5</i>	Decreased	Mitochondrial respiration
<i>tbc-2</i>	Decreased	Yolk protein storage
<i>rme-1</i>	Decreased	Yolk protein storage
<i>rme-6</i>	Decreased	Yolk protein storage
<i>bec-1/Atg6/Vps30/Bec1</i>	Decreased	Autophagy
<i>pha-4/FOXA</i>	Decreased	Energy homeostasis
<i>gpb-2/Gβ5</i>	Decreased	G protein signaling
<i>hsf-1/HSF</i>	Decreased	Heat shock response
<i>cep-1/p53</i>	Decreased	DNA repair
<i>pcm-1/PCMT1</i>	Decreased	Damaged protein repair

The loss-of-function mutant L1 starvation survival phenotype is indicated along with the general function of the gene product. The gain-of-function allele *skn-1(lax188)* and *skn-1* over-expression are reported to have decreased and increased L1 starvation survival phenotypes, respectively (Paek *et al.* 2012)

PHA-4/FOXA, SKN-1/Nrf, and starvation survival

L1 arrest provides an excellent model to investigate the genetics of starvation survival in an animal. A genome-wide screen to identify genes required for starvation survival has not been performed, but a limited number of important genes participating in a variety of processes are known (Table 3). For example, the forkhead transcription factor *pha-4*/FOXA is required for caloric restriction to extend adult lifespan (Panowski *et al.* 2007), and *pha-4* mutants are sensitive to starvation (Zhong *et al.* 2010). Furthermore, overexpression of *pha-4* extends starvation survival, suggesting a physiological role in energy homeostasis as opposed to a developmental defect compromising survival of the mutant (Zhong *et al.* 2010). The bZip transcription factor *skn-1*/Nrf is also required for caloric restriction to extend adult lifespan as well as resistance to a variety of stressors (Bishop and Guarente 2007; Wang *et al.* 2010), and overexpression of *skn-1* also extends L1 starvation survival (Paek *et al.* 2012). A role for *pha-4*/FOXA and *skn-1*/Nrf in starvation survival is particularly significant, given their well-characterized roles in controlling the development of the pharynx (the feeding organ) and the digestive tract, respectively, during embryonic development (Bowerman *et al.* 1992; Mango *et al.* 1994; Gaudet and Mango 2002; Maduro and Rothman 2002).

Mutations that increase starvation survival

Loss-of-function mutants with increased starvation survival highlight the role of insulin-like signaling during L1 arrest (Table 3). Mutation of *daf-2*/InsR or *age-1*/PI3K extend adult lifespan (Friedman and Johnson 1988; Kenyon *et al.* 1993; Kimura *et al.* 1997), and these mutants also have extended

starvation survival (Munoz and Riddle 2003; Baugh and Sternberg 2006). Furthermore, mutations effecting *unc-31*/CAPS, which is required for dense core vesicle secretion of insulin-like peptides, also extend starvation survival during L1 arrest (Lee and Ashrafi 2008). Increased starvation survival of *daf-2* and *unc-31* mutants depends on *daf-16*/FOXO (Baugh and Sternberg 2006; Lee and Ashrafi 2008). *unc-31* functions in the chemosensory neurons ASH and ADL to affect starvation survival (see *Insulin-like peptide secretion*), and mutants that disrupt function of these and other ciliated chemosensory neurons (*e.g.*, *osm-6*/IFT52) also extend survival (Lee and Ashrafi 2008). The effect of *asna-1*, which encodes a conserved ATPase that is required for insulin-like peptide secretion (see *Insulin-like peptide secretion*) (Kao *et al.* 2007) on starvation survival has not been reported. The only other gene reported to extend starvation survival when mutated is *ife-2*/eIF4E (Lee *et al.* 2012). *ife-2* encodes a translation initiation factor that when mutated also increases adult lifespan and heat resistance (Hansen *et al.* 2007). Other genes known to extend adult lifespan when disrupted have not been systematically examined for effects on starvation survival, though such an analysis would elucidate the extent of overlap in the genetic basis of these two traits.

DAF-16/FOXO, DAF-18/PTEN, AMPK, and starvation survival

Starvation survival depends on a variety of genes that have no obvious phenotypic effect in fed animals. The effector of insulin-like signaling *daf-16*/FOXO is required for developmental arrest of somatic cells during starvation, and disruption of *daf-16* also reduces starvation survival (Baugh and Sternberg 2006). The

Table 4 Known sites of action for genes that contribute to regulation of L1 arrest

Tissue	Gene	Phenotype affected	Function
Nervous system	<i>daf-16/FOXO</i>	Developmental arrest	Insulin-like signaling
Nervous system	<i>aak-2/AMPK</i>	Starvation survival	Energy homeostasis
Sensory neurons	<i>ocr-2/TRPV</i>	Starvation survival	Sensory perception
Sensory neurons	<i>asna-1</i>	Developmental arrest	Insulin-like peptide secretion
Sensory neurons	<i>unc-31/CAPS</i>	Starvation survival	Insulin-like peptide secretion
Glial cells	<i>mir-235</i>	Developmental arrest	Downstream of insulin-like signaling
Intestine	<i>asna-1</i>	Developmental arrest	Insulin-like peptide secretion
Intestine	<i>aak-2/AMPK</i>	Starvation survival	Energy homeostasis
Intestine	<i>daf-18/PTEN</i>	Starvation survival	Insulin-like signaling
Intestine	<i>ain-1</i>	Starvation survival	miRISC component
Epidermis	<i>daf-18/PTEN</i>	Starvation survival	Insulin-like signaling
Epidermis	<i>mir-235</i>	Developmental arrest	Downstream of insulin-like signaling
Germ cells	<i>aak-2/AMPK</i>	Germ cell arrest	Energy homeostasis
Germ cells	<i>daf-18/PTEN</i>	Germ cell arrest	Insulin-like signaling
Somatic blast cells	<i>cki-1/p27</i>	Developmental arrest	Cell cycle regulation

Anatomical sites and genes are given along with the phenotype affected by their disruption and their general function.

negative regulator of the insulin-like/PI3K signaling pathway *daf-18/PTEN* is required for germ cell arrest during starvation (see *Germline arrest*), and *daf-18* mutants are even more sensitive to starvation than *daf-16* (Fukuyama *et al.* 2006, 2012). *aak-2*, which encodes an α -subunit of AMPK, is also arrest defective in the soma and germline, and it is sensitive to starvation (Baugh and Sternberg 2006; Fukuyama *et al.* 2012; Lee *et al.* 2012). Double mutation of *aak-1* and *aak-2*, disrupting two α -subunits of AMPK, enhances the arrest defect and causes severe starvation sensitivity comparable to mutation of *daf-18* (Fukuyama *et al.* 2012). Notably, the effects of *daf-18* and AMPK on starvation survival are independent of *daf-16*. They converge on TOR complex I for regulation of germ cell arrest, but the results of epistasis analysis does not suggest the same conclusion for starvation survival, though this could be due to incomplete penetrance of RNAi (Fukuyama *et al.* 2012).

Regulation of developmental arrest and starvation survival is anatomically complex (Table 4). Both *aak-2* and *daf-18* function in the intestine to promote starvation survival, but *daf-18* also functions in the epidermis though *aak-2* does not, and *aak-2* functions in neurons though *daf-18* does not (Fukuyama *et al.* 2012). These results indicate that AMPK and *daf-18/PTEN* function by at least partially distinct mechanisms to regulate starvation survival. In addition, transgenic rescue of *aak-2* function in intestine or neurons does not suppress the germ cell arrest defect of *aak-2* mutants (see above), suggesting distinct sites of action for regulation of starvation survival and germ cell quiescence. Curiously, *aak-2* functions in the epidermis and excretory cell to promote survival of dauer larvae, but function in these tissues does not affect survival of L1 arrest (Narbonne and Roy 2009; Fukuyama *et al.* 2012). Taken together these results reveal distinct contributions of different tissues to energy homeostasis, with critical differences between dauer and L1 arrest.

miRNAs and starvation survival

miRNAs contribute to regulation of L1 arrest by targeting the insulin-like pathway. *ain-1* encodes a component of the

miRISC, and mutation of *ain-1* reduces miRISC function. Han and coworkers found that *ain-1* mutants are sensitive to starvation and have reduced survival of L1 arrest (Zhang *et al.* 2011). They screened 72 miRNA mutants (affecting 87 miRNA genes) for starvation survival during L1 arrest and identified 10 mutants with reduced survival. *mir-71* is particularly sensitive to starvation, and *age-1/PI3K* and *unc-31/CAPS* are both epistatic to it (Zhang *et al.* 2011). *UNC-31* is required for dense core vesicle secretion and functions upstream of *DAF-2/InsR*, *AGE-1*, and the rest of the insulin-like signaling pathway (Figure 2; see *Insulin-like peptide secretion*). GFP-based miRNA sensors for the *age-1* and *unc-31* 3' UTRs were used to show that *Mir-71* directly targets *age-1/PI3K* and *unc-31/CAPS* (Zhang *et al.* 2011). In contrast to *daf-16*, the *mir-71* mutant is apparently not L1 arrest defective, though it is starvation sensitive. However, it is possible that an L1 arrest defect would have been detected if buffer S-basal had been used with 0.1% ethanol rather than without, since the added carbon source is necessary for the *daf-16* mutant arrest-defective phenotype (Baugh and Sternberg 2006; see *Environmental factors affecting starvation survival*). Nevertheless, vulval development is disrupted in the *mir-71* mutant after recovery from L1 arrest, but not in animals that had not been arrested, and the authors suggested that *mir-71* functions in maintenance of L1 arrest or recovery but not initiation (Zhang *et al.* 2011).

miRNA expression has been comprehensively examined during L1 arrest (Karp *et al.* 2011). Five miRNA genes including *mir-71* were found to be up-regulated during L1 arrest, and some but not all of them are also up-regulated in dauer larvae. The four in addition to *mir-71* were not reported to affect starvation survival (Zhang *et al.* 2011). *mir-235* is also up-regulated during L1 arrest, and mutation of *mir-235* causes an L1 arrest-defective phenotype without affecting starvation survival (Kasuga *et al.* 2013). Surprisingly, this result suggests that the L1 arrest-defective phenotype can be uncoupled from the starvation survival phenotype.

Other genes that affect dauer formation and lifespan

Insulin-like signaling regulates dauer formation and adult lifespan (Kenyon *et al.* 1993; Kimura *et al.* 1997; Hu 2007), but other pathways that regulate these processes do not affect starvation survival during L1 arrest. Loss of the TGF- β ligand *daf-7* or its receptor *daf-1* causes a constitutive dauer formation phenotype, and loss of the co-SMAD *daf-3* or the nuclear hormone receptor *daf-12* prevents dauer formation (Hu 2007). In contrast, disruption of these genes does not affect starvation survival (Lee and Ashrafi 2008; Lee *et al.* 2012). Mutation of *eat-2* causes defective feeding, providing a genetic model for extension of adult lifespan by caloric restriction (Raizen *et al.* 1995; Lakowski and Hekimi 1998). *isp-1*/ISP and *clk-1*/COQ7/CAT5 are involved in mitochondrial respiration, and *isp-1* and *clk-1* mutants have extended adult lifespan as well (Lakowski and Hekimi 1996; Feng *et al.* 2001). *eat-2*, *isp-1*, and *clk-1* mutants each extend lifespan independently of *daf-16*/FOXO, but none of them extend starvation survival. In fact, each of these mutants actually reduces starvation survival (Lee and Ashrafi 2008). These results demonstrate that regulation of starvation survival is somewhat distinct from regulation of dauer formation and adult lifespan, though insulin-like signaling plays a prominent role in each case.

Energy availability

Starvation survival depends on the availability of maternal energy stores and autophagy. Mutation of *tbc-2*, *rme-1*, or *rme-6* reduces embryonic yolk protein abundance, and each mutant also has reduced starvation survival (Chotard *et al.* 2010). It is unknown if starvation survival of these yolk mutants can be rescued by ethanol in the buffer, which can be used as a carbon source for fatty acid synthesis (see *Environmental factors affecting starvation survival*) (Castro *et al.* 2012). Autophagy also appears to provide energy during starvation, as RNAi of *bec-1*/Atg6/Vps30/Beclin1 reduces starvation survival (Kang *et al.* 2007). However, excessive autophagy can also compromise survival as well as recovery from arrest (see *MAPK, autophagy, and feeding*) (Kang *et al.* 2007).

Stress resistance

Stress resistance is increased during L1 arrest, and stress response genes promote starvation survival. For example, mutation of the heat shock factor *hsf-1* reduces survival comparable to *daf-16* (Baugh and Sternberg 2006). Mutation of the p53 ortholog *cep-1*, which participates in DNA damage response, also reduces starvation survival (Derry *et al.* 2001). Mutation of *pcm-1*/PCMT1, which encodes an L-isopartate O-methyltransferase that repairs damaged proteins, reduces starvation survival as well (Gomez *et al.* 2007). These results are consistent with starvation and other stresses causing common forms of molecular damage.

Adaptation to starvation during L1 arrest results in resistance to a variety of environmental stresses. Adult lifespan

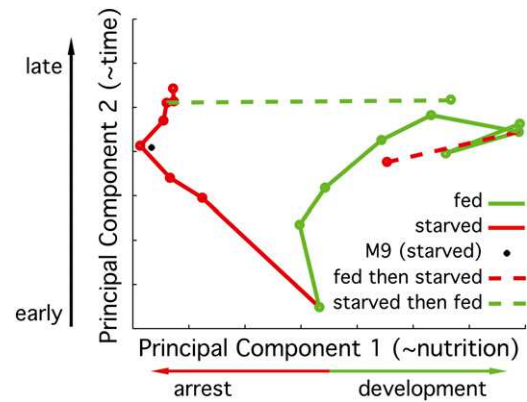


Figure 4 Gene expression dynamics during L1 arrest and development. mRNA abundance was analyzed in a pair of staged populations that hatched in the presence (green) or absence (red) of food (*E. coli*), and the first two principal components are plotted (Baugh *et al.* 2009). Samples from the starved population were fed to initiate recovery 12 hr after hatching and analyzed 3 hr later (green dashes), and samples from the fed population were starved 12 hr after hatching and analyzed 3 hr later (red dashes). Larvae starved in M9 buffer (black dot; no ethanol) are indistinguishable from larvae starved in S-basal (red; 20 mM ethanol) 3 hr after hatching.

is normal even after several days of L1 arrest, extending total lifespan (Johnson *et al.* 1984). The concept of “hormesis” posits that mild doses of a stressor increase fitness, and possibly lifespan, by the activation of stress response pathways (Gems and Partridge 2008). This ageless state during L1 arrest suggests that starvation induces hormesis, providing cross-tolerance to a variety of stresses.

The insulin-like signaling pathway affects resistance to multiple stresses during starvation. Arrested L1’s are resistant to freezing, enabling long-term storage of stocks (Lewis 1995). L1 arrest also increases heat resistance (Kang and Avery 2009b). Heat resistance requires *daf-16*/FOXO. Furthermore, *daf-2*/InsR, *age-1*/PI3K, and other mutants that extend lifespan further extend heat resistance during L1 arrest, consistent with a common genetic basis between lifespan and starvation-induced heat resistance (Munoz and Riddle 2003). Oxidative stress resistance is also increased during L1 arrest (Weinkove *et al.* 2006; Kang and Avery 2009b). Again, increased resistance to oxidative stress requires *daf-16*/FOXO as well as *daf-18*/PTEN, and mutations affecting *age-1*/PI3K further increase resistance, suggesting a common genetic basis between lifespan and starvation-induced oxidative stress resistance (Weinkove *et al.* 2006). Addition of leucine to the starvation buffer decreases starvation survival and resistance to heat and oxidative stress, providing additional evidence that resistance to multiple stresses is part of the starvation response (Kang and Avery 2009a). Furthermore, arrested L1 larvae are more resistant to anoxia than fed L1, L2, L3, and L4 larvae or adults (Padilla *et al.* 2002), and *daf-2*/InsR mutants are also more resistant to hypoxia than wild type (Scott *et al.* 2002). Mutation of *daf-2*/InsR or *age-1*/PI3K also confers resistance to hypertonic stress in *daf-16*/FOXO-dependent fashion (Lamitina and Strange 2005), implying increased resistance to hypertonic

stress during L1 arrest. Stress response genes are up-regulated during L1 arrest (Baugh *et al.* 2009), and regulators of different stress responses are required for starvation survival (see above), suggesting that adaption to starvation involves many of the same genes required to resist other stresses. Nevertheless, as with lifespan and starvation survival, the genetic basis of starvation survival and resistance to various environmental stresses have not been systematically compared.

Gene Expression

Gene expression dynamics

Nutrient availability has a profound impact on gene expression. Gene expression dynamics during L1 arrest and L1 development were characterized by high-density oligonucleotide microarray analysis (Baugh *et al.* 2009). Cultures were staged so that larvae hatched synchronously in the presence or absence of food, and arrested larvae were fed and fed larvae were starved 12 hr after hatching (Figure 4). At the most stringent level of statistical significance, 27% of protein-coding genes were affected by nutrient availability, which is more than the number affected during 24 hr of larval development. At a nominally significant threshold, over half of the genes were affected by nutrient availability. Time series analysis revealed that the full response to starvation occurs within 3–6 hr of hatching without food, and the expression profile is roughly constant between 6 and 24 hr of starvation. Destabilized reporter gene analysis of transcription factors up-regulated during starvation revealed active transcription for at least 2 days, suggesting that this constant expression profile reflects active maintenance of the starvation response at steady state. However, the expression profile changes rapidly in response to feeding during recovery from arrest (Figure 4), including up- and down-regulation of gene expression (Baugh *et al.* 2009). Expression dynamics during recovery were analyzed more extensively by mRNA-seq, revealing that the vast majority of genes that will be up-regulated within 6 hr of recovery are up-regulated within the first hour (Maxwell *et al.* 2012).

Alternative mRNA isoform expression

mRNA-seq analysis during L1 arrest and recovery revealed nutritional control of mRNA isoform expression (Maxwell *et al.* 2012). Splicing factors and other regulators of mRNA metabolism were themselves regulated by alternative splicing. Ribosomal proteins as well as factors regulating translation displayed alternative isoform expression. Signaling was also affected by alternative isoform expression, as evidenced by alternative inclusion of predicted phosphorylation sites (Maxwell *et al.* 2012). A similar, pervasive impact of alternative splicing on protein phosphorylation potential was subsequently found in vertebrates (Merkin *et al.* 2012), suggesting that this under-appreciated mode of regulation is fundamental. Taken together, analysis of alternative mRNA isoform expression revealed a variety of nutrient-dependent, post-transcriptional

regulatory mechanisms affecting mRNA splicing and metabolism, translation, and signaling networks.

Operons and rapid recovery

Approximately 20% of *C. elegans* genes are organized in operons (Blumenthal and Gleason 2003; Blumenthal 2005). These operons are transcribed to produce a single polycistronic transcript that is then processed to produce monocistronic transcripts for translation. Though operons are relatively exotic in metazoans, they are common among nematodes and also present in the chordates *Ciona intestinalis* and *Oikopleura dioica* (Ganot *et al.* 2004; Guiliano and Blaxter 2006; Ghedin *et al.* 2007; Abad *et al.* 2008; Satou *et al.* 2008). Given lack of evidence for horizontal gene transfer in metazoans, the existence of operons has been enigmatic. It has been suggested that operons facilitate coregulation of related genes, but the correlation of expression between pairs of operon genes is not much greater than that of closely linked nonoperon genes, and expression of genes in different operons is also correlated (Lercher *et al.* 2003; Zaslaver *et al.* 2011). In addition, operons tend to contain genes encoding “housekeeping” functions that are essential for growth (Blumenthal and Gleason 2003; Blumenthal 2005; Zaslaver *et al.* 2011).

Zaslaver *et al.* (2011) proposed that metazoan operons are evolutionarily adaptive because they bundle multiple growth genes under the regulation of a single promoter, extending transcriptional resources when they are limiting. They showed that genes encoding transcriptional machinery, which tend to be in operons, are expressed at relatively low levels during L1 and dauer arrest, and that expression of these genes increases immediately during recovery. They also showed that transcriptional resources are limiting during L1 arrest, making growth after arrest more sensitive to treatment with the transcriptional inhibitor α -amanitin than continuous growth without arrest (Zaslaver *et al.* 2011). Mathematical modeling suggests that a relatively small increase in effective transcriptional resources could significantly accelerate recovery from arrest (Zaslaver *et al.* 2011). Consistent with this model, operon expression in general increases dramatically during recovery from L1 and dauer arrest (Zaslaver *et al.* 2011; Maxwell *et al.* 2012). This pattern is conserved after metamorphosis in *C. intestinalis*, which is also a growth-intensive period in the lifecycle (Zaslaver *et al.* 2011). Operons are also highly expressed in the germline (Reinke and Cutter 2009), which grows rapidly and continuously to produce gametes; and they are expressed at relatively low abundance during embryogenesis, which is not associated with growth (Zaslaver *et al.* 2011). To summarize, operons appear to have evolved in metazoans at least in part to extend limiting transcriptional resources during periods of intense growth, for example during recovery from L1 arrest.

Postrecruitment regulation of RNA polymerase II

Gene expression changes rapidly in response to feeding during recovery from L1 arrest (Figure 4) (Baugh *et al.* 2009; Maxwell

et al. 2012). This led Baugh *et al.* (2009) to speculate that nutrient availability affects RNA polymerase II (Pol II) regulation postrecruitment. Recruitment of Pol II to the promoter was thought to be the principal point of transcriptional regulation in eukaryotes for decades. It was discovered that a few *Drosophila* genes are regulated postrecruitment during the early elongation phase of transcription, but they were regarded as exceptional (Rougvie and Lis 1988, 1990). In particular, Pol II is recruited to the heat-shock response gene Hsp70, initiates transcription, and then pauses elongation in the promoter-proximal region in noninducing conditions. Pol II resumes elongation in response to heat shock, completing synthesis of the Hsp70 transcript (Rougvie and Lis 1988; O'Brien and Lis 1991). This postrecruitment regulation of elongation ("pausing") is thought to facilitate a rapid response to heat shock.

Genome-wide Pol II chromatin immunoprecipitation studies in *Drosophila* and mammalian cells revealed that pausing is actually widespread, as evidenced by accumulation of Pol II in the promoter-proximal region of many genes (Kim *et al.* 2005; Guenther *et al.* 2007; Muse *et al.* 2007; Zeitlinger *et al.* 2007). Nascent transcript sequencing demonstrated that Pol II accumulated in the promoter-proximal region had initiated elongation (Core *et al.* 2008; Nechaev *et al.* 2010). Paused Pol II prohibits binding of a nucleosome in the promoter-proximal region, contributing to the maintenance of an open chromatin state permissive to rapid induction (Gilchrist *et al.* 2010). The multimeric negative elongation factor complex (NELF) contributes to pausing, and NELF knockdown increases nucleosome binding and reduces expression of many paused genes, consistent with pausing potentiating rather than repressing transcription (Gilchrist *et al.* 2008, 2010). NELF subunit homologs have not been discovered in the *C. elegans* or *Saccharomyces cerevisiae* genomes, and *bona fide* pausing has not been demonstrated in either organism.

Pol II accumulates at the 5' end of many growth and development genes in *C. elegans* during L1 arrest (Baugh *et al.* 2009). Baugh *et al.* (2009) hypothesized that this 5' accumulation facilitates a rapid and coordinated response to feeding. Postrecruitment regulation of Pol II could also enable quiescent cells to retain their identity. Consistent with this hypothesis, the ratio of Pol II binding density at 5' ends compared to coding regions decreases within 1 hr of recovery, and genes with 5' accumulation of Pol II increase average expression during recovery from arrest (Baugh *et al.* 2009). Pol II binding was measured but not activity, so it is unclear if 5' accumulation actually reflects pausing of elongation or an earlier, postrecruitment point of regulation (e.g., initiation). Interpretation was further confounded by the fact that transcription start sites remain largely unknown in *C. elegans* given 5' *trans*-splicing. These results are reminiscent of gene regulation in *S. cerevisiae*. Pol II accumulates in intergenic regions during stationary phase in *S. cerevisiae*, and it shifts to coding regions in response to fresh media during recovery from quiescence (Radonjic *et al.* 2005), but there is no evidence that this postrecruitment regulation of

Pol II reflects pausing and resumption of elongation in particular. In summary, nutrient-dependent postrecruitment regulation of Pol II is significant in *C. elegans* (Baugh *et al.* 2009), presenting an opportunity to complement studies in other metazoan systems.

Gene regulatory networks

Although the dynamics of gene expression during L1 arrest have been well characterized, functional analysis of gene regulation is limited. A set of 15 transcription factors most significantly up-regulated during L1 arrest were identified and studied by destabilized reporter gene analysis (Baugh *et al.* 2009). The activity of a gene product can certainly be affected by nutrient availability independent of effects on mRNA expression; for example, DAF-16/FOXO nuclear localization is regulated in response to nutrient availability (Henderson and Johnson 2001). Nevertheless, increased mRNA abundance is a convenient way to identify putative regulators. The identity and known function of transcription factors up-regulated during L1 arrest suggests that many of the factors involved in the starvation response also function during development. In addition, these factors are expressed in a variety of tissues, reflecting a complex anatomical response to starvation (Baugh *et al.* 2009). In addition to the 15 transcription factors studied, the majority of the unusually large nuclear hormone receptor family (284 members) are up-regulated during L1 arrest, including *nhr-49* (Baugh *et al.* 2009). *nhr-49* and its coactivator *mdt-15*/MED15 are known to regulate metabolic gene expression (see *Intermediary metabolism*) (Van Gilst *et al.* 2005a; Taubert *et al.* 2006), but their function has not been analyzed genome-wide. The transcription factor *hlh-13* is thought to be a direct target of DAF-16/FOXO; it is also up-regulated during L1 arrest, and it affects recovery from arrest (Liachko *et al.* 2009), but its function has also not been investigated genome-wide.

The forkhead transcription factors *daf-16*/FOXO and *pha-4*/FOXA are both required for starvation survival during L1 arrest. PHA-4 regulation has been examined with chromatin immunoprecipitation (ChIP-seq) of PHA-4::GFP during L1 arrest (Zhong *et al.* 2010). ChIP-seq identified 4621 putative PHA-4 target genes. This is a surprisingly large number of putative targets, and it is unclear how many are actually regulated by PHA-4. Nevertheless, PHA-4 targets differ between embryos and L1 arrest, where the gene has developmental and physiological functions, respectively, and PHA-4::GFP binding correlates with expression (Zhong *et al.* 2010). Furthermore, the number of putative PHA-4 target genes is in line with 21 other factors examined by the modENCODE consortium (Niu *et al.* 2011). In contrast to *pha-4*, mRNA expression has been analyzed in a *daf-16* mutant, and DamID, and alternative to ChIP, has been used to analyze DAF-16 binding (Murphy *et al.* 2003; Schuster *et al.* 2010). These experiments were done in young adults without starvation, and disruption of *daf-2*/InsR function by either mutation or RNAi was used to modulate DAF-16 activity. DamID

identified 907 putative DAF-16 targets, but integrative analysis of the gene expression data suggests that DAF-16 has detectable regulatory function at only 65 targets and that it functions as an activator but not repressor (Schuster *et al.* 2010). Caveats remain regarding the false-positive and -negative rates for each of these assays, and additional work is needed to characterize the regulatory function of these factors and others during L1 arrest.

Related Arrest Phenomena

Many different genetic and environmental perturbations can cause developmental arrest at the L1 larval stage. Zygotic mutation of many essential genes results in a terminal L1 arrest phenotype, since embryogenesis can often be completed with maternal gene product alone. However, starvation-induced L1 arrest is a reversible physiological response to nutrient availability that is mechanistically distinct from loss of essential gene function.

Starvation at other developmental stages triggers related arrest phenotypes. Other larval stages arrest development in response to starvation, and they can survive for at least as long as larvae in L1 arrest (Johnson *et al.* 1984; Van Gilst *et al.* 2005b; Ruaud and Bessereau 2006; Kang *et al.* 2007). Starvation of reproductive animals causes reversible shrinkage of the germline and slows down oogenesis dramatically (Angelo and Van Gilst 2009; Seidel and Kimble 2011). None of these arrest phenomena have been well characterized mechanistically, but presumably they are regulated in similar fashion to L1 arrest.

Other stresses besides starvation can also result in developmental arrest at the L1 stage. In many cases, embryos can hatch under the same conditions; perhaps the lack of growth during embryogenesis or the egg case renders them less sensitive. For example, larvae that manage to hatch at 30° arrest as L1 larvae (Munoz and Riddle 2003; Baugh and Sternberg 2006). In addition, larvae hatching in 200 mM NaCl arrest at the L1 stage. Nematodes are capable of adaptation to high salt (Lamitina *et al.* 2004; Frazier and Roth 2009), and development resumes within a day as larvae adapt, ultimately producing progeny that do not arrest upon hatching in 200 mM NaCl (L. R. Baugh, unpublished results). Anoxia causes immediate developmental arrest in embryos and larvae, as well as behavioral quiescence, and the mechanism for arrest is distinct from that of L1 arrest (Padilla *et al.* 2002). Likewise, exposure to relatively large amounts of diacetyl, a product of bacterial fermentation, also causes behavioral quiescence and developmental arrest (Hoffmann *et al.* 2010). It is unknown if or how developmental arrest is regulated in response to each of these stresses. Feeding is inhibited in response to relatively mild doses of heat shock, ethanol, and other stressors (Jones and Candido 1999), suggesting that developmental arrest could result indirectly from starvation due to altered behavior. Comparative analysis of the mechanisms governing behavior, arrest, and resistance in response to different stresses will elucidate direct and indirect physiological consequences of different stresses.

Summary and Unanswered Questions

Research on L1 arrest has brought many insights into how postembryonic development is governed in response to nutrient availability (Table 2, Table 3, and Table 4). Insulin-like and AMPK signaling are key regulators of developmental arrest, starvation survival, and stress resistance. Germline and somatic cells arrest at different points in the cell cycle, both under the control of these signaling pathways, but with different downstream effectors. Monomethyl branched-chain fatty acid synthesis is necessary for development, with its disruption triggering arrest independent of the insulin-like signaling pathway, as if it functions as a metabolic sensor. Feeding behavior is modified during L1 arrest via MAPK activation in the pharynx, and pharyngeal autophagy is necessary to survive starvation but can also limit recovery. Multiple tissues contribute to systemic regulation of developmental arrest and starvation survival, including chemosensory and other neurons as well as the intestine. Essential amino acid sensation reduces stress resistance and starvation survival, as if it is normally sensed in association with food. Ethanol is used as a carbon source during starvation, increasing survival without triggering development. Gene expression is dramatically different during L1 arrest and development, reflecting a shift in metabolism and increased stress resistance in response to starvation. Expression patterns are relatively stable after a few hours of starvation but change rapidly in response to feeding. Operons and postrecruitment regulation of RNA Pol II both appear to promote rapid induction of growth gene expression during recovery from arrest.

The considerable progress made toward mechanistic understanding of L1 arrest raises several major questions. For example, site(s) of action of insulin-like signaling and its integration is unclear. To the extent that insulin-like signaling functions cell-nonautonomously, downstream effectors are unknown. We also do not know which of the 40 predicted insulin-like peptides regulate L1 arrest, which cells express and secrete them, and how they are regulated. Other signaling pathways also contribute to regulation, but it is unclear how they are coordinated at the cellular and organismal levels. Many but not all of the genes that affect adult lifespan also affect starvation survival and stress resistance during L1 arrest, but without genome-wide screens and systematic comparisons we are left to speculate how these phenomena are mechanistically related. L1 arrest and recovery provides an excellent metazoan model to investigate regulation of gene expression in response to nutrient availability, but whole animals have been used for genomic analysis to date, obscuring tissue-specific regulation. Furthermore, the gene regulatory network mediating nutritional control of expression has hardly been characterized. It is also unclear how regulation of L1 arrest compares to related stress- and starvation-induced arrest phenomena. Nevertheless, with growing interest in the subject and emerging technologies, research on L1 arrest promises more insights into how animals survive feast and famine.

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