OXFORD GENETICS

GENETICS, 2022, 220(1), iyab202
https://doi.org/10.1093/genetics/iyab202
Advance Access Publication Date: 12 November 2021
Investigation

Insulin signaling and osmotic stress response regulate arousal and developmental progression of *C. elegans* at hatching

Emily A. Bayer [†], Katarina M. Liberatore,[‡] Jordan R. Schneider, Evan Schlesinger, Zhengying He, Susanna Birnbaum, and Bruce Wightman*

Biology Department, Muhlenberg College, Allentown, PA 18104, USA

*Corresponding author: Biology Department, Muhlenberg College, 2400 Chew St., Allentown, PA 18104, USA. Email: wightman@muhlenberg.edu
[†]Present address: Biozentrum, University of Basel, CH-4056 Basel, Switzerland.
[‡]Present address: Meyer Cancer Center, Weill Cornell Medicine, New York, NY 10021, USA.

Abstract

The progression of animal development from embryonic to juvenile life depends on the coordination of organism-wide responses with environmental conditions. We found that two transcription factors that function in interneuron differentiation in *Caenorhabditis elegans*, *fax-1*, and *unc-42*, are required for arousal and progression from embryogenesis to larval life by potentiating insulin signaling. The combination of mutations in either transcription factor and a mutation in *daf-2* insulin receptor results in a novel perihatching arrest phenotype; embryos are fully developed but inactive, often remaining trapped within the eggshell, and fail to initiate pharyngeal pumping. This pathway is opposed by an osmotic sensory response pathway that promotes developmental arrest and a sleep state at the end of embryogenesis in response to elevated salt concentration. The quiescent state induced by loss of insulin signaling or by osmotic stress can be reversed by mutations in genes that are required for sleep. Therefore, countervailing signals regulate late embryonic arousal and developmental progression to larval life, mechanistically linking the two responses. Our findings demonstrate a role for insulin signaling in an arousal circuit, consistent with evidence that insulin-related regulation may function in control of sleep states in many animals. The opposing quiescent arrest state may serve as an adaptive response to the osmotic threat from high salinity environments.

Keywords: sleep; diapause; insulin signaling; developmental progression

Introduction

Coordinated execution of developmental programs is essential for transition through animal life stages. In mammals, changes in circulatory, immunological, and gastrointestinal functions accompany the transition from fetus to neonate and broad hormonal effects on development accompany the onset of puberty (Henning 1981; Sisk and Foster 2004). Given the need for an organism-wide response, it is unsurprising that transition between stages is under endocrine control. For example, gonadotrophin-releasing hormone plays a central role in triggering body-wide developmental changes associated with puberty in mammals (Herbison 2016), and prothoracicotropic hormone and ecdysteroids trigger arthropod morphogenesis (Hiruma and Kaneko 2013). In *Caenorhabditis elegans*, insulin peptide and steroid signaling regulate entry into the dauer larva stage, a diapause that can interrupt continuous reproductive development for multiple months in response to adverse environmental conditions (Kimura *et al.* 1997; Hu 2007). The study of developmental transitions in invertebrates has been profitable in identifying conserved mediators of developmental control. For example, the heterochronic gene *lin-28*, first described in *C. elegans* (Ambros and Horvitz 1984), plays a role in regulating entry into puberty in mammals (Faunes and Larraín 2016). A thorough understanding of developmental progression in invertebrates has also played a key role in identifying molecular pathways that are relevant to human pathologies such as diabetes mellitus.

Regulation of the transition between developmental stages is as essential for development as specification of the stage itself, and in *C. elegans* this is associated with periods of quiescence. Nematodes enter a sleep state associated with molting between larval stages (Raizen *et al.* 2008). Quiescence at lethargus involves inactivity, reduced awareness, and cessation of feeding, similar to sleep in other animals (Campbell and Tobler 1984; Trojanowski and Raizen 2016; Bringmann 2018). Given the reduced activity and absence of nutritional intake, sleep could also be a mechanistic component of developmental arrest.

The nervous system is a critical site for the coordinated response to arrest cues via the insulin-signaling pathway; neuronal expression of the DAF-16 FOXO transcription factor, the mediator of the transcriptional response to DAF-2 insulin receptor, is sufficient to inhibit larval development (Baugh 2013). Although the sensory neurons that function in reception of environmental cues to orchestrate developmental transitions have

Received: September 29, 2021. **Accepted:** November 03, 2021
© The Author(s) 2021. Published by Oxford University Press on behalf of Genetics Society of America. All rights reserved.
For permissions, please email: journals.permissions@oup.com

been well-characterized in *C. elegans* (Thomas 1993), relatively little is known about interneuron regulation downstream of the initial sensory response.

A low-penetrance developmental delay phenotype associated with mutations in the *C. elegans* transcription factor genes *fax-1* and *unc-42* prompted us to explore a potential role for interneurons in regulating developmental transitions. *fax-1* encodes a conserved nuclear hormone receptor (PNR/NR2E3 in vertebrates) and *unc-42* encodes a paired-class homeodomain protein (Baran *et al.* 1999; Much *et al.* 2000; Wightman *et al.* 2005; Pereira *et al.* 2015). Both genes function in the specification of a limited number of discrete interneuron identities through an apparent combinatorial mechanism. Here, we report that combining mutations in either gene with mutations in *daf-2* insulin receptor causes a highly penetrant arrest phenotype at hatching. Arrested animals are inactive, fail to initiate pharyngeal pumping, and fail to execute the transition from an embryonic state to a hatched, free-swimming larva. Therefore, insulin signaling, abetted by interneuron function, is required for arousal, initiation of pharyngeal feeding behavior, and developmental progression at hatching, demonstrating that sleep, pharyngeal pumping, and developmental arrest are mechanistically linked.

Burton *et al.* (2018) have shown that nematodes arrest at hatching in response to elevated osmotic stress. The osmotic stress pathway involves an apparent neuroendocrine signal produced from sensory neurons. Genetic analysis of the interneuron-insulin pathway demonstrates that the insulin and osmotic stress signaling systems are antagonistic, with insulin signaling favoring arousal and progression and the osmotic stress response signal favoring quiescence and developmental arrest. We find that insulin-mediated arousal and osmotic stress-mediated sleep depend on neuropeptide sleep pathways, coupling the stress response to a prosleep physiological response.

Materials and methods

Nematode culture and genetics

Caenorhabditis elegans were cultured and Mendelian crosses performed using standard procedures on NGM plates fed OP50 bacteria at 15°C or 20°C (Brenner 1974; Stiernagle 2006). Mutations in *fax-1* and *unc-42* were followed in crosses and confirmed by their distinctive mobility defects. Mutations in *daf-2* were followed by identification of dauer larvae at 25°C. Other mutations were identified and confirmed by direct amplification genotyping or RFLP analysis (see Supplementary Table S5 for primers). A list of strains used in this study is shown in Supplementary Table S6.

Analysis of developmental arrest

To define the transient arrest phenotype of *fax-1* and *unc-42* mutants, we allowed adult animals to lay eggs on individual seeded NGM plates for 1 h at 20°C, then removed the adults. Plates were allowed to grow for 12, 24, 36, or 48 h. Once animals reached the desired time point, we picked all of the animals from the plate to 5 mM sodium azide on a 2% agarose pad and visualized by DIC Nomarski microscopy using a Nikon UD microscope.

Our standard perihatching arrest assay was performed by moving animals to be tested from 15°C or 20°C culture temperature to 25°C for 30 min to 1 h. Preseeded NGM test plates were preincubated for at least 30 min at 25°C in parallel. For each trial, six to eight gravid hermaphrodites were picked onto test plates and allowed to lay eggs at 25°C for 2 h before being removed, resulting in approximately 25–100 freshly-laid synchronized eggs per plate. Plates were incubated for 48 h at 25°C and examined under a Zeiss Stemi dissecting microscope at 100× magnification (arrested L1 larvae are difficult to spot in the food lawn at lower magnification). Over this time period, wild-type animals progress to L4 or early adult stages, whereas *daf-2* mutants reach L3 and arrest as dauer larvae. Animals that were still entrapped in eggshells or were at the L1 stage after 48 h were scored as perihatching arrested. L2 stage larvae, while clearly developmentally delayed, were not scored as arrested. Most double and triple mutant combinations that include a *daf-2* mutation arrested as dauer larvae when perihatching arrest was suppressed, indicating a higher threshold for insulin function in dauer formation when compared with perihatching arrest. The exceptions include combinations with *daf-16* or *daf-18* mutations, both of which suppress the dauer-constitutive phenotype of *daf-2* mutations.

Temperature-shift experiments were performed by preincubating NGM plates for 1 h at either 15°C or 25°C. We then prepared eggs by bleach treatment (Stiernagle 2006) and washing three times in S basal medium. Embryos were incubated in 0.5 ml S basal with gentle agitation at 15°C or 25°C for 24 h, ensuring that all embryos complete embryogenesis. Animals that hatch in the absence of food arrest as L1 larvae. Animals at both embryonic incubation temperatures were transferred to NGM plates at 25°C and assessed for perihatching arrest after 24 h as described above. Approximately 1% of embryos were apparently killed by bleach treatment, based on the absence of the typical perihatching threefold embryo morphology and were not counted in our analysis.

Salt-dependent arrest was assessed on NGM plates supplemented with NaCl to concentrations ranging from 100 to 500 mM. Standard NGM plates, with a NaCl concentration of 57 mM provided controls. Because high salt inhibits egg-laying, we prepared egg populations by preincubating the strains to be tested at 25°C for 1 h on NGM plates then isolating eggs by bleach treatment (Stiernagle 2006) or by direct dissection of eggs from living hermaphrodites by excision with a pair of 18G hypodermic needles. Both treatments can result in embryonic death, so all trials were verified by parallel controls on standard seeded NGM plates. Any trial with >5% embryonic lethality on control plates was discarded. We noted a general increase in embryonic arrest on higher salinity plates when eggs were prepared by bleach treatment. Strains on NaCl plates and control plates were grown at 25°C for 24 h and scored for perihatching arrest as described above.

Arousal and response latency were examined by mounting late-stage embryos or newly hatched L1 animals on agarose pads in M9 media. After a 15- to 30-min rest period, quiescent animals were stimulated by illumination with an ultraviolet lamp on a Nikon UD microscope, filtered by a EGFP filter cube (470 nm excitation), with maximum illumination. Response was measured in seconds using Ethotimer to record events.

Pharyngeal pumping was measured by mounting newly hatched L1 animals into a generous aliquot of OP50 bacteria in M9 medium onto an agarose pad. After a 30-min rest period, animals were observed using DIC microscopy and posterior corpus pharyngeal contraction events recorded using Ethotimer software.

Construction of fusion gene and transgenics

We constructed a core *gfp::fax-1* construct by amplifying a 1.2 kb *fax-1* cDNA with NheI and BspEI sites appended and ligating this fragment into plasmid pPD117.01, creating a promoterless in-frame *gfp::fax-1* fusion with synthetic introns in the GFP coding region (pFAX1GFPNB). Into this plasmid, we ligated an amplified

1.6 kb fragment upstream of the *glr-5* gene, using appended PstI and XmaI sites. The resulting construct was injected into wild-type *C. elegans* along with a pRF4 Rol-6 marker to create transgenic arrays *bwEx193–bwEx196* (Evans 2006). We confirmed expression of GFP::FAX-1 in neurons by GFP fluorescence using a Nikon UD fluorescence microscope. Arrays were crossed into a *fax-1(gm83); daf-2(e1370)* background and assessed for rescue of perihatching arrest as described above. Some rescued dauer larvae were non-Rol, but had detectable GFP fluorescence, indicating presence of the transgene.

Statistical analysis

Pairwise comparisons of arrest phenotypes were assessed for statistical analysis using a two-sample unequal variance two-tailed *T* test. *P* values of <0.05, <0.01, and <0.001 are indicated in figures and tables, where relevant. Experiments described were performed in triplicate at a minimum. Data reported in Supplementary Tables S1–S3 represent aggregate totals from multiple individual experiments, some of which are reported in other figures. Control experiments from Figure 2B are duplicated in Figures 3–5 to allow direct comparison to a larger, representative control dataset.

Reagent sharing plan

Strains created will be stored at the *Caenorhadbitis* Genetics Center, when accepted according to their criteria, or stored in the Wightman Lab collection at Muhlenberg College and made available on request. DNA constructs referenced are stored at the Wightman Lab collection at Muhlenberg College and available upon request.

Results

Mutations in *fax-1* and *unc-42* cause a low penetrance developmental delay phenotype

Routine culturing of *fax-1* mutant strains suggested that the overall growth rate of *fax-1* mutant populations was somewhat slow compared with wild-type or strains carrying other mutations that affect the nervous system. Examination of staged populations revealed that 9–16% of *fax-1* mutant animals lagged behind the population as a whole (Figure 1, A and B). The incomplete penetrance of the developmental delay phenotype is not due to hypomorphic allele "leakiness" because both *fax-1* alleles used in this study are null mutations (Wightman *et al.* 2005). While the most developed animals after 12 h of growth were late L1, just as for wild-type, some *fax-1* mutant animals were still in an arrested early L1 state, with morphology identical to newly hatched animals, despite the presence of food. After 24 h of feeding, laggards were no longer arrested as early L1 animals, but continued to trail behind the wild-type animals. A conservative estimate indicates an average delay for *fax-1* mutants of about 7 h of developmental time after 12 h of feeding, 10 h average delay after 24 h of feeding, and 13 h average delay after 48 h of feeding (Figure 1B). We observed extraordinary animals (<1%) that were arrested in early L1 even after 48 h of being in the presence of food. The length of the L3 stage for *fax-1* mutant larvae was 332 min, similar to 328 min for wild-type ($N = 10$). In addition, the mean time spent in L4 lethargus for *fax-1* mutants was 138 min, similar to 130 min for wild-type ($N = 14$). Based on these observations we conclude that the developmental delay in *fax-1* mutants stems from a transient arrest primarily by newly hatched larvae, although affected animals may also be somewhat slower in progressing through later larval stages.

We previously showed that mutations in *unc-42* cause overlapping phenotypes with *fax-1*, and that UNC-42 coordinately regulates some neuron specific genes with FAX-1 (Wightman *et al.* 2005). Given this, we examined *unc-42* mutants to see if they also displayed a developmental delay phenotype. Similar to *fax-1* mutants, a 15% subset of the population of *unc-42* mutants lagged behind the population as a whole, with a transient early L1 arrest (Figure 1C). Combining mutations in both *fax-1* and *unc-42* did not increase the severity of the transient arrest phenotype (Figure 1C), consistent with *fax-1* and *unc-42* functioning in a linear pathway.

Mutations in *fax-1* and *unc-42* cause a synthetic perihatching arrest phenotype in combination with mutations in the *daf-2* insulin receptor

Given the well-established role of the insulin-signaling pathway in mediating developmental transitions and arrest (Baugh and Sternberg 2006), we explored whether mutations in *fax-1* and *unc-42* would interact genetically with mutations in the known insulin-signaling pathway. Strong mutations in the *daf-2* insulin receptor cause L1 arrest at 25°C, while weaker mutations in *daf-2* cause nearly 100% arrest at the alternative L3 dauer stage (Gems *et al.* 1998). Due to inherent temperature-sensitivity of the arrest process, both of these mutations resist arrest when grown at 15°C. Strains carrying mutations in *fax-1* or *unc-42* caused a highly penetrant arrest phenotype in combination with a weak *daf-2(e1370)* allele when grown at 25°C just before or after hatching (Figure 2 and Table 1). We refer to the timing of this arrest as "perihatching," since animals arrest on one side or the other of hatching—either as fully formed animals still coiled inside the eggshell or as early L1 with posthatching morphology.

A similar perihatching arrest phenotype was observed when *fax-1* mutations were combined with other weak *daf-2* mutations (Supplementary Table S1). Mutations in *daf-2* have been characterized as "Class 1" and "Class 2" based on their enhancement and suppression interactions with other insulin pathway mutations (Gems *et al.* 1998). Both Class 2 alleles tested, *e1370* and *m579*, caused a similar high-penetrance synthetic perihatching arrest, when combined with a *fax-1* mutation, but two Class 1 alleles did not. However, a third Class 1 allele (*m41*) caused a modest penetrance synthetic arrest, indicating that the phenomenon is allele-specific but not strictly correlated with the two classes previously described.

Because dauer formation is temperature-sensitive, we explored whether perihatching arrest was similarly dependent on temperature. Both *daf-2; fax-1* and *daf-2; unc-42* double mutants did not display perihatching arrest when grown at the permissive temperature of 15°C (Supplementary Table S1). Therefore, the perihatching arrest phenotype is temperature-sensitive, just like the dauer arrest phenotype. Perihatching arrested double mutants grown at the nonpermissive 25°C for 48 h could be "rescued" by dropping the temperature for 96 h to the permissive temperature 15°C (Figure 2C), demonstrating that the phenotype is indeed a developmental arrest as opposed to an irreversible lethality. Furthermore, double mutant embryos that were grown until hatching at 15°C and then transferred after hatching to 25°C did not arrest until dauer, indicating that perihatching arrest is an embryonic function, rather than a posthatching larval function (Figure 2). These results distinguish perihatching arrest from the previously described posthatching L1 arrest that results from starvation and strong *daf-2* insulin receptor mutations (Baugh and Sternberg 2006).

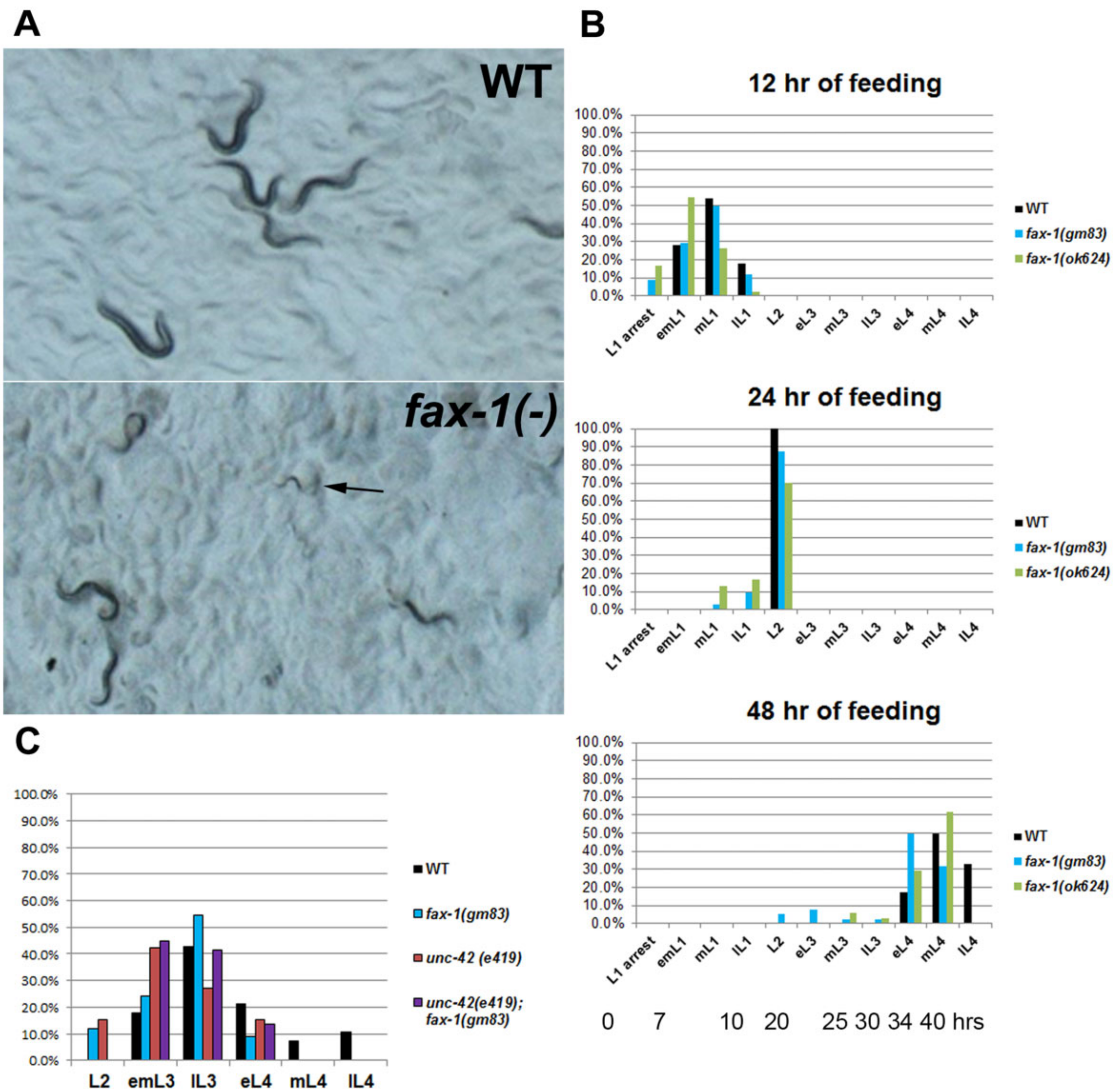

Figure 1 Developmental delay in transcription factor mutants. Development is delayed in a subpopulation of nematodes carrying mutations in *fax-1* or *unc-42*. (A) Micrograph showing wild-type and *fax-1(gm83)* mutant strains grown as a synchronized culture at 20°C for 48 h with 40X magnification. Arrow identifies a younger larva illustrating developmental delay within the population. (B) Progressive analysis of populations of wild-type and *fax-1* mutants after 12, 24, and 48 h of feeding at 20°C. Each genotype is illustrated by a single experiment with a representative population. Numbers at bottom of panel define approximate wild-type times after hatching for each stage at 20°C. (C) Stages of populations in *fax-1(gm83)*, *unc-42(e419)*, and double mutants after 48 h of feeding at 20°C. Data shown are the distributions of representative single populations for each genotype, with each time-point of analysis evaluating a population of 26–59 animals. Staging definitions: L1 arrest = L1 stage larvae with anatomy as at hatching; emL1 = early and middle-stage L1 larvae with divisions of ventral nerve cord cells and Z2/Z3 germ-line cells; lL1 = late-stage L1 larvae with divided Z1/Z4 somatic gonad cells; L2 = L2 larvae; eL3 = early L3 larvae with extended gonad arms; mL3 = mid-L3 larvae with anchor cell of gonad positioned over vulval precursor cells; lL3 = late L3 larvae with divided vulval precursor cells; eL4 = early L4 larvae with initial vulval invagination; mL4 = mid-L4 larvae with "Christmas tree" morphology invaginated vulva; lL4 = late L4 larvae with collapsing vulva.

To begin to define the site of *fax-1* function in controlling perihatching arrest, we used the promoter of the *glr-5* glutamate receptor gene to drive expression of a *gfp::fax-1* fusion gene in a limited number of interneurons that overlap with *fax-1* and *unc-42* expression and function (Brockie *et al.* 2001) (Supplementary Figure S1). The *Pglr-5::gfp::fax-1* was able to rescue the perihatching arrest phenotype of *fax-1(gm83); daf-2(e1370)* mutant embryos at 25°C (Figure 2B), but did not rescue the dauer-formation defect caused by the *daf-2* mutation. This result argues that *fax-1* function in one or more of the neuron classes common to the expression of *fax-1* and *glr-5* is the site of *fax-1* function in regulating arrest at hatching. This analysis implicates the interneuron classes AVA, AVB, AVE, AVK, DVA, RIC, and SIB (Supplementary Figure S1). Our efforts to delimit this requirement further have not revealed a specific neuronal site of action, leaving open the possibility that function in a combination of neurons is necessary.

We considered the possibility that *daf-2*-dependent perihatching arrest might be a general or trivial effect of nervous system developmental or physiological disruption. Previous studies have identified various synthetic effects of mutations in nervous system genes with *daf-2* mutations (Ailion and Thomas 2003). We examined the effect of mutations in other transcription factors that perturb nervous system development, known downstream targets of *fax-1* and *unc-42*, genes that are required generally for axon pathfinding, and genes that function in cellular neurophysiology (Supplementary Table S2). Mutations in most other transcription factors caused no or very low penetrance perihatching

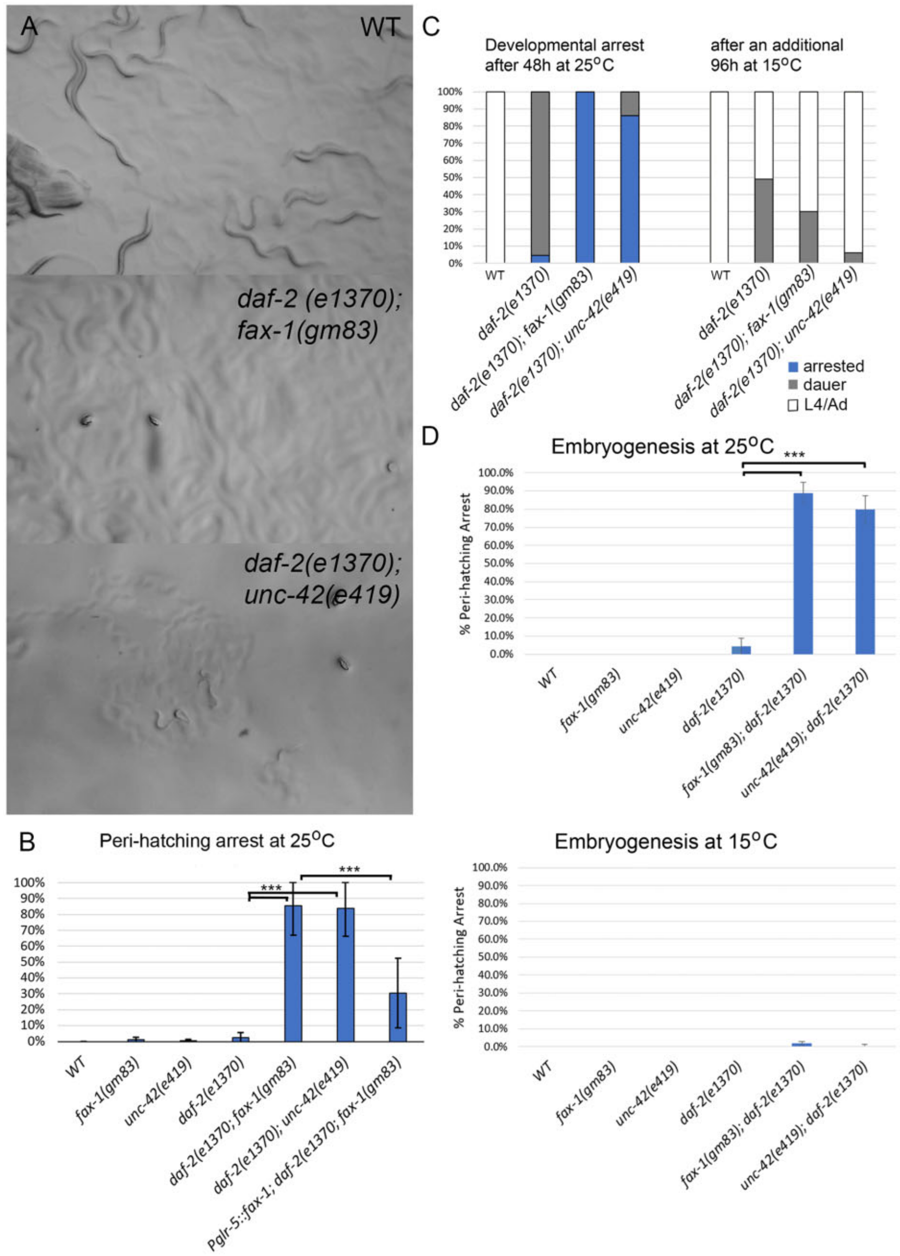

Figure 2 Perihatching arrest in double mutants. The perihatching arrest phenotype in *daf-2/InsR;* transcription factor double mutants is shown. (A) Micrographs from staged populations of embryos after 24 h at 25°C. Top panel shows late L1 and early L2 larvae in wild-type; middle and lower panel shows arrested late embryos and newly hatched L1 larvae in *fax-1* and *unc-42* mutants. 50X magnification. (B) Penetrance of perihatching arrest in standard 48 h at 25°C assays. Animals were considered arrested if they were still encased within an eggshell or arrested in the L1 stage. ***Pairwise comparisons are significantly different at $P \ll 0.001$. (C) Recovery of strains arrested at 25°C by incubation for 96 h at the permissive temperature of 15°C. (D) Temperature shift experiment in which embryos were allowed to develop at 25°C (top panel) or 15°C (bottom panel), shifted to 25°C after hatching and then assayed for arrest after 24 h. Error bars indicate standard deviation among replicate experiments.

arrest. One exception was the *unc-30 (e318)* mutation, however, synthetic perihatching arrest was not seen with *unc-30 (e191)*, suggesting that this arrest could be due to a genetic background effect. Mutations in known downstream targets of *fax-1* and *unc-42* did not cause a synthetic arrest. Mutations in genes required for various aspects of neural signaling caused low to modest penetrance perihatching arrest. In particular, this includes genes required for neuropeptide processing or release (*egl-3, egl-21*, and *unc-31*), which is not surprising given the dependence of perihatching arrest on insulin signaling. Disrupting chemical synapses (*unc-64*) and gap junctions (*unc-9*) caused a modest penetrance phenotype that was similar to perihatching arrest, raising the possibility that arrest depends in part on chemical neurotransmitter or gap junction connectivity. Compromising acetylcholine, GABA, and octopamine signaling pathways did not cause a synthetic arrest. Mutations in two genes that function in axon pathfinding, *unc-34* and *unc-69*, did cause a synthetic arrest, however the time of arrest for *unc-69* was well after hatching and neither synthetic effect was suppressible by *ssu-1* (Supplementary Table S2), on which the perihatching arrest pathway depends (see below). These results indicate that *unc-34* and *unc-69* mediate arrests that are mechanistically distinct from the pathway dependent on *fax-1* and *unc-42*. Overall, this analysis is consistent with the notion that perihatching synthetic arrest is a

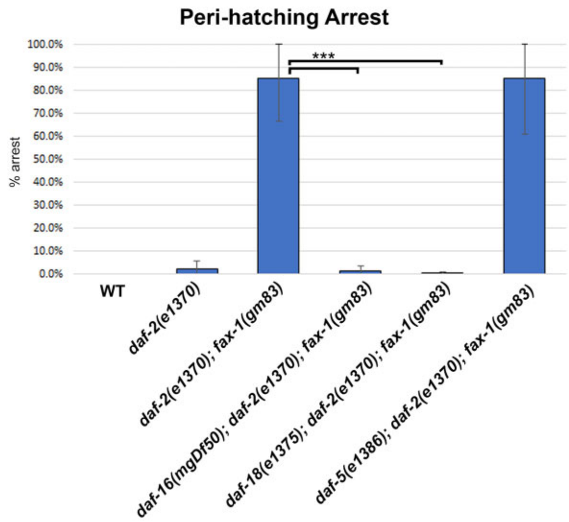

Figure 3 Suppression of perihatching arrest by insulin pathway mutations. Mutations in *daf-16/FoxO* and *daf-18/PTEN* fully suppress the perihatching arrest of *daf-2; fax-1* double mutants. In contrast, a *daf-5* TGFβ pathway mutation fails to suppress. Figure shows average results of standard 48 h at 25°C assays from multiple independent trials. Data shown are also included in Table 2. ***Pairwise differences significant at $P \ll 0.01$. Error bars indicate standard deviation among replicate experiments.

specific phenomenon tied to the development of particular neurons, rather than a general result of disrupting axon pathfinding in the context of reduced insulin signaling.

Perihatching arrest does not require the TGFβ or steroid pathways

Mutations in the TGF-β pathway *daf-1*, *daf-7*, and *daf-8* genes all cause a loss-of-function dauer-constitutive (Daf-c) phenotype, similar to *daf-2*. *daf-1* encodes a TGF-β receptor, *daf-7* encodes a TGF-β peptide precursor protein, and *daf-8* encodes an SMAD protein that functions in the TGF-β response (Hu 2007; Fielenbach and Antebi 2008). We examined whether *fax-1* and *unc-42* mutations would also cause an L1 arrest phenotype in combination with Daf-c mutations in the TGF-β pathway. For all three TGF-β pathway genes, the addition of *fax-1* or *unc-42* mutations did not cause a synthetic L1 arrest (Table 1). Therefore, in contrast to Daf-c mutations in insulin receptor, the synthetic perihatching arrest phenotype does not occur with Daf-c TGF-β pathway mutations.

Mutations in *daf-9* 3-keto-sterol-26-monooxygenase, which is required for production of dafachronic acids, and gain-of-function mutations in *daf-12* nuclear receptor also cause a Daf-c phenotype (Jia *et al.* 2002; Hu 2007). Just as observed for the TGF-β pathway, these mutations did not cause a synthetic L1 arrest when paired with either *fax-1* or *unc-42* (Table 1). Therefore, the TGF-β pathway and steroid pathways, although important for dauer arrest, do not appear to function in perihatching arrest.

Table 1 Developmental arrest at 25°C

Genotype	% perihatching arrest	% dauer	% L4/Ad	N
Wild type	0.0	0.0	100.0	512
fax-1(gm83)	1.1	0.0	98.9	648
fax-1(ok624)	2.5	0.0	97.5	197
unc-42(e419)	3.8	0.0	96.2	416
unc-42(e623)	4.0	0.0	96.0	122
daf-2(e1370)	2.3	97.7	0.0	1152
daf-2(e1370); fax-1(gm83)	85.6*	14.4	0.0	1226
daf-2(e1370); fax-1(ok624)	82.7*	17.3	0.0	150
daf-2(e1370); unc-42(e419)	83.9*	16.1	0.0	709
daf-2(e1370); unc-42(e623)	97.7*	2.3	0.0	128
daf-7(ok3125)	0.0	98.3	1.7	234
daf-7(ok3125); fax-1(gm83)	0.0	98.1	1.9	160
daf-7(ok3125); unc-42(e419)	1.0	94.8	4.1	97
daf-1(m40)	0.0	97.9	2.1	380
daf-1(m40); fax-1(gm83)	3.6	95.4	1.0	194
daf-1(m40); unc-42(e419)	10.0	90.0	0.0	70
daf-8(e1393)	0.0	95.7	4.3	225
daf-8(e1393); fax-1(gm83)	1.6	94.5	3.9	307
daf-8(e1393); unc-42(e419)	2.9	95.4	1.7	241
daf-9(m540)	0.6	93.6	5.8	171
daf-9(m540) fax-1(gm83)	0.0	100.0	0.0	140
daf-12(rh274)	4.9	69.1	25.9	81
daf-12(rh274) fax-1(gm83)	1.4	81.6	16.9	207

The percentage of animals with a given genotype that arrest at hatching (% perihatching arrest), arrest as L3 dauer (% dauer) and develop into L4 larvae and eventually adults (%L4/Ad) after 48 h of growth at 25°C.
*Significantly different from *daf-2(e1370)* mutants at $P \ll 0.001$.

Perihatching arrest depends on canonical insulin pathway mediators

The roles of *fax-1* and *unc-42* in perihatching arrest in different pathways can be tested by the ability of Daf-d mutations to suppress arrest caused by *fax-1; daf-2* and *unc-42; daf-2* double mutants. The *daf-16* FOXO transcription factor is a key mediator of the response to insulin signaling; mutations in *daf-16* suppress the Daf-c and L1 arrest phenotypes of insulin pathway Daf-c mutations (Vowels and Thomas 1992; Tissenbaum 2018). The *daf-16 (mgDf50)* mutation was able to suppress the perihatching arrest phenotype of *fax-1; daf-2* and *unc-42; daf-2* double mutants, with most animals avoiding arrest at hatching or dauer (Figure 3 and Table 2). Similarly, *daf-18* encodes a PTEN tyrosine phosphatase, which functions downstream of *daf-2* insulin receptor in the canonical insulin-signaling response (Ogg and Ruvkun 1998). As observed for *daf-16*, the addition of a *daf-18* mutation fully suppressed the perihatching arrest of *fax-1; daf-2* double mutants (Figure 3). Taken together, these data indicate that perihatching arrest is a function of canonical insulin signaling in responding cells.

In contrast, a gene required for TGF-β signaling response was not able to suppress the perihatching arrest phenotype. The *daf-5* gene encodes a cytoplasmic component that is required for TGF-β signaling response (de Graca *et al.* 2004). Mutations in *daf-5* cause a dauer-defective phenotype and are able to suppress the Daf-c phenotype of upstream TGF-β pathway mutations such as those in *daf-7* TGF-β and *daf-1* TGF-β receptor. Mutations in *daf-5* failed to suppress the perihatching arrest phenotype of both *fax-1; daf-2* and *unc-42; daf-2* double mutants (Figure 3 and Table 2). Combined with the failure of Daf-c TGF-β pathway mutations to cause a synthetic arrest phenotype, these data demonstrate that perihatching arrest is a function of insulin signaling but not TGF-β signaling, thereby distinguishing it from global control of dauer formation and arrest.

Mutations in *fax-1* and *unc-42* also reduce insulin-like signaling associated with dauer larva formation

The *daf-2 (e979)* mutation causes a stronger loss-of-function in insulin receptor function than the canonical *e1370* mutation (Gems *et al.* 1998). Homozygous *daf-2 (e979)* mutants form dauers at 15°C and arrest during embryogenesis or during the L1 stage after hatching at 25°C. To determine if *fax-1* or *unc-42* mutations enhance this stronger insulin receptor mutation, we constructed double mutants with *daf-2 (e979)*. Both *fax-1 (gm83); daf-2 (e979)* and *unc-42 (e419); daf-2 (e979)* strains displayed an enhanced dauer formation defect at 15°C (Supplementary Table S3). The dauer-constitutive phenotype of *fax-1 (gm83); daf-2 (e979)* was sufficiently severe (99%) that the strain could not be maintained in double homozygous form even at the normally permissive temperature of 15°C (rare animals that exit from the dauer stage produce nearly all dauers in the next generation). The dauer entry decision is made in late L1, and dauer exit depends on pathways that operate in the alternative L3 dauer stage. Therefore, both *fax-1* and *unc-42* mutations also compromise an insulin-dependent signal later in later larval development. In contrast, the increased longevity of *daf-2* mutants was not enhanced by *fax-1* mutations (Supplementary Figure S2), thus separating the function of insulin signaling in developmental progression from that of aging. Taken together, the evidence that these two transcription factors enhance the severity of several different insulin pathway functions indicates that *fax-1* and *unc-42* normally potentiate multiple aspects of insulin signaling, further supporting the conclusion that loss of *fax-1* and *unc-42* decreases insulin signaling.

Perihatching arrested animals are in a quiescent sleep state

Microscopic examination of perihatching arrested animals revealed that they were in an inactive, qiescent state. Arrested *fax-1; daf-2* and *unc-42; daf-2* animals had grossly normal late embryonic morphology and anatomy (cuticle and alae), but were immobile in a state of quiescence (Figure 4A). While wild-type embryos actively roll within the eggshell throughout the second half of embryogenesis, double-mutant arrested animals were either entirely inactive or displayed only occasional sluggish attempts at forward, backward, or axial movement. About half of the arrested animals were still enclosed in an eggshell. Others had a partially digested eggshell still around them, with a tail poking out one end, as if their inactivity rendered them incapable of escaping the eggshell after hatching. Still other double mutants hatched as L1 animals that were inactive and were sometimes found folded in a fourfold "pretzel" contortion—the posture of late embryogenesis. Greater than 90% of arrested double mutant embryos and L1 animals displayed defective pharyngeal pumping: either no pharyngeal contraction at all or only a weak contraction of the posterior pharyngeal bulb every 5–10 s. Less than 10% of double mutant embryos hatched and displayed sluggish movement and pharyngeal pumping—they presumably represent the small escaper population in double mutants that arrest as dauer larvae (Figure 2 and Supplementary Table S1).

Table 2 Effect of Daf-d mutations on developmental arrest at 25°C

Genotype	% perihatching arrest	% dauer	% L4/Ad	N
Wild type	0.0	0.0	100.0	512
fax-1(gm83)	1.1	0.0	98.9	648
unc-42(e419)	3.8	0.0	96.2	416
daf-2(e1370)	2.3	97.7	0.0	1152
daf-2(e1370); fax-1(gm83)	85.6	14.4	0.0	1226
daf-2(e1370); unc-42(e419)	83.9	16.1	0.0	709
daf-16(mgDf50)	0.0	0.0	100.0	149
daf-16(mgDf50); daf-2(e1370)	0.0	0.0	100.0	239
daf-16(mgDf50); fax-1(gm83)	4.4	0.0	90.4	135
daf-16(mgDf50); unc-42(e419)	1.8	0.0	96.5	228
daf-16(mgDf50); daf-2(e1370); fax-1(gm83)	4.9*	0.0	87.3	102
daf-16(mgDf50); daf-2(e1370); unc-42(e419)	1.3*	0.0	98.3	236
daf-18(e1375)	0.0	0.0	100.0	43
daf-18(e1375); daf-2(e1370)	2.7	2.7	94.6	111
daf-18(e1375); fax-1(gm83)	0.0	0.0	100.0	132
daf-18(e1375); daf-2(e1370); fax-1(gm83)	0.0*	0.0	100.0	346
daf-5(e1386)	11.3	0.0	88.7	97
daf-5(e1386); daf-2(e1370)	1.5	96.9	1.5	65
daf-5(e1386); fax-1(gm83)	6.3	0.0	91.5	176
daf-5(e1386); fax-1(gm83); daf-2(e1370)	94.4	5.6	0.0	107
daf-5(e1386); unc-42(e419)	10.5	0.0	83.0	153
daf-5(e1386); unc-42(e419); daf-2(e1370)	89.3	10.7	0.0	178

The percentage of animals with a given genotype that arrest at hatching (% perihatching arrest), arrest as L3 dauer (% dauer), and develop into L4 larvae and eventually adults (%L4/Ad) after 48 h of growth at 25°C. The table totals data from two or more independent trials.
*Significantly different from matched double-mutant controls at $P \ll 0.001$.

Given that both *fax-1* and *unc-42* mutations compromise the ability of larvae and adults to move, we examined *fax-1; daf-2* and *unc-42; daf-2* double mutant embryos by time-lapse from the "comma stage," which initiates morphogenesis at approximately 6 h of embryonic development, through the time of hatching at approximately 12 h (Figure 4B). Double mutant embryos completed morphogenesis normally, with twitching apparent at the twofold stage, just as in wild-type, and moved actively during threefold and quickening (9–11 h). Active movement similar to wild-type persisted through the period of cuticle synthesis at approximately 11 h, before coming to a halt as the embryo proceeds to the final stages of hatching. wild-type embryos initiated pharyngeal pumping inside the eggshell about 30–60 min before hatching. Double-mutant embryos ceased movement by this time and did not initiate pharyngeal pumping. These observations indicate that morphogenesis and embryonic movement is normal in perihatching arrested animals until the last 1–2 h of embryonic development, at which time they enter a state of quiescence.

If insulin signaling is required for arousal at hatching, then we might expect stronger mutations in *daf-2* insulin receptor to display a perihatching arrest phenotype similar to the *fax-1; daf-2* and *unc-42; daf-2* double mutants. The *daf-2 (e979)* mutation has the strongest phenotype of *daf-2* alleles that are homozygous viable (Gems *et al.* 1998). Previous reports have described a defect in morphogenesis of *daf-2 (e979)* homozygous embryos (Suresh and Wightman 2020) and an L1 arrest phenotype after hatching (Baugh and Sternberg 2006). We examined *daf-2 (e979)* mutants at 25°C and discovered that 23.5% of embryos that undergo normal morphogenesis arrest as perihatching arrested embryos similar to *fax-1; daf-2* and *unc-42; daf-2* double-mutants (Figure 4, B and C) or as the previously described active arrested L1 larvae. Like the double mutants, *daf-2(e979)* mutant embryos that arrest at the end of embryogenesis are in a quiescent state and fail to initiate pumping. The complexity of the *daf-2(e979)* phenotype indicates multiple times and processes for insulin function in development: morphogenesis, arousal at hatching, and developmental progression after hatching. Therefore, the *fax-1; daf-2* and *unc-42; daf-2* double mutants appear to reveal a specific component of insulin-signaling function in the hour before hatching.

One property of sleep is its reversibility by potent sensory stimulus (such as sound or light), despite lowered overall sensory awareness (Borbély 1982; Huber *et al.* 2004; Raizen *et al.* 2008; Trojanowski and Raizen 2016). To test if arrested embryos could be aroused by sensory stimulus, we applied blue wavelength light

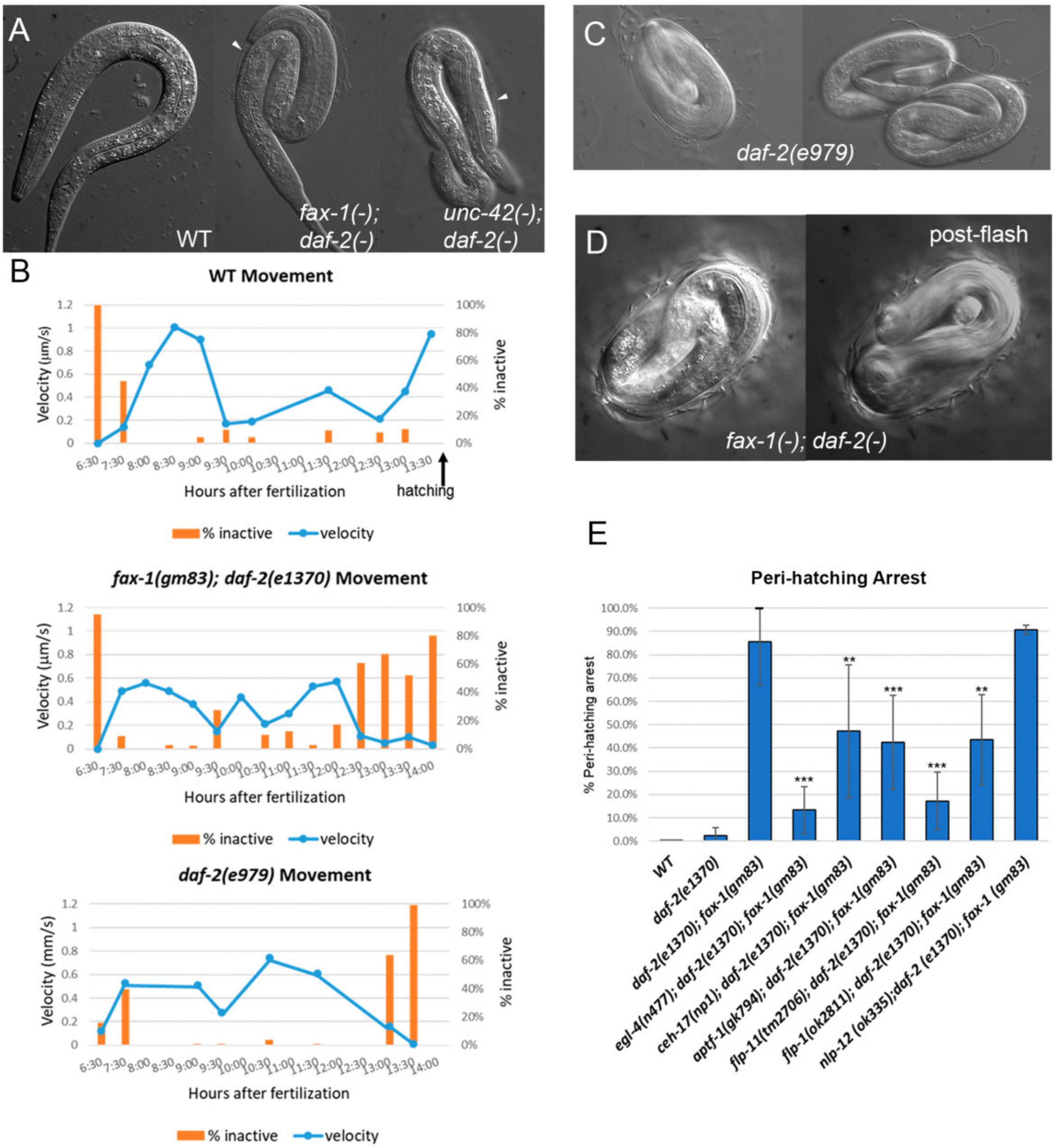

Figure 4 Arrested embryos are in a sleep state. (A) Micrographs of wild-type and arrested *fax-1(gm83); daf-2(e1370)* and *unc-42(e419); daf-2(e1370)* animals. Arrowheads identify visible segments of eggshell trapping the immobile animals. (B) Developmental time course of activity for individual representative embryos. Animals were filmed for 10–20 s intervals at various times, beginning with the "comma" stage, shortly before movement commences. For each time point, we calculated the average velocity of embryo movement and percentage of time spent without moving during the interval (% inactive). (C) Early elongation (left) and arrested late (right) embryos of *daf-2(e979)* mutants. Both panels are 3 s exposures. Note the blurriness of the early elongation embryo, indicative of active movement, when compared with the clarity of the immobile late embryos. (D) Reversal of quiescence in an arrested embryo by application of blue light. An arrested, quiescent *fax-1(gm83); daf-2(e1370)* embryo before (left) and after (right) the application of blue light for 10 s. Blurriness in the right panel reveals vigorous movement in response to the aversive stimulus. (E) Suppression of perihatching arrest by mutations in genes required for sleep in *C. elegans*. Figure shows standard 48-h arrest assays. These data are also reported in Supplementary Table S4. *egl-4*Pairwise differences are significant at $^{**}P < 0.01$ and $^{***}P \ll 0.001$. Error bars indicate standard deviation among replicate experiments.

to perihatching arrested embryos for pulses of up to 35 s. Blue light is an aversive stimulus for *C. elegans* (Ward *et al.* 2008). Arrested *fax-1; daf-2, unc-42; daf-2*, and *daf-2(e979)* embryos could be provoked to active movement by the application of blue light (Figure 4D). The response to blue light showed an increased latency [22.9 s for *fax-1(gm83); daf-2(e1370)* arrested embryos when compared with 6.6 s for wild-type, N = 14, $P \ll 0.01$], another property of sleep. While blue light prompted arousal, it did not prompt pharyngeal pumping activity over the time of observation. These findings indicate that the quiescent state of perihatching arrested animals is indeed a sleep state based on these criteria.

Caenorhabditis elegans exhibit a sleep state associated with lethargus during larval development at the initiation of each cuticular molt at L2, L3, L4, and adult (Raizen *et al.* 2008). Molecular and physiological analysis of lethargus indicates that it has properties in common with mammalian sleep (Kayser and Biron 2016; Tojanowksi and Raizen 2016; Bringmann 2018). Sensory neurons and two key interneurons, ALA and RIS, define key neurological pathways for the regulation of sleep during larval development (Van Buskirk and Sternberg 2007; Turek *et al.* 2013). Several genes have been shown to have prosleep activities: the *egl-4* cGMP-dependent kinase in sensory neurons, the *ceh-17* homeobox transcription factor in the ALA interneuron, and the *aptf-1* transcription factor and *flp-11* neuropeptide in the RIS interneuron (Raizen *et al.* 2008; Van Buskirk and Sternberg 2010; Turek *et al.* 2013, 2016).

We found that perihatching arrest can be suppressed by mutations in genes required for sleep. Mutations in *egl-4, aptf-1* and *flp-11* were able to significantly suppress perihatching arrest caused by *fax-1; daf-2* and *unc-42; daf-2* double mutants (Figure 4E and Supplementary Table S4), although the effect was not as strong as the suppression caused by the insulin pathway components *daf-16* or *daf-18* (Figure 3). These findings suggest that perihatching arrest represents a sleep state, dependent on signaling from sensory neurons and the interneuron RIS. The case for involvement of ALA is less clear; a mutation in *ceh-17* provided modest, but significant, suppression of *fax-1; daf-2* double arrest, but did not suppress *unc-42; daf-2* (Supplementary Table S4). Neither *fax-1* nor *unc-42* are expressed in RIS or ALA indicating that the key arousal neurons dependent on *fax-1* and *unc-42* function upstream or downstream of the RIS and ALA sleep-promoting neurons. Therefore, insulin-signaling function in promoting arousal in late embryogenesis is opposed by the activity of known sleep pathways—when insulin signaling is compromised, late embryos fall into a sleep state.

Osmotic stress and insulin-signaling pathways control arousal and progression

Burton *et al.* (2017, 2018) have described a *C. elegans* arrest in response to osmotic stress immediately before or after hatching. Therefore, we considered the possibility that quiescent perihatching arrest is a manifestation of the osmotic stress response pathway. Wild-type embryos grown on 325 mM or 500 mM NaCl and *daf-2* mutants grown on 300 mM NaCl arrested in a condition that is similar to *fax-1; daf-2* and *unc-42; daf-2* double mutants, consistent with this possibility (Figure 5). Like the *fax-1; daf-2* and *unc-42; daf-2* double mutants, *daf-2(e1370)* and wild-type embryos on high salinity plates were active in midembryogenesis, but fell into a quiescent state prior to hatching and fail to activate pharyngeal pumping. Wildtype embryos in 500 mM NaCl were not roused by blue light stimulus as easily as the *fax-1; daf-2* and *unc-42; daf-2* double mutants (8 of 14 wild-type embryos tested on 500 mM NaCl responded with sluggish movement with a latency of 25 s), suggesting that the quiescent state of embryos in high salt environments is more difficult to reverse.

To examine osmotic stress further, we tested *fax-1* and *unc-42* single mutants for sensitivity to osmotic arrest on high sodium chloride-containing plates. Compared with wild-type, both *fax-1* and *unc-42* mutations displayed an increased sensitivity to perihatching arrest on 275, 300, and 325 mM NaCl plates (Figure 5, A and B). The effect was not as strong as that observed with *daf-2(e1370)*, consistent with the idea that *fax-1* and *unc-42* reduce insulin signaling, but to a lesser extent than compromising insulin receptor.

Genetic analysis by Burton *et al.* (2018) demonstrated that osmotic arrest depends on two key genes: *ssu-1*, which encodes a cytosolic sulfatase that is expressed solely in the ASJ sensory neurons, and *nhr-1*, which encodes a nuclear hormone receptor that appears to function cell-autonomously in cells throughout the body. Given that vertebrate cytosolic sulfatases function in processing of steroids, Burton *et al.* (2018) have proposed that osmotic arrest is mediated by an unidentified lipophilic hormone produced in ASJ neurons by the SSU-1 sulfatase and that NHR-1 may help mediate the response to the hormone. We tested the possibility that perihatching arrest also depends on *ssu-1* and *nhr-1* by making triple mutants that combined a mutation in either gene with *fax-1; daf-2* and *unc-42; daf-2* double mutants. In all cases, the presence of a mutation in either *ssu-1* or *nhr-1* strongly suppressed perihatching arrest (Figure 5C), similar to mutations in *daf-16*. Salt-induced arrest of wild-type embryos activates the expression of a superoxide dismutase *sod-5::gfp* reporter gene, consistent with the notion that arrest is part of a stress response (Burton *et al.* 2018). Double mutant *fax-1; daf-2* embryos similarly induce *sod-5::gfp* expression at low salt concentration: 90.8% of *fax-1(gm83); daf-2(e1370)* perihatching animals expressed detectable GFP when compared with 51.2% of *daf-2(e1370)* controls ($P < 0.01$) (Figure 5D). Finally, osmotic arrest depends on *daf-2* activity in the intestine (Burton *et al.* 2017), as does *fax-1; daf-2* arrest, although we found that providing *daf-2* function in neurons could also weakly rescue perihatching arrest (Figure 5E). Taken together, these observations support the hypothesis that perihatching arrest is a manifestation of the normal osmotic stress response, revealed by the loss of proarousal, proprogression signaling via the insulin response.

The connection between arousal and osmotic stress arrest raises the possibility that compromising sleep pathways might also suppress osmotic arrest. To address this, we examined whether mutations in *aptf-1* and *flp-11*, both of which are required for perihatching arrest and for nematode sleep states promoted by the RIS interneuron, can suppress the osmotic arrest of *daf-2(e1370)* mutants on 300 mM NaCl. Under these culturing conditions, wild-type nematodes do not arrest, but *daf-2(e1370)* mutant embryos arrest at high penetrance (Burton *et al.* 2017). Neither *aptf-1* nor *flp-11* mutations were able to suppress the arrest phenotype, however, they were both able to suppress the quiescence phenotype, demonstrating that the quiescence and perihatching arrest can be uncoupled (Figure 5F). When *daf-2(e1370)* embryos were placed onto 300 mM NaCl plates, the embryos arrested inside the eggshell in a quiescent condition. In contrast, both *aptf-1; daf-2* and *flp-11; daf-2* embryos hatched, yielding mobile L1 larvae. However, the active L1 animals remained arrested with morphology similar to a newly hatched L1 animal, indicating that simply preventing a sleep state is not sufficient to reverse arrest. Arrested *flp-11; daf-2* double mutants on 300 mM NaCl also failed to activate pharyngeal pumping (70% did not pump at all; 30% pumped weakly and inconsistently; $N = 27$). These results argue that salt-induced quiescence is a sleep state, under the control of the prosleep RIS interneuron.

The separation of developmental arrest from quiescence raises the possibility that the arrest component of the phenotype may reflect a critical role for pharyngeal pumping in developmental progression. The intake of nutrition after hatching has long been known to be required for activating the transition from embryonic to larval developmental events (Baugh 2013). Therefore, it is possible that the arrest component of perihatching arrest might arise mostly or entirely as a consequence of the failure to feed. Consistent with this possibility, we documented a modest, but significant, reduction in pharyngeal pumping in both *fax-1* and *unc-42* mutants. Under standard culture conditions, *fax-1(gm83)* L1 larvae pump at the rate of 2.28 pumps/sec and *unc-42(e419)* L1 larvae pump at 1.83 pumps/s, when compared with 2.98 pumps/s for wild-type (Supplementary Figure S3). To explore this possibility further, we examined the effect of food source and pharmacological manipulation on perihatching arrest. Previous work has shown significant differences on growth and developmental progression with alternative bacterial food sources (So *et al.* 2011; Avery and You 2012). Some of this effect is due to physiological limitations of pharyngeal pumping and some

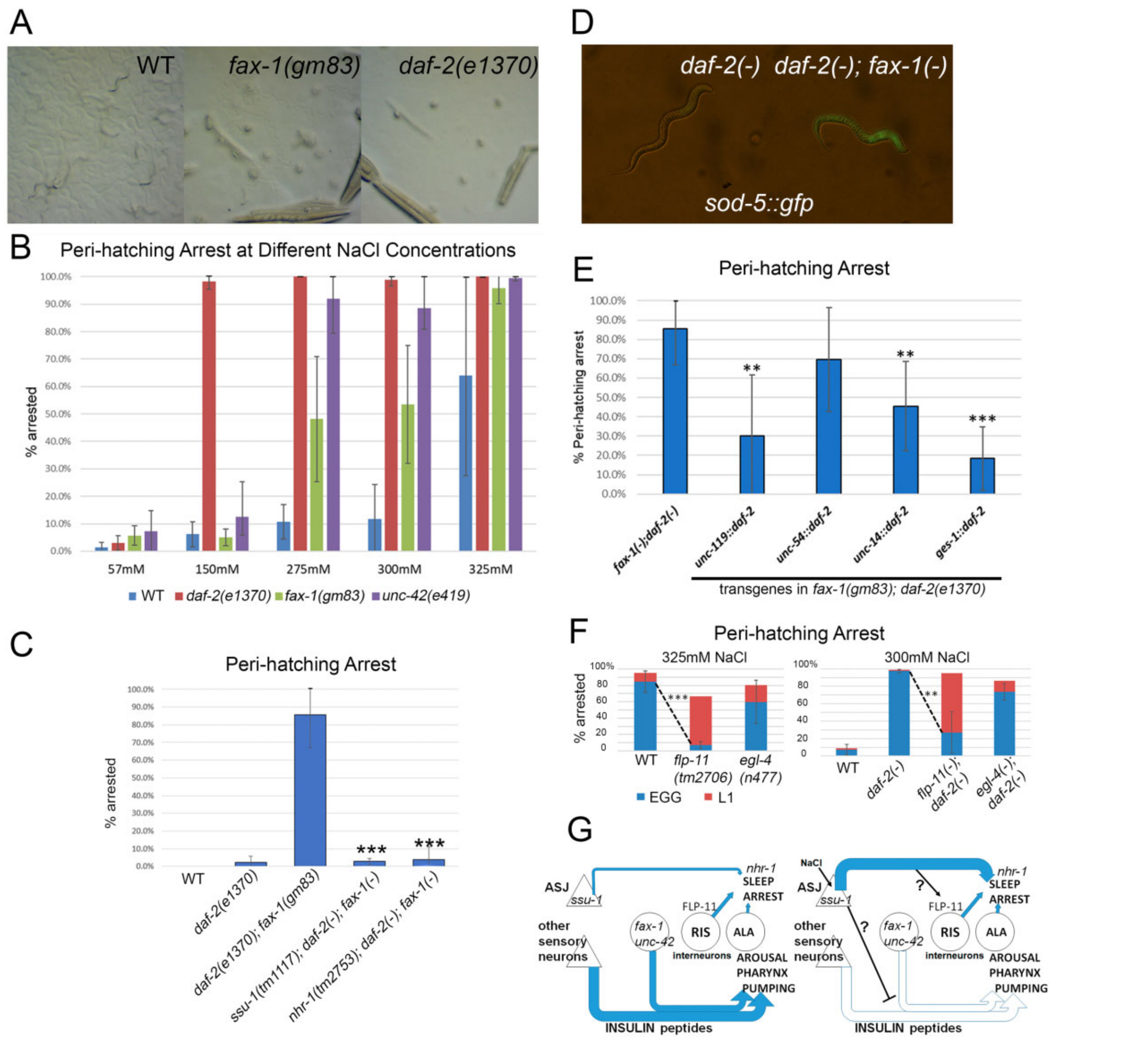

Figure 5 Osmotic stress opposes insulin-dependent arousal. (A) *fax-1 (gm83)* and *daf-2(e1370)* embryos arrest at hatching when placed on 300 mM NaCl plates for 24 h at 25°C. (B) Dose–response analysis of perihatching arrest of wild-type, *daf-2*, *fax-1* and *unc-42* mutant embryos on different salt concentrations at 25°C. (C) Suppression of perihatching arrest of *daf-2; fax-1* double mutant embryos by mutations in the *ssu-1* and *nhr-1* genes required for osmotic arrest. (D) Induction of a *sod-5::gfp* reporter in a *fax-1; daf-2* arrested L1 larvae. Left panel shows *daf-2(e1370)* as a control, right panel a *daf-2(e1370); fax-1(gm83)* double mutant. (E) Rescue of perihatching arrest by transgenes driving tissue-specific expression of wild-type *daf-2* in *daf-2(e1370); fax-1(gm83)* mutant backgrounds. *unc-119* is expressed broadly in several tissues, *unc-54* in body muscle, *unc-14* in neurons, and *ges-1* in the intestine (Wolkow *et al.* 2000). (F) Mutations in *flp-11*, which is required for RIS-dependent sleep, can suppress quiescence of otherwise wild-type animals at 325 mM salt (left), and suppress the quiescence defect in *daf-2(e1370)* mutant embryos grown on 300 mM salt (right). Immobile perihatching animals remain within the egg (blue), while motile L1's hatch and are scored as L1 (red). The alleles shown are *daf-2(e1370)*, *flp-11(tm2706)*, and *egl-4(n477)*. (G) Schematic illustrated one model for relating the opposed insulin and osmotic stress pathways. Some of these data are also included in Supplementary Table S4. Differences in pair-wise comparisons to controls are significant at *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$. Error bars indicate standard deviation among replicate experiments.

pumping-defective mutants can be "rescued" by growing them on the more easily-eaten HB101 *E. coli* strain instead of the standard OP50 strain (Davis *et al.* 1995; Avery and Shtonda 2003). We observed a slight decrease in perihatching arrest of *daf-2; fax-1* double mutants, but no rescue of *daf-2; unc-42* double mutants when eggs were laid on HB101 bacteria (Supplementary Table S4). Agonists of serotonin and acetylcholine transmission, both of which stimulate pharyngeal pumping (Avery and Horvitz 1990; Song and Avery 2012), were not able to rescue perihatching arrest in *fax-1; daf-2* double mutants, *daf-2* mutants on 300 mM NaCl, or *daf-2; flp-11* double mutants on 300 mM NaCl (Supplementary Table S4). Hatched animals that were arrested as L1 in *daf-2; flp-11* double mutants were able to move and forage, but pumped weakly or not at all, even in the presence of carbachol, an acetylcholine agonist that normally stimulates pumping ($N = 26$). Taken together, these observations suggest that developmental arrest at hatching may not be entirely a simple consequence of the inability to feed, although we cannot rule out that possibility.

Discussion

Opposition between insulin and osmotic stress pathways controls developmental progression

The analysis of insulin pathway and osmotic stress response argues that two countervailing pathways regulate animal developmental arrest and arousal as embryos approach hatching

(Figure 5G). The ASJ sensory neuron senses salt concentration in the environment (Burton *et al.* 2017, 2018)—presumably once the eggshell is compromised near the end of the embryogenesis. The *ssu-1* gene encodes a cytosolic sulfotransferase, which is expected to modify a hydrophobic molecule based on known biochemical activities, suggesting that the ASJ may produce a steroid-like neuroendocrine signal in response to a detected osmotic threat (Burton *et al.* 2018). Many, or perhaps all, cells respond to the presumptive ASJ signal via the nuclear receptor *nhr-1*, which activates a program that includes an induced sleep state, cessation of pharyngeal pumping, and immediate developmental arrest.

The ASJ osmotic response pathway is opposed by a canonical insulin-signaling pathway that promotes arousal, pharyngeal pumping, and developmental progression (Figure 5G). The existence of this function and its opposition to the osmotic stress response pathway is demonstrated by the fact that *daf-2* mutations at modest salt concentrations cause a perihatching arrest phenotype that phenocopies wild-type arrest at high salt (as well as the arrest of *fax-1; daf-2* and *unc-42; daf-2* doubles at low salt), and that these arrests are equally suppressed by compensatory mutations in the insulin pathway (*daf-16* or *daf-18*) and the osmotic response pathway (*ssu-1* or *nhr-1*). Therefore, when insulin pathway function is compromised by mutation, the underlying osmotic stress signal is no longer opposed, leading to developmental arrest and quiescence. We do not know whether there is an endogenous level of *ssu-1* signaling even at low salt concentrations that is "revealed" by the loss of opposing insulin signaling (as hypothesized in Figure 5G) or if the loss of insulin signaling also increases the *ssu-1*-dependent signal or a downstream prosleep response. The promotion of arousal, pharyngeal pumping, and developmental progression is potentiated by a signal from interneurons that depend on *fax-1* and *unc-42*. The body-wide insulin response appears to be integrated in the nervous and gustatory systems (Figure 5E).

Insulin signaling has been previously implicated in promoting developmental progression. The *daf-2* gene was first identified on the basis of its promotion of developmental progression in opposition to dauer larvae arrest (also a relatively quiescent state that ceases pharyngeal pumping; Vowels and Thomas 1992; Gems *et al.* 1998; Hu 2007). The insulin pathway is also used to bypass developmental arrest checkpoints at other larval stages (Schindler *et al.* 2014). When larvae are starved they arrest development, accumulating at distinctive stages in the early L3 or early L4 stages, just as one would expect for a conventional "checkpoint." Avoiding arrest at these stages also depends on insulin signaling, clearly establishing another developmental progression role. The role of insulin signaling in perihatching arrest is nicely aligned with these later roles in overcoming developmental checkpoints, and the possibility of an end-of-embryogenesis developmental checkpoint is an attractive possibility. However, we have not been able to decisively separate the pharyngeal pumping defect from the arrest defect, so it remains possible in this case that developmental arrest at the end of embryogenesis is a secondary consequence of the inability to browse and feed, rather than a bona-fide checkpoint.

Perihatching arrest constitutes a novel sleep state

The reversal of quiescence, failure to pump, increased latency of the response, along with the dependence on known prosleep genes argues that perihatching arrested animals are in a sleep state. This finding ties the osmotic stress response to the genetics and physiology of animal sleep. The perihatching sleep state may be analogous to, or mechanistically related to, stress-induced sleep mechanisms in *C. elegans* (Hill *et al.* 2014; Honer *et al.* 2020). This sleep state is opposed by arousal signals that depend on insulin signaling and interneuron function. The conclusion that perihatching arrest is a sleep state is supported by its reversibility, its increased latency in response to aversive stimuli, and its dependence on genes that have known prosleep activities. The hatching of *daf-2; flp-11* animals argues that arrest within the eggshell is a direct consequence of late embryo inactivity that can be reversed by elimination of a prosleep neuropeptide. Unlike the well-studied larval sleep states associated with lethargus, perihatching arrest is not experienced by wild-type animals in low salinity environments. Furthermore, it does not seem to be associated with cuticle synthesis (like larval lethargus sleep), since the initial embryonic cuticle synthesis occurs an hour or more before perihatching quiescence.

Previous studies have demonstrated an important role for insulin signaling in promoting wakefulness in *C. elegans*. The insulin pathway mediator *daf-16/FOXO* plays a role in regulating sleep homeostasis (Driver *et al.* 2013) and mediates compensatory sleep states during larval lethargus-associated sleep (Bennett *et al.* 2018). Compromising *daf-2* function in adults leads to a quiescent, hibernation-like state with dramatically reduced pharyngeal pumping that is reminiscent of perihatching arrest (Gaglia and Kenyon 2009). Similarly, Wu *et al.* (2018) have demonstrated multiple sleep states throughout larval and adult life, all dependent on the RIS interneuron and insulin signaling, Skora *et al.* (2018) have used brain-wide imaging to detail how insulin signaling promotes wakefulness in response to absence of food, and You *et al.* (2008) have linked *egl-4* function to insulin signaling and coordinate control of arousal and satiation. Our study, in combination with these previous analyses, indicate a physiological link among arousal, food and osmotic sensation, and pharyngeal activity.

Insulin signaling has been implicated in promoting arousal in vertebrates: the proarousal orexin hormones increase insulin secretion in vertebrates (Sutcliffe and de Lecea 2000; Park *et al.* 2015) and direct application of insulin to rats can promote arousal (Tkacs *et al.* 2007). However, other studies from vertebrates and invertebrates suggest the opposite relationship. For example, metabolic insulin secretion in vertebrates is a response to satiation, which is associated with increased sleepiness (Fernstrom and Wurtman 1971). Likewise, in *Drosophila* the loss of insulin-related signaling leads to arousal rather than quiescence (Cong *et al.* 2015). Taken together, these apparently contradictory findings suggest that insulin superfamily neuroendocrine signaling may have a complicated relationship to arousal, with proarousal functions in some contexts and prosleep functions in others.

Interneurons contribute to insulin-dependent arousal

The synthetic arrest exhibited by *fax-1* and *unc-42* mutants, as well as their enhanced sensitivity to osmotic stress, suggest that interneurons that depend on these two transcription factors have functions that favor arousal, pharyngeal pumping, and developmental progression. It is possible that the synthetic effect is due to a cellular-level "gain-of-function," however, we were unable to generate the same synthetic phenotype with mutations that perturb axon pathfinding generally (Supplementary Table S2), suggesting that it is not a simple consequence of wiring defects. Given that both *fax-1* and *unc-42* also enhanced the insulin pathway dauer phenotype (Supplementary Table S3) and that a stronger *daf-2* mutant can cause the same phenotype (Figure 4), the simplest explanation is that interneuron function acts to increase insulin signaling. This could occur by a direct contribution to insulin signaling, such as increasing the production of an insulin agonist or decreasing the production of an insulin antagonist, or it could potentiate the response more broadly, downstream of *daf-2/InsR*. Such an effect could be mediated by a neuroendocrine output from *fax-1* and *unc-42* interneurons. We note that the two transcription factors could be required for key neuronal identity functions at the time of their differentiation in midembryogenesis, with the effect on insulin signaling in mutants a later consequence of an earlier defect, or the two transcription factors could actively maintain neuronal states and be directly involved in regulating the expression of proarousal functions at hatching.

Rescue of the perihatching arrest defect by expression of *fax-1* under the control of a *glr-5* promoter helps identify candidate interneurons for mediating the proarousal function. These include the interneuron classes AVA, AVB, AVE, AVK, DVA, RIC, and SIB (Supplementary Figure S1). Most of these neurons have previously been implicated in positively regulating arousal states (Chalfie 1985, Hums *et al.* 2016; Chew *et al.* 2018; Oranth *et al.* 2018; Skora *et al.* 2018; Cianciulli *et al.* 2019; Maluck *et al.* 2020) and/or pharyngeal pumping (Rogers *et al.* 2001). All produce neuropeptides that could be mediators, and all except DVA and SIB express at least one insulin-related neuropeptide (Taylor *et al.* 2021). The AVK interneurons are physically adjacent to the RIS interneuron, and receive multiple postsynaptic inputs from RIS, a neuron that regulates arousal in larva by production of the FMRFamide-related neuroendocrine peptide FLP-11 (Turek *et al.* 2016), a prosleep peptide on which perihatching arrest depends. The relevant activity might be produced by just one of these neuron classes or some combination of them. While *fax-1* and *unc-42* behave similarly in most experiments, there is evidence that *unc-42* arrest might be somewhat stronger. Mutations in *ceh-17* (on which the prosleep function of ALA neurons depend) and feeding HB101 bacteria were unable to rescue *unc-42; daf-2* double mutants, but were able to weakly rescue *fax-1; daf-2* doubles. In addition, *unc-42* mutants were somewhat more sensitive to salt (Supplementary Table S4 and Figure 5D). While these are only slight differences, they suggest a possible mechanistic difference between *fax-1* and *unc-42*.

Perihatching arrest is a novel potential diapause

The embryonic timing, sleep state, and lack of pharyngeal pumping distinguish perihatching arrest from the well-studied potential L1 arrest, which is a response to the lack of food and also depends on insulin signaling (Baugh and Sternberg 2006; Baugh 2013; Kaplan and Baugh 2016). Given the requirement for insulin signaling, it is possible that the two arrest points are mechanistically linked to each other, with perihatching arrest an earlier response to osmotic stress and L1 arrest a later response to the absence of food. In either case, the phenomenon prevents the progression of development under unfavorable environmental conditions. Insulin signaling also promotes arousal and development later in larval life, by preventing dauer, L3 and L4 arrest, in these cases in response to crowding and starvation (Hu 2007; Schindler *et al.* 2014). Therefore, the overall net effect of insulin signaling seems to consistently favor developmental progression over diapause.

Quiescence and developmental arrest may provide an adaptive mechanism by which terrestrial nematodes respond to the hazards imposed by high osmotic stress environments. *Caenorhabditis elegans* is a widespread species found in multiple environments, but is thought to be reproductive most often in rotting fruit, mushroom beds, and organic compost (Kiontke and Sudhaus 2006). Terrestrial nematodes face significant environmental chemical hazards, including those presented by high saline conditions, as well as periods of desiccation and rehydration. In coastal or estuarine environments, they may become exposed to seawater. Under culture conditions, *C. elegans* experience body shrinkage and significant mortality at NaCl concentrations above 200 mM (Lamitina *et al.* 2004). Therefore, the ability to sense salt, perhaps at the moment of eggshell rupture, is an opportunity for the organism to enter an arrested, nonfeeding state until conditions are more favorable to life and reproduction. Previous studies have hinted at a role for insulin superfamily signaling in the response of other animals to osmotic stress. For example, insulin signaling is reduced in response to osmotic stress in the crab *Neohelice granulate* (Trapp *et al.* 2018) and changes to IGF-1 levels are associated with osmotic change as steelhead trout are moved from freshwater to saltwater (Liebert and Schreck 2006). Our analysis of the regulation of osmotic response in *C. elegans*, along with these studies suggest a possible conserved and adaptive role for insulin signaling in the response to cellular stress. It will be particularly interesting to learn if osmotic stress responses in other invertebrates also include a sleep state.

Data availability

All key quantitative data are represented in article, tables, figures, and supplemental materials. This study includes no large datasets deposited in public databases.

Supplementary material is available at *GENETICS* online.

Acknowledgments

We appreciate the technical assistance of Sheila Clever, Diane Dologite, and Katherine Esbenshade and administrative support of Christina Wallitsch. Muhlenberg College students Arianna Mesrobian, Danny Monzo, Long Nguyen, and Danielle Silver made technical contributions to this project. Nick Burton, David Raizen, Ilya Ruvinsky, Meera Sundaram, and the anonymous reviewers at *Genetics* provided helpful guidance and feedback. Many strains were provided by the CGC, which is funded by NIH Office of Research Infrastructure Programs (P40 OD010440), and by the National Bioresource Project of Japan.

Funding

This work was funded by the Muhlenberg College Biology Department, the Crist Family Research Fellowship, the Lake Road Research Fellowship, the Vaughan Award, and grant 1R15GM107799 from the National Institutes of Health.

Conflicts of interest

The authors declare that there is no conflict of interest.

Literature cited

Ailion M, Thomas JH. 2003. Isolation and characterization of high-temperature-induced Dauer formation mutants in *Caenorhabditis elegans*. Genetics. 165:127–144.

Ambros V, Horvitz HR. 1984. Heterochronic mutants of the nematode *Caenorhabditis elegans*. Science. 226:409–416. doi:10.1126/science.6494891.

Avery L, Horvitz HR. 1990. Effects of starvation and neuroactive drugs on feeding in *Caenorhabditis elegans*. J Exp Zool. 253:263–270. doi:10.1002/jez.1402530305.

Avery L, Shtonda BB. 2003. Food transport in the *C. elegans* pharynx. J Exp Biol. 206:2441–2457. doi:10.1242/jeb.00433.

Avery L, You YJ. 2012. *C. elegans* feeding. WormBook. 1–23. doi: 10.1895/wormbook.1.150.1.

Baran R, Aronoff R, Garriga G. 1999. The *C. elegans* homeodomain gene *unc-42* regulates chemosensory and glutamate receptor expression. Development. 126:2241–2251.

Baugh LR. 2013. To grow or not to grow: nutritional control of development during *Caenorhabditis elegans* L1 arrest. Genetics. 194: 539–555. doi:10.1534/genetics.113.150847.

Baugh LR, Sternberg PW. 2006. DAF-16/FOXO regulates transcription of cki-1/Cip/Kip and repression of *lin-4* during *C. elegans* L1 arrest. Curr Biol. 16:780–785. doi:10.1016/j.cub.2006.03.021.

Bennett HL, Khoruzhik Y, Hayden D, Huang H, Sanders J, *et al.* 2018. Normal sleep bouts are not essential for *C. elegans* survival and FoxO is important for compensatory changes in sleep. BMC Neurosci. 19:10. doi:10.1186/s12868-018-0408-1.

Borbély AA. 1982. A two process model of sleep regulation. Hum Neurobiol. 1:195–204.

Brenner S. 1974. The genetics of *Caenorhabditis elegans*. Genetics. 77: 71–94.

Bringmann H. 2018. Sleep-active neurons: conserved motors of sleep. Genetics. 208:1279–1289. doi:10.1534/genetics.117.300521.

Brockie PJ, Madsen DM, Zheng Y, Mellem J, Maricq AV. 2001. Differential expression of glutamate receptor subunits in the nervous system of *Caenorhabditis elegans* and their regulation by the homeodomain protein UNC-42. J Neurosci. 21:1510–1522. doi: 10.1523/JNEUROSCI.21-05-01510.2001.

Burton NO, Dwivedi VK, Burkhart KB, Kaplan REW, Baugh LR, *et al.* 2018. Neurohormonal signaling via a sulfotransferase antagonizes insulin-like signaling to regulate a *Caenorhabditis elegans* stress response. Nat Commun. 9:5152. doi:10.1038/s41467-018–07640-w.

Burton NO, Furuta T, Webster AK, Kaplan REW, Baugh LR, *et al.* 2017. Insulin-like signalling to the maternal germline controls progeny response to osmotic stress. Nat Cell Biol. 19:252–257. doi: 10.1038/ncb3470.

Campbell SS, Tobler I. 1984. Animal sleep: a review of sleep duration across phylogeny. Neurosci Biobehav Rev. 8:269–300. doi: 10.1016/0149-7634(84)90054-x.

Chalfie M, Sulston JE, White JG, Southgate E, Thomson JN, *et al.* 1985. The neural circuit for touch sensitivity in *Caenorhabditis elegans*. J Neurosci. 5:956–964. doi:10.1523/JNEUROSCI.05-04-00956.1985.

Chew YL, Grundy LJ, Brown AEX, Beets I, Schafer WR. 2018. Neuropeptides encoded by *nlp-49* modulate locomotion, arousal and egg-laying behaviours in *Caenorhabditis elegans* via the receptor SEB-3. Phil Trans R Soc B. 373:20170368. doi:10.1098/rstb.2017.0368.

Cianciulli A, Yoslov L, Buscemi K, Sullivan N, Vance RT, *et al.* 2019. Interneurons regulate locomotion quiescence via cyclic adenosine monophosphate signaling during stress-induced sleep in *Caenorhabditis elegans*. Genetics. 213:267–279. doi:10.1534/genetics.119.302293.

Cong X, Wang H, Liu Z, He C, An C, *et al.* 2015. Regulation of sleep by insulin-like peptide system in *Drosophila melanogaster*. Sleep. 38: 1075–1083. doi:10.5665/sleep.4816.

Davis MW, Somerville D, Lee RY, Lockery S, Avery L, *et al.* 1995. Mutations in the *Caenorhabditis elegans* Na,K-ATPase alpha-subunit gene, eat-6, disrupt excitable cell function. J Neurosci. 15: 8408–8418. doi:10.1523/JNEUROSCI.15-12-08408.1995.

da Graca LS, Zimmerman KK, Mitchell MC, Kozhan-Gorodetska M, Sekiewicz K, *et al.* 2004. DAF-5 is a Ski oncoprotein homolog that functions in a neuronal TGF beta pathway to regulate *C. elegans* dauer development. Development. 131:435–446. doi:10.1242/dev.00922.

Driver RJ, Lamb AL, Wyner AJ, Raizen DM. 2013. DAF-16/FOXO regulates homeostasis of essential sleep-like behavior during larval transitions in *C. elegans*. Curr Biol. 23:501–506. doi:10.1016/j.cub.2013.02.009.

Evans TC. 2006. Transformation and microinjection. In: The *C. elegans* Research Community, WormBook, editor. WormBook. doi/10.1895/wormbook.1.108.1. http://www.wormbook.org.

Faunes F, Larraín J. 2016. Conservation in the involvement of heterochronic genes and hormones during developmental transitions. Dev Biol. 416:3–17. doi:10.1016/j.ydbio.2016.06.013.

Fernstrom JD, Wurtman RJ. 1971. Brain serotonin content: increase following ingestion of carbohydrate diet. Science. 174:1023–1025. doi:10.1126/science.174.4013.1023.

Fielenbach N, Antebi A. 2008. *C. elegans* dauer formation and the molecular basis of plasticity. Genes Dev. 22:2149–2165. doi: 10.1101/gad.1701508.

Gaglia MM, Kenyon C. 2009. Stimulation of movement in a quiescent, hibernation-like form of *Caenorhabditis elegans* by dopamine signaling. J Neurosci. 29:7302–7314. doi:10.1523/JNEUROSCI.3429-08.2009.

Gems D, Sutton AJ, Sundermeyer ML, Albert PS, King KV, *et al.* 1998. Two pleiotropic classes of *daf-2* mutation affect larval arrest, adult behavior, reproduction and longevity in *Caenorhabditis elegans*. Genetics. 150:129–155. PMC1460297.

Henning SJ. 1981. Postnatal development: coordination of feeding, digestion, and metabolism. Am J Physiol. 241G199–G214. doi: 10.1152/ajpgi.1981.241.3.G199.

Herbison AE. 2016. Control of puberty onset and fertility by gonadotropin-releasing hormone neurons. Nat Rev Endocrinol. 12:452–466. doi:10.1038/nrendo.2016.70.

Hill AJ, Mansfield R, Lopez JM, Raizen DM, Van Buskirk C. 2014. Cellular stress induces a protective sleep-like state in *C. elegans*. Curr Biol. 24:2399–2405. doi:10.1016/j.cub.2014.08.040.

Hiruma K, Kaneko Y. 2013. Hormonal regulation of insect metamorphosis with special reference to juvenile hormone biosynthesis. Curr Top Dev Biol. 103:73–100. doi:10.1016/B978-0-12-385979-2.00003-4.

Honer M, Buscemi K, Barrett N, Riazati N, Orlando G, *et al.* 2020. Orcokinin neuropeptides regulate sleep in *Caenorhabditis elegans*. J Neurogenet. 34:440–452. doi:10.1080/01677063.2020.1830084.

Hu PJ. 2007. Dauer. WormBook. 8:1–19.

Huber R, Hill SL, Holladay C, Biesiadecki M, Tononi G, *et al.* 2004. Sleep homeostasis in Drosophila melanogaster. Sleep. 27: 628–639. doi:10.1093/sleep/27.4.628.

Hums I, Riedl J, Mende F, Kato S, Kaplan HS, *et al.* 2016. Regulation of two motor patterns enables the gradual adjustment of locomotion strategy in *Caenorhabditis elegans*. Elife. 5:e14116. doi: 10.7554/eLife.14116.

Jia K, Albert PS, Riddle DL. 2002. DAF-9, a cytochrome P450 regulating *C. elegans* larval development and adult longevity. Development. 129:221–231. PMID: 11782415.

Kaplan RE, Baugh LR. 2016. L1 arrest, *daf-16/FoxO* and nonautonomous control of post-embryonic development. Worm. 5: e1175196.doi:10.1080/21624054.2016.1175196.

Kayser MS, Biron D. 2016. Sleep and development in genetically tractable model organisms. Genetics. 203:21–33. doi:10.1534/genetics.116.189589.

Kimura KD, Tissenbaum HA, Liu Y, Ruvkun G. 1997. daf-2, an insulin receptor-like gene that regulates longevity and diapause in *Caenorhabditis elegans*. Science. 277:942–946. doi:10.1126/science.277.5328.942.

Kiontke K, Sudhaus W. 2006. Ecology of *Caenorhabditis* species. In: The C. elegans Research Community, WormBook, editor. WormBook. doi/10.1895/wormbook.1.37.1. http://www.wormbook.org.

Lamitina ST, Morrison R, Moeckel GW, Strange K. 2004. Adaptation of the nematode *Caenorhabditis elegans* to extreme osmotic stress. Am J Physiol Cell Physiol. 286:C785–C791. doi:10.1152/ajpcell.00381.2003.

Liebert AM, Schreck CB. 2006. Effects of acute stress on osmoregulation, feed intake, IGF-1, and cortisol in yearling steelhead trout (*Oncorhynchus mykiss*) during seawater adaptation. Gen Comp Endocrinol. 148:195–202. doi:10.1016/j.ygcen.2006.03.002.

Maluck E, Busack I, Besseling J, Masurat F, Turek M, *et al.* 2020. A wake-active locomotion circuit depolarizes a sleep-active neuron to switch on sleep. PLoS Biol. 18:e3000361.doi:10.1371/journal.pbio.3000361.

Much JW, Slade DJ, Klampert K, Garriga G, Wightman B. 2000. The fax-1 nuclear hormone receptor regulates axon pathfinding and neurotransmitter expression. Development. 127:703–712.

Ogg S, Ruvkun G. 1998. The *C. elegans* PTEN homolog, DAF-18, acts in the insulin receptor-like metabolic signaling pathway. Mol Cell. 2:887–893. doi:10.1016/s1097-2765(00)80303-2.

Oranth A, Schultheis C, Tolstenkov O, Erbguth K, Nagpal J, *et al.* 2018. Food sensation modulates locomotion by dopamine and neuropeptide signaling in a distributed neuronal network. Neuron. 100: 1414–1428.e10. doi:10.1016/j.neuron.2018.10.024.

Park JH, Shim HM, Na AY, Bae JH, Im SS, *et al.* 2015. Orexin A regulates plasma insulin and leptin levels in a time-dependent manner following a glucose load in mice. Diabetologia. 58:1542–1550. doi:10.1007/s00125-015-3573-0.

Pereira L, Kratsios P, Serrano-Saiz E, Sheftel H, Mayo AE, *et al.* 2015. A cellular and regulatory map of the cholinergic nervous system of *C. elegans*. Elife. 4:e12432.doi:10.7554/eLife.12432. PubMed PMID: 26705699; PubMed Central PMCID: PMC4769160.

Raizen DM, Zimmerman JE, Maycock MH, Ta UD, You YJ, *et al.* 2008. Lethargus is a *Caenorhabditis elegans* sleep-like state. Nature. 451: 569–572. doi:10.1038/nature06535.

Rogers CM, Franks CJ, Walker RJ, Burke JF, Holden-Dye L. 2001. Regulation of the pharynx of *Caenorhabditis elegans* by 5-HT, octopamine, and FMRFamide-like neuropeptides. J Neurobiol. 49: 235–244. doi:10.1002/neu.1078.

Schindler AJ, Baugh LR, Sherwood DR. 2014. Identification of late larval stage developmental checkpoints in *Caenorhabditis elegans* regulated by insulin/IGF and steroid hormone signaling pathways. PLoS Genet. 10:e1004426.doi:10.1371/journal.pgen.1004426.

Sisk CL, Foster DL. 2004. The neural basis of puberty and adolescence. Nat Neurosci. 7:1040–1047. doi:10.1038/nn1326.

Skora S, Mende F, Zimmer M. 2018. Energy scarcity promotes a brain-wide sleep state modulated by insulin signaling in *C. elegans*. Cell Rep. 22:953–966. doi:10.1016/j.celrep.2017.12.091.

So S, Miyahara K, Ohshima Y. 2011. Control of body size in *C. elegans* dependent on food and insulin/IGF-1 signal. Genes Cells. 16: 639–651. doi:10.1111/j.1365-2443.2011.01514.x.

Song BM, Avery L. 2012. Serotonin activates overall feeding by activating two separate neural pathways in *Caenorhabditis elegans*. J Neurosci. 32:1920–1931. doi:10.1523/JNEUROSCI.2064-11.2012.

Stiernagle T. 2006. Maintenance of *C. elegans* (February 11, 2006). In: The C. elegans Research Community, WormBook, editor. WormBook. doi/10.1895/wormbook.1.101.1. http://www.wormbook.org.

Suresh A, Wightman B. 2020. The *daf*-2 insulin receptor functions in *C. elegans* embryo elongation. MicroPubl Biol. 10.17912/micropub.biology.000117. doi: 10.17912/micropub.biology.000117. PMID: 32550500; PMCID: PMC7252343.

Sutcliffe JG, de Lecea L. 2000. The hypocretins: excitatory neuromodulatory peptides for multiple homeostatic systems, including sleep and feeding. J Neurosci Res. 62:161–168. doi: 10.1002/1097-4547(20001015)62:2<161::AID-JNR1>3.0.CO;2-1.

Taylor SR, Santpere G, Weinreb A, Barrett A, Reilly MB, *et al.* 2021. Molecular topography of an entire nervous system. Cell. 184: 4329–4323. doi:10.1016/j.cell.2021.06.023.

Thomas JH. 1993. Chemosensory regulation of development in *C. elegans*. Bioessays. 15:791–797. doi:10.1002/bies.950151204.

Tissenbaum HA. 2018. DAF-16: FOXO in the context of *C. elegans*. Curr Top Dev Biol. 127:1–21. doi:10.1016/bs.ctdb.2017.11.007.

Tkacs NC, Pan Y, Sawhney G, Mann GL, Morrison AR. 2007. Hypoglycemia activates arousal-related neurons and increases wake time in adult rats. Physiol Behav. 91:240–249. doi: 10.1016/j.physbeh.2007.03.003.

Trapp M, Valle SC, Pöppl AG, Chittó ALF, Kucharski LC, *et al.* 2018. Insulin-like receptors and carbohydrate metabolism in gills of the euryhaline crab *Neohelice granulata*: effects of osmotic stress. Gen Comp Endocrinol. 262:81–89. doi:10.1016/j.ygcen.2018.03.017.

Trojanowski NF, Raizen DM. 2016. Call it worm sleep. Trends Neurosci. 39:54–62. doi:10.1016/j.tins.2015.12.005.

Turek M, Besseling J, Spies JP, König S, Bringmann H. 2016. Sleep-active neuron specification and sleep induction require FLP-11 neuropeptides to systemically induce sleep. Elife. 5: e12499.doi:10.7554/eLife.12499.

Turek M, Lewandrowski I, Bringmann H. 2013. An AP2 transcription factor is required for a sleep-active neuron to induce sleep-like quiescence in *C. elegans*. Curr Biol. 23:2215–2223. doi: 10.1016/j.cub.2013.09.028.

Van Buskirk C, Sternberg PW. 2007. Epidermal growth factor signaling induces behavioral quiescence in *Caenorhabditis elegans*. Nat Neurosci. 10:1300–1307.

Van Buskirk C, Sternberg PW. 2010. Paired and LIM class homeodomain proteins coordinate differentiation of the *C. elegans* ALA neuron. Development. 137:2065–2074. doi:10.1242/dev.040881.

Vowels JJ, Thomas JH. 1992. Genetic analysis of chemosensory control of dauer formation in *Caenorhabditis elegans*. Genetics. 130: 105–123.

Ward A, Liu J, Feng Z, Xu XZ. 2008. Light-sensitive neurons and channels mediate phototaxis in *C. elegans*. Nat Neurosci. 11:916–922. doi:10.1038/nn.2155.

Wightman B, Ebert B, Carmean N, Weber K, Clever S. 2005. The *C. elegans* nuclear receptor gene *fax-1* and homeobox gene *unc-42* coordinate interneuron identity by regulating the expression of glutamate receptor subunits and other neuron-specific genes. Dev Biol. 287:74–85.

Wolkow CA, Kimura KD, Lee MS, Ruvkun G. 2000. Regulation of *C. elegans* life-span by insulinlike signaling in the nervous system. Science. 290:147–150. doi:10.1126/science.290.5489.147.

Wu Y, Masurat F, Preis J, Bringmann H. 2018. Sleep counteracts aging phenotypes to survive starvation-induced developmental arrest in *C. elegans*. Curr Biol. 28:3610–3624.e8. doi:10.1016/j.cub.2018.10.009.

You YJ, Kim J, Raizen DM, Avery L. 2008. Insulin, cGMP, and TGF-beta signals regulate food intake and quiescence in *C. elegans*: a model for satiety. Cell Metab. 7:249–257. doi:10.1016/j.cmet.2008.01.005.

Communicating editor: B. Conradt