Report

Current Biology

The *C. elegans* Hypodermis Couples Progenitor Cell Quiescence to the Dietary State

Highlights

- The hypodermis controls nutrient-responsive reactivation of quiescent blast cells
- The hypodermis integrates signals from insulin-like ligands and amino acids
- Feeding ethanol and amino acids upregulates expression of insulin-like genes
- The amino-acid-responsive Rag-TORC1 pathway also reactivates quiescent blast cells

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In Brief

Fukuyama et al. show that the combination of ethanol and amino acids can act as a dietary cue to trigger quiescent neural and mesodermal progenitor cells to reactivate their developmental programs in the nematode *C. elegans*. The hypodermis (skin) conveys the dietary state to progenitor cells via the insulin/IGF- and TORC1 signaling pathways.

Fukuyama et al., 2015, Current Biology *25*, 1241–1248
May 4, 2015
http://dx.doi.org/10.1016/j.cub.2015.03.016

CellPress

Current Biology
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The *C. elegans* Hypodermis Couples Progenitor Cell Quiescence to the Dietary State

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http://dx.doi.org/10.1016/j.cub.2015.03.016

SUMMARY

The nutritional status of an organism can greatly impact the function and behavior of stem and progenitor cells [1]. However, the regulatory circuits that inform these cells about the dietary environment remain to be elucidated. Newly hatched *C. elegans* larvae (L1s) halt development in "L1 arrest" or "L1 diapause" until ample food is encountered and triggers stem and progenitor cells to exit from quiescence [2]. The insulin/insulin-like growth factor signaling (IIS) pathway plays a key role in this reactivation [3, 4], but its site(s) of action have not been elucidated nor have the nutrient molecule(s) that stimulate the pathway been identified. By tissue-specifically modulating the activity of its components, we demonstrate that the IIS pathway acts in the hypodermis to regulate nutrition-responsive reactivation of neural and mesodermal progenitor cells. We identify ethanol, a likely component of the natural *Caenorhabditis* habitat, and amino acids as nutrients that synergistically reactivate somatic progenitor cells and upregulate expression of insulin-like genes in starved L1 larvae. The hypodermis likely senses the availability of amino acids because forced activation of the amino-acid-responsive Rag-TORC1 (target of rapamycin complex 1) pathway in this tissue can also release somatic progenitor cell quiescence in the presence of ethanol. Finally, there appears to be crosstalk between the IIS and Rag-TORC1 pathways because constitutive activation of the IIS pathway requires Rag to promote reactivation. This work demonstrates that ethanol and amino acids act as dietary cues via the IIS and Rag-TORC1 pathways in the hypodermis to coordinately control progenitor cell behavior.

RESULTS AND DISCUSSION

Ethanol Supplementation Is Required for Reactivating Quiescent Somatic Progenitor Cells in *daf-18* and *daf-16* Mutants

De-repression of the IIS pathway through loss of its negative regulators *daf-18*/PTEN and *daf-16*/FOXO (Figure S1A) releases germline stem cells and somatic progenitor cells, respectively, from quiescence during L1 diapause [3, 4]. These findings imply that the IIS pathway is sufficient to promote reactivation. However, *C. elegans* media typically contain cholesterol added from an ethanol stock, and prior studies used nutritionally deficient media containing ethanol (M9 containing 0.08% [v/v] ethanol [3] and S basal [4]) to induce L1 arrest. Because ethanol can be incorporated into fatty acids and amino acids during L1 diapause [5], we assessed the effects of ethanol on the failure of *daf-18* and *daf-16* mutants to maintain quiescence. *daf-18*-null mutants also failed to arrest germline proliferation when animals were cultured for 2 days in M9 lacking ethanol (72.6% ± 13.2% [average ± SD] animals have greater than two germ cells; n = 403), indicating that ethanol supplementation is not required for the exit from germline quiescence. We next tested whether ethanol alters the behavior of somatic cells, focusing on P neuroblasts and the M mesoblast, progenitor cells that initiate postembryonic development during the L1 stage in fed animals [6]. P cell reactivation begins with a ventral migration, whereas M initiates with cell division, which is easily monitored using a P*hlh-8*::*gfp* reporter [7] (Figure 1A). Wild-type animals cultured in M9 plus ethanol maintained developmental arrest of both P and M cells for at least 1 week. In contrast, P and M cells were reactivated in *daf-18* and *daf-16* mutants cultured in these conditions (Figures 1B–1E), similar to results obtained with animals hatched in S basal media [4] (Figures S1B and S1C). In M9 or S basal lacking ethanol (and cholesterol), *daf-18* and *daf-16* mutant larvae underwent progressive tissue degeneration, and this, as well as the lethality associated with loss of *daf-18* (Figure S1D), restricted analysis to the behavior of the M mesoblast in *daf-16* mutants. M failed to divide in *daf-16* mutants cultured for 7 days in either M9 (0%; n = 500) or S basal (Figure S1C) lacking supplementation. Furthermore, cholesterol is not a key trigger of release from quiescence, because when *daf-18* and *daf-16* mutants were cultured in S basal from which cholesterol, but not ethanol, had been omitted, both P and M blast cells became reactivated at a frequency similar to that of L1s cultured in complete S basal (Figures S1B and S1C).

Supplementation of M9 with glucose, an alternative carbon and energy source, was also insufficient to reactivate P and M cells in wild-type animals. Although glucose suppressed the lethality of *daf-18* and *daf-16* mutants at least as well as ethanol (Figure S1E), it triggered P cell migration in a much smaller percentage of animals (Figure 1C). Glucose was also less effective in triggering M division in *daf-18* mutants (Figure 1E). These observations argue that ethanol does not promote blast cell

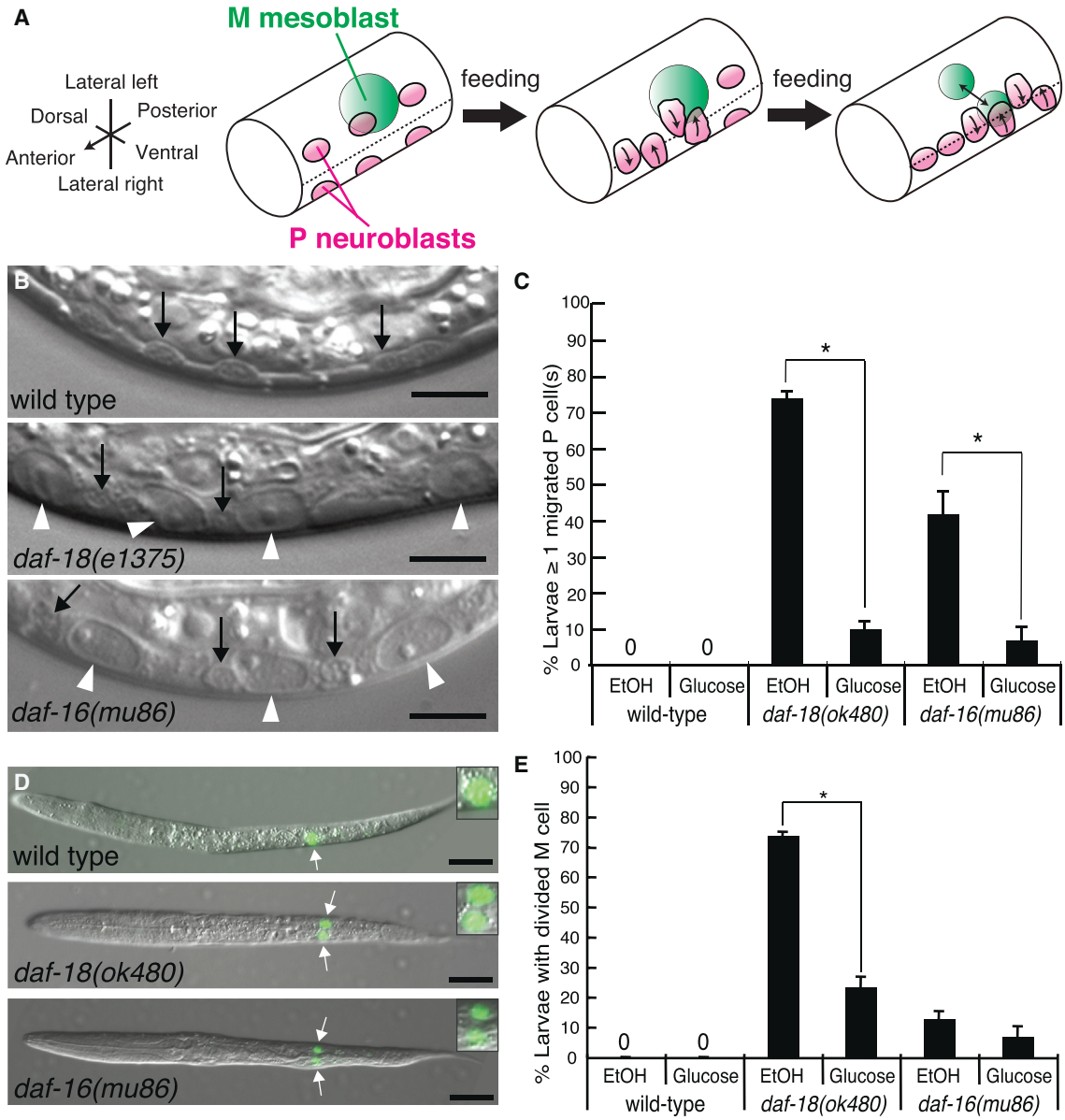

Figure 1. Effects of Ethanol and Glucose on Reactivation of Quiescent P and M Blast Cells

(A) Schematic of early L1 behaviors of P neuroblasts and the M mesoblast. The nuclei of P neuroblasts and the M mesoblast are shown in pink and green, respectively. Adapted from [8].

(B) Micrographs of wild-type, *daf-18*(*e1375*), and *daf-16*(*mu86*) mutant L1 larvae showing neurons (black arrows) and P neuroblasts (white arrowheads) in the ventral nerve cord after 7, 3, and 5 days of L1 starvation, respectively, in M9 plus 0.08% (v/v) ethanol. Times reported as "X days of L1 starvation" indicate that the animals were hatched and cultured in the indicated medium for X days following embryo isolation by alkali/bleach treatment (see the Experimental Procedures). In wild-type animals, P cells suspend the ventral migration program and remain on the lateral surface, out of view. Reactivated P cells in *daf-18*(*ok480*) L1 larvae apparently divide right after arriving at the ventral midline. Thus, a *daf-18*(*e1375*) animal is used here to show the migrated but undivided P cells. The scale bars represent 5 μm.

(C) P cell reactivation after 7 days of L1 starvation in M9 plus 0.08% (v/v) ethanol or 5% (w/v) glucose. *daf-18* and *daf-16* mutant animals used here and in (D) and (E) contain *ayIs6*, an integrated array including P*hlh-8*::*gfp*, which programs nuclear localization of GFP in the M lineage [7]. Data here and in (E) are represented by mean + SEM. *$p < 0.01$ for Student's t test.

(D) Micrographs showing the M cell and its descendants marked by P*hlh-8*::*gfp* (arrows) in animals after 7 days of L1 starvation in M9 plus 0.08% (v/v) ethanol. The scale bars represent 20 μm.

(E) Effects of ethanol and glucose on M cell reactivation after 7 days of L1 starvation. *$p < 0.001$ for Student's t test.

See also Figure S1.

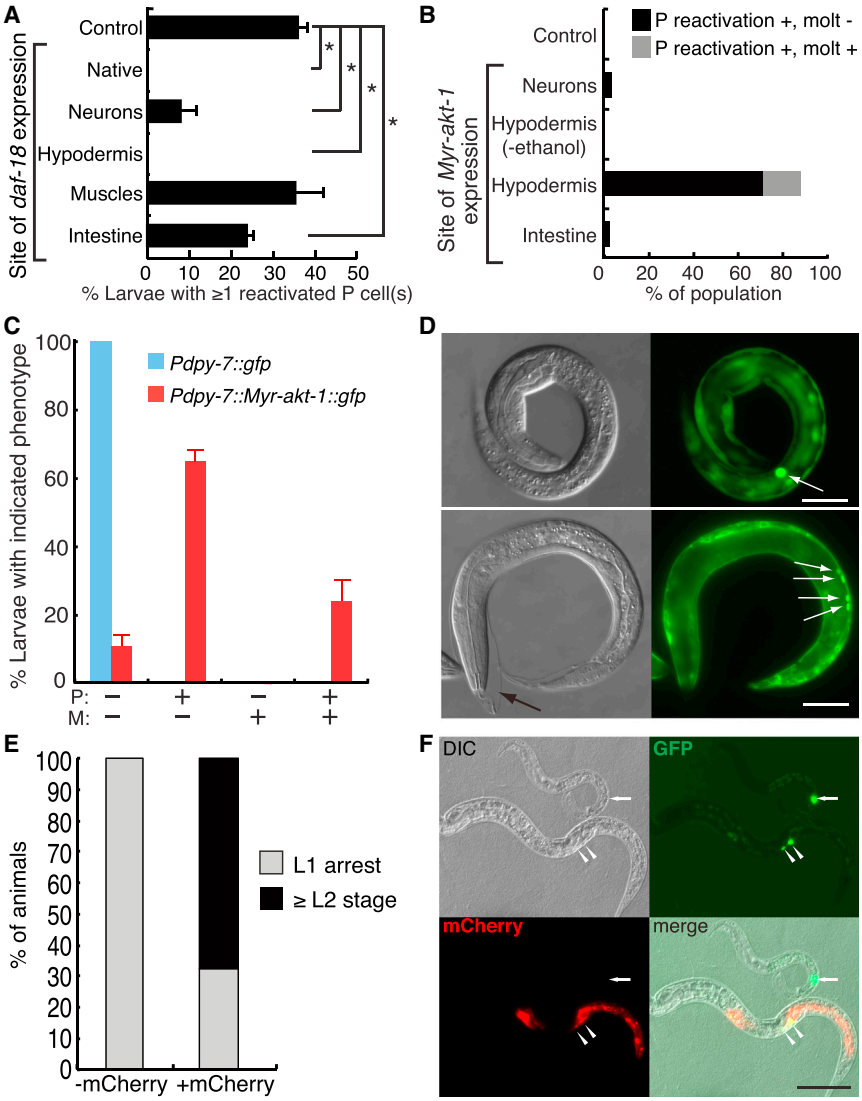

Figure 2. IIS Pathway Activity in the Hypodermis Reactivates P and M Blast Cells

(A) Effects of tissue-specific *daf-18* expression on P cell reactivation in *daf-18*(*ok480*) mutants. Transgenic *daf-18*(*ok480*) L1 larvae were scored for P cell reactivation after 7 days of starvation in M9 containing 0.08% (v/v) ethanol. *dpy-7*, *rgef-1*, *myo-3*, and *ges-1* promoters were used to drive *daf-18* expression specific to hypodermis, neurons, body wall muscle, and intestine, respectively. Data are represented by mean + SEM. Animals transformed with only the co-injection marker P*str-1*::*gfp* served as a negative control. Native indicates *daf-18* expressed from its endogenous promoter (see the Supplemental Experimental Procedures). *$p < 0.01$ for Student's t test.

(B–D) Effect of constitutively active AKT-1 expression on progenitor cell quiescence. A constitutively active form of AKT-1 was generated by adding the avian Src myristoylation sequence to the AKT-1 N terminus (*Myr-akt-1*) as was done for mouse AKT, targeting the protein to the membrane and resulting in its activation [9]. P*dpy-7*::*gfp* was used for negative controls. Transgenic L1 larvae were scored for the presence (+) or absence (−) of blast cell reactivation and molting after 5 days of starvation in M9 containing 0.08% (v/v) ethanol. (B) *dpy-7*, *rgef-1*, and *pgp-1* promoters were used to drive *Myr-akt-1* expression in the hypodermis, neurons, or intestine, respectively. Data are presented as the mean. (C) Effect of hypodermal *Myr-akt-1* expression on reactivation of P and M blast cells scored in the same individuals. P*hlh-8*::*gfp* was present to allow visualization of the M lineage. Data are presented as mean + SEM. (D) Micrographs of starved L1 animals expressing P*hlh-8*::*gfp* and either P*dpy-7*::*gfp* (control, upper panels) or P*dpy-7*::*Myr-akt-1*::*gfp* (lower panels) in the hypodermis. White arrows indicate the M cell (upper right) or its descendants (lower right). Black arrow (bottom left) indicates L1 cuticle in the process of being shed. The scale bars represent 20 μm.

(E and F) *daf-2* activity in the hypodermis controls initiation of L1 development. *daf-2*(*e979*); P*hlh-8*::*gfp* animals with or without an extrachromosomal array that expresses P*dpy-7*::*daf-2*(+)::*venus* in the hypodermis and P*pgp-1*::*mCherry* in the intestine were cultured at 25.5°C for ≥48 hr after egg-laying. DAF-2(+)::VENUS was not visible in most animals with mCherry expression, probably due to low expression levels. The *daf-2a* isoform was used to provide wild-type *daf-2* activity [10]. (E) Percentage of animals with (+mCherry; n = 56) or without (−mCherry; n = 43) the *daf-2*(+) array that arrested in the L1 stage. All animals scored as "L1 arrest" lacked reactivation of P and M and failed to molt (judged by the presence of L1-specific cuticle containing alae). (F) Micrographs showing that hypodermal *daf-2*(+) expression restores M reactivation. The L1 larva (top worm) lacks the *daf-2*(+)::*venus* array (no intestinal mCherry expression) and has an undivided M cell (arrow). In contrast, the bottom worm contains the *daf-2*(+) array (mCherry+), has molted to the L2 stage, and has a pair of migrating sex myoblasts (arrowheads). The scale bars represent 50 μm.

See also Figure S2.

reactivation simply by supporting survival of *daf-18* and *daf-16* mutants; rather, they suggest that metabolite(s) derived from ethanol and glucose act synergistically with activation of the IIS pathway to stimulate the release of P and M cells from quiescence.

Hypodermal Activation of the IIS Pathway Reactivates Somatic Progenitor Cells, but Not Germline Stem Cells

An important unanswered question is which cell(s) or tissue(s) require IIS pathway components to properly control stem and progenitor cell quiescence. To address this issue, we expressed *daf-18*(+) from tissue-specific promoters and scored for restored P cell quiescence in *daf-18* mutants. *daf-18* expression driven by its native promoter or the hypodermal-specific *dpy-7* promoter effectively rescued the P cell reactivation defect (Figure 2A). In contrast, neuronal and intestinal expression resulted in a reduced ability to rescue, and muscle expression had no significant effect.

To confirm that the hypodermis plays a key role in the control of P cell reactivation, we forced hypodermal activation of the IIS pathway by modulating AKT-1, a kinase that inhibits DAF-16/FOXO downstream of DAF-2, the worm insulin receptor (IR)

(Figure S1A). A constitutively active form of AKT-1 expressed as a *gfp* fusion (*Myr-akt-1*::*gfp*; see Figure 2 legend) from the *dpy-7* promoter efficiently triggered P cell migration in wild-type animals in an ethanol-dependent manner (Figure 2B). In contrast, intestinal or neuronal expression of *Myr-akt-1*::*gfp* had little, if any, effect on quiescence. Hypodermal *Myr-akt-1*::*gfp* expression also triggered M cell division and execution of the L1 molt in some animals (Figures 2B–2D). However, there was not complete release from diapause in these animals. The germline stem cells, Z2 and Z3, and the somatic gonad progenitors, Z1 and Z4, remained quiescent even in animals that had molted into L2-stage larvae (n = 46).

P Cell Activation of AKT-1 Is Incapable of Promoting Reactivation

The *dpy-7* promoter used is active in the main body hypodermis (hyp7), P cells of the ventral hypodermis, and small hypodermal syncytia of the head and tail, indicating that IIS pathway activity in one or more of these cell types likely regulates the decision between L1 quiescence and initiation of blast cell development. Among the animals expressing *Myr-akt-1*::*gfp* from the *dpy-7* promoter were two animals that retained the array in P cells on one side of the animal, but not the other. In these animals, only the P cells on the side that retained the array migrated (Figure S2A), raising the possibility that *Myr-akt-1* activity in P cells might cell autonomously contribute to neuroblast reactivation. However, both P and M cells remained quiescent in nearly all animals expressing *Myr-akt-1*::*gfp* in at least 10 of 12 P cells, but not other hypodermal cells, from a *hlh-3*::*pes-10* hybrid promoter [8] (P cells, 93.9%, n = 33; M cells, 100%, n = 30; Figure S2B), and none of these animals was observed to molt. These results suggest that both P and M cells require IIS pathway activation in hypodermal cells outside of the P lineage for their exit from quiescence, and they imply that the hypodermis communicates with P neuroblasts via an IIS-independent pathway.

Hypodermal *daf-2* Activity Controls Reactivation of P and M

Because receptor tyrosine kinases other than the insulin receptor can also antagonize DAF-18/PTEN and activate *akt* kinases, it was important to test whether *daf-2*/IR is required to regulate progenitor cell reactivation. Inactivation of DAF-2/IR as well as impaired secretion of insulin-like peptides causes L1 stage developmental arrest even under replete conditions [4, 11, 12]. However, the developmental status of stem and progenitor cells in these arrested animals has not been reported. To test directly whether DAF-2/IR is required for reactivation, we hatched wild-type and *daf-2*(*e979*ts) embryos at the nonpermissive temperature (25.5°C) and cultured them for 48 hr in the presence of food (see the Experimental Procedures). All wild-type animals (n = 107) grew into L4 larvae or fertile young adults with functional vulvae and apparently normal locomotion, reflecting normal P cell and germline development. In contrast, *daf-2* mutants arrested as L1s, with quiescent P, Z1/Z4, and Z2/Z3 cells (n = 117). In addition, all *daf-2*(*e979*); P*hlh-8*::*gfp* animals examined failed to activate M cell division when grown in replete conditions (n = 43). These observations indicate that IIS pathway activity is necessary to reactivate P, M, and Z1/Z4 progenitor cells as well as germline stem cells.

We next asked whether hypodermal activity of *daf-2* promotes P and M cell reactivation, using the *dpy-7* promoter to express *daf-2*(+)::*venus* in *daf-2*(*e979*); P*hlh-8*::*gfp* animals. When these animals were hatched and cultured for 48 hr in the presence of food at 25.5°C, more than half of the transgenic animals grew beyond the L1 stage (Figure 2E). The animals ranged in stage from L2 to adult and had multiple M cell descendants and vulval development (if mid-L3 stage or later), indicating that M and P progenitor cells exited from quiescence (Figure 2F; data not shown). Thus, activity of the IIS pathway in the hypodermis plays an instructive role in reactivating P and M cells.

Cell-Autonomous Activation of M Cell Division Does Not Trigger P Cell Migration

M reactivation is not solely controlled by IIS pathway activity in the hypodermis. Expression of *Myr-akt-1*::*mcherry* in M cell-autonomously initiated its division, and this was significantly enhanced by the presence of ethanol (Figures S2C and S2D). This result is in contrast to *Myr-akt-1* expression in P and suggests that regulation of M and P differs. However, M expression of *Myr-akt-1* failed to activate P cell migration, division of Z1/Z4 and Z2/Z3, or molting (0%; n = 111), suggesting that IIS activity can control M cell quiescence in both cell-autonomous and non-autonomous manners but that M cell activation of the pathway is insufficient to release other cells from L1 arrest.

Ethanol and Amino Acids Are Sufficient to Reactivate Somatic Progenitor Cells

One downstream target of the IIS pathway in mammals is mTORC1, a protein complex whose characteristic components are the serine/threonine kinase mTOR (mammalian or mechanistic TOR) and its cofactor Raptor [13]. In addition to being insulin and IGF-1 responsive, the kinase activity of mTORC1 is also stimulated by amino acids via Rag GTPases [13] (Figure S1A). mTOR, Raptor, and Rag orthologs are present in *C. elegans*, prompting us to test whether amino acids contribute to the release of stem and progenitor cell quiescence.

Wild-type L1 larvae were hatched and cultured in M9 containing ethanol, amino acids, or both, and several lineages were examined for initiation of development. Ethanol alone triggered division of stem-cell-like hypodermal blast cells known as seam cells (63% ± 3%; n > 100), but other lineages including P, M, Z1/Z4, and Z2/Z3 remained developmentally arrested (Figure 3A). Supplementation with amino acids alone was even less effective and failed to initiate postembryonic development in any lineage examined. However, when wild-type animals were cultured in M9 containing ethanol plus amino acids, the P, M, and Z1/Z4 lineages were released from developmental arrest in most animals and the majority molted to become L2 larvae (Figures 3A and 3B). Consistently, *lin-4* miRNA, which accumulates in the mid-to-late L1 stage of fed animals [14], was also dramatically upregulated by these nutrients (Figure 3C). Z2/Z3 rarely became reactivated in the presence of ethanol and amino acids, possibly reflecting the requirement for another nutrient (Figures 3A and 3B).

Interestingly, the combinations of events observed in individual animals (Figure 3B) suggest that release from developmental arrest occurs in a strict order: P, M, molting and Z1/Z4, and finally, Z2/Z3, because the latter events were usually accompanied by

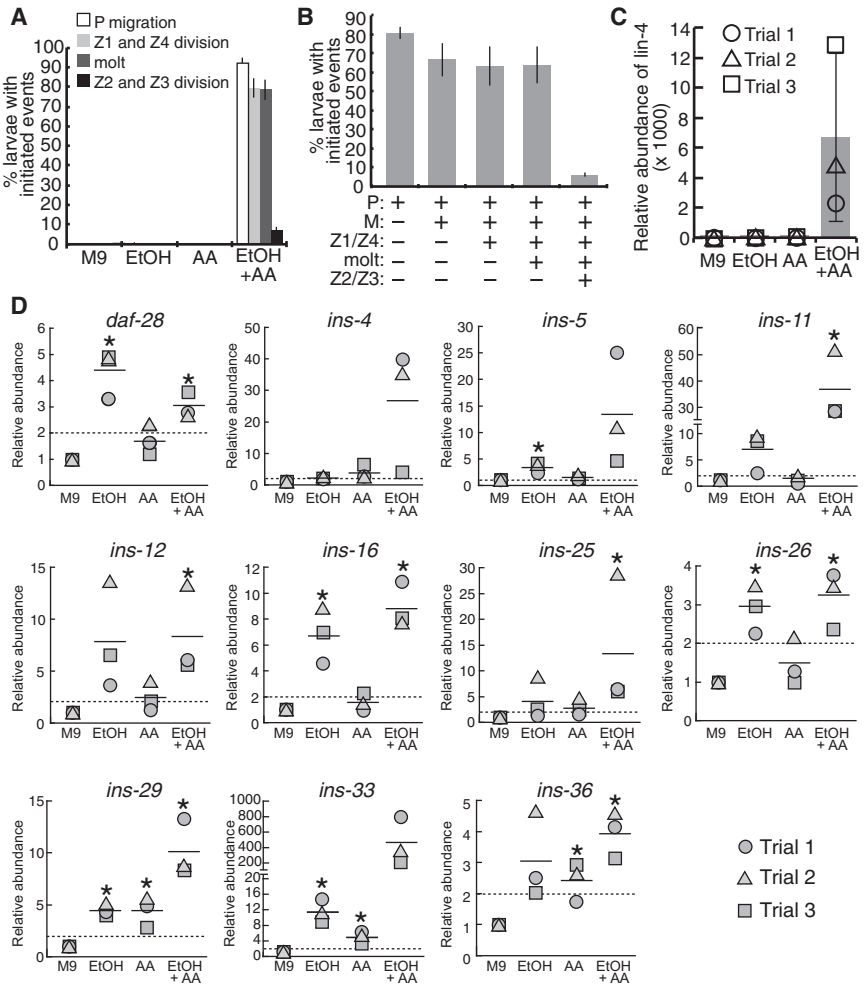

Figure 3. Ethanol and Amino Acids Can Activate Multiple L1 Developmental Events

(A and B) The percent of larvae that have initiated reactivation of the indicated stem and progenitor cells or molting are shown. Data are presented by mean ± SEM. (A) Wild-type animals were hatched and cultured for 1 week in M9 alone or containing the indicated supplements (see the Experimental Procedures) and scored for the noted events. EtOH, ethanol; AA, amino acids. (B) Animals transgenic for P*hlh-8*::*gfp* were hatched and cultured for 1 week in M9 plus EtOH and amino acids and then scored for initiation of the indicated events. The combinations of events observed are indicated underneath the percentage of animals having those combinations. For example, P cells had migrated in 81% of the animals, and of these, 67% also had a divided M cell.

(C) Relative abundance of *lin-4* miRNA after 7 days of L1 starvation in indicated media was determined using a TaqMan microRNA assay (Life Technologies) with the small nucleolar RNA sn2841 as an internal control. Although variability in the magnitude of *lin-4* miRNA expression was observed between biological replicates, the increase seen in EtOH plus AAs was always >2,000-fold. Data are presented as mean ± SD.

(D) *ins* genes whose expression was induced in newly hatched L1 larvae cultured in M9 plus EtOH and/or AA are shown. Total RNA was prepared from wild-type animals after 4 days of L1 starvation in the indicated conditions. The y axis of each graph indicates the relative abundance of transcript levels determined by qRT-PCR using three independent biological samples indicated by the circle, triangle, and square (see the Experimental Procedures), with a horizontal bar to indicate the mean. Because the y axis differs between graphs, a dotted line is included in each to indicate 2-fold. *The mean expression in test culture relative to M9 alone is more than 2-fold higher, and $p < 0.05$ for a paired t test. Note that the mean expression of *ins-4* and *ins-33* in animals cultured in ethanol plus amino acids relative to M9 alone was always more than 2-fold higher but was not judged to be statistically significant due to the deviation.

See also Figure S3.

the former. Similarly, reactivation of P, M, and molting apparently also occurs in this order in animals expressing *Myr-akt-1*::*gfp* in the hypodermis (Figure 2C; data not shown). These observations suggest the existence of a mechanism that regulates the order of stem and progenitor cell activation.

Ethanol and Amino Acids Can Activate Expression of Insulin-like Peptides

To release somatic lineages from quiescence, ethanol and amino acids act, at least in part, by altering the expression of insulin-like peptide genes. Either ethanol or amino acids alone was sufficient to increase *daf-28*, *ins-5*, *ins-12*, *ins-16*, *ins-24*, *ins-26*, *ins-30*, and *ins-36* message levels in starved L1s, whereas in contrast, *ins-4*, *ins-11*, *ins-25*, *ins-28*, *ins-29*, and *ins-33* were additively or synergistically induced by these components (Figures 3D and S3). Strikingly, five *ins* genes induced by the combination of amino acids and ethanol (*daf-28*, *ins-4*, *ins-5*, *ins-26*, and *ins-33*) are also induced when L1s feed on *E. coli* [15], and two (*daf-28* and *ins-4*) can trigger M cell division when overexpressed under nutritionally poor conditions [16], further supporting the notion that ethanol and amino acids reactivate progenitor cells at least partly via the IIS pathway.

Amino Acid Signaling in the Hypodermis Reactivates both P and M Blast Cells

The observation that both amino acids and hypodermal activation of the IIS pathway have similar effects on reactivation of somatic progenitor cells in the presence of ethanol suggests that the hypodermis plays a direct role in sensing amino acids. To test this hypothesis, we focused on two GTPases, Rag, which mediates amino acid signaling that converges on the IIS pathway via mTORC1, and Rheb, which relays the activation of the IIS pathway to mTORC1 (Figure S1A) [13].

Rag GTPases can be divided into two subfamilies, one containing orthologs of mammalian RagA and RagB and the other composed of RagC and RagD orthologs, and are known to act as obligate heterodimers, consisting of one member of each subfamily [13]. Cell culture studies suggest that constitutively active forms of RagA/B and Rheb remain GTP-bound, whereas activated RagC/D proteins are restricted to GDP-bound

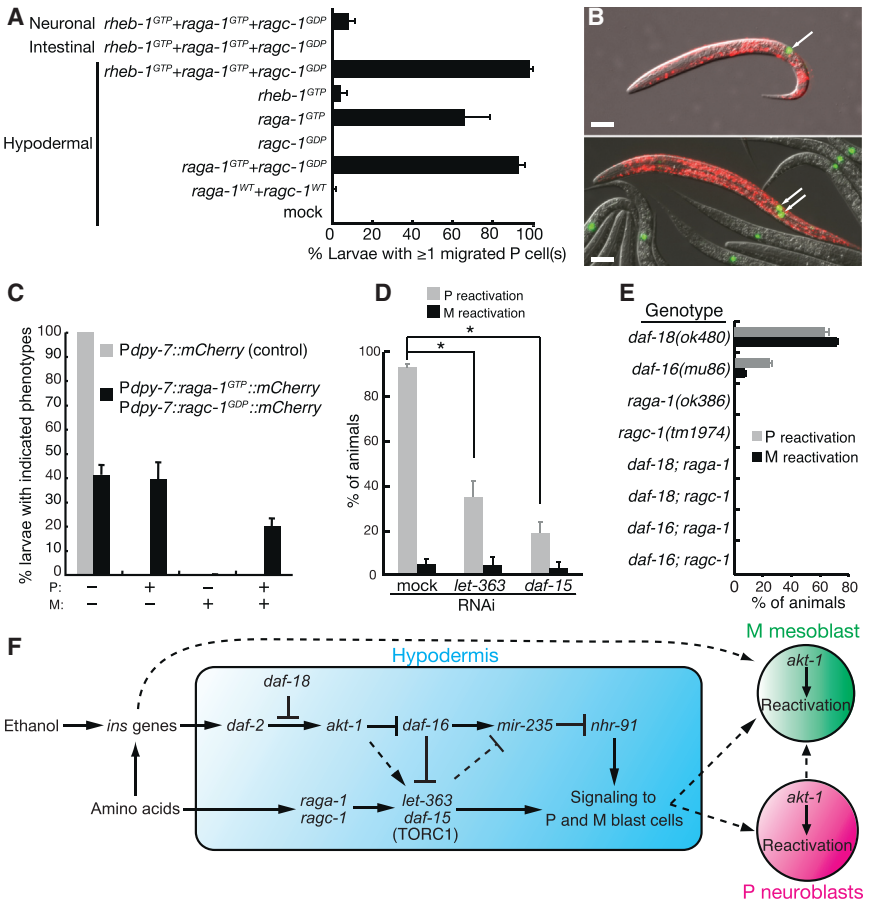

Figure 4. Hypodermal Activation of Rag Genes Reactivates Quiescent P and M Blast Cells

(A) Effects of tissue-specific expression of constitutively active forms of *rag* and *rheb* genes (*raga-1*^{GTP}, *ragc-1*^{GDP}, and *rheb-1*^{GTP}) on P cell reactivation after 5-day L1 starvation. The superscript *WT* indicates a wild-type version of the gene. A transgenic line carrying both P*dpy-7*::*gfp* and P*dpy-7*::*mCherry* was used as a control. *dpy-7*, *rgef-1*, and *pgp-1* promoters were used to drive *rheb-1*, *raga-1*, and *ragc-1* expression in the hypodermis, neurons, and intestine, respectively. Data here and in (C)–(E) are represented by mean ± SEM.

(B) Hypodermal activation of Rag genes can reactivate the M cell. P*dpy-7*::*mCherry* serves as a control and does not reactivate M, which is marked by P*hlh-8*::*gfp* (arrow, upper panel). In contrast, M has divided in an animal expressing constitutively activated *raga-1* and *ragc-1* as mCherry fusions from the *dpy-7* promoter (arrows, bottom panel) but has not divided in siblings lacking these fusions. Photographs and data were taken after 5 days of L1 starvation. The scale bars represent 20 μm.

(C) Quantification of P and M blast cell reactivation (+) or its absence (–) in animals expressing the indicated transgenes in the hypodermis.

(D) P cell reactivation caused by constitutively active Rag genes is dependent on *let-363*/TOR and *daf-15*/RAPTOR. Animals transgenic for hypodermally expressed *raga-1*^{GTP} and *ragc-1*^{GDP} were grown on bacteria expressing dsRNA for *let-363* or *daf-15*, or carrying an empty L4440 vector ("mock"), and their F2 embryos were used for the phenotypic analysis (see the Experimental Procedures). *$p < 0.01$ for Student's t test.

(E) Ectopic reactivation of P and M cells in *daf-18*(*ok480*) and *daf-16*(*mu86*) animals is suppressed by loss-of-function mutations of the Rag genes. Phenotypes were scored after 7 days of L1 starvation. All strains carried P*hlh-8*::*gfp*.

(F) Model for the nutrient-dependent reactivation of P and M blast cells. Ethanol and amino acids upregulate expression of a subset of *ins* genes, resulting in activation of the IIS pathway in the hypodermis. Amino acids also likely activate the RAGA-1/RAGC-1 complex in the hypodermis. Stimulation of both the IIS pathway and Rag might lead to full activation of TORC1, similar to observations in mammalian studies [19]. Hypodermal activation of the IIS and Rag-TORC1 pathways eventually signals to P and M blast cells to trigger their exit from developmental quiescence. *mir-235* acts in the hypodermis to suppress reactivation of P and M blast cells, partly through its target *nhr-91* [8]. Expression of *mir-235* is upregulated partly by *daf-16* and downregulated by feeding via *daf-2*, suggesting that *mir-235* acts downstream of the IIS pathway. However, whether TORC1 is also involved in regulation of *mir-235* and *nhr-91* remains to be investigated. Reactivation of P neuroblasts almost always precedes that of the M cell, implying a temporal order to these processes and perhaps communication from P to M. *akt-1* activity within P cells may contribute to their reactivation, concomitant with its activation in the hypodermis surrounding these progenitor cells (Figure S2A). Because *Myr-akt-1* can reactivate M cell autonomously (Figures S2C and S2D), insulin-like ligands may also directly stimulate this blast cell. Dashed lines indicate proposed interactions.

conformations. Expression of these activated proteins can overcome the inhibition of mTORC1 activity caused by amino acid starvation [17–19]. *C. elegans* has one member of each subfamily, *raga-1* [20] and *ragc-1* [21], and a single Rheb homolog, *rheb-1* [22]. We tested whether activated versions of these proteins could substitute for amino acids and reactivate blast cells in L1 larvae hatched and cultured in M9 plus ethanol. When expressed together in the hypodermis, RAGA-1^{GTP}, RAGC-1^{GDP}, and RHEB-1^{GTP} triggered P cell migration, whereas their neuronal or intestinal expression did not (Figure 4A). When assayed singly, only RAGA-1^{GTP} significantly stimulated P cell development. Activated Rag was required to release P cells from quiescence, because the P cells failed to migrate in animals expressing wild-type Rag transgenes.

P cell migration in animals expressing RAGA-1^{GTP} and RAGC-1^{GDP} in the hypodermis was significantly suppressed by RNAi against either *let-363*/TOR or *daf-15*/RAPTOR (Figure 4D), suggesting that TORC1 acts downstream of the Rag genes to control this process. Because mammalian Rag proteins directly activate mTORC1 in response to extracellular amino acids [19], the *C. elegans* hypodermis might directly monitor amino acids levels in the body fluids to control progenitor cell reactivation.

Hypodermal expression of the activated Rag genes also caused M cell division (Figures 4B and 4C), and all animals with a divided M cell contained migrated P cells, as was observed for *Myr-akt-1* expression. Neither Z2/Z3 nor Z1/Z4 exited from quiescence when the three activated GTPases were co-expressed in the hypodermis, intestine, or neurons

(0%; n > 100 animals), supporting the idea that quiescence of reproductive organ progenitors (the germline and somatic gonad) is regulated in a manner distinct from that of P and M blast cells. Expression of the activated Rag genes in M failed to release P, M, Z1/Z4, and Z2/Z3 from quiescence (one of 107 animals had a divided M cell), further supporting a role for the hypodermis in amino acid sensing.

mTORC1 is hyperactivated in PTEN-deficient mouse cells [23]. Thus, in *daf-18*/PTEN mutant worms, activation of *let-363*/TOR may contribute to failure to inhibit reactivation of somatic progenitor cells during L1 diapause, as observed for germline stem cells [21]. Consistent with this idea, mutation of either *raga-1* or *ragc-1*, whose mammalian orthologs are essential for full activation of mTORC1 [18, 19], suppressed the reactivation of P and M blast cells in starved *daf-18* mutants (Figure 4E). *C. elegans daf-16*/FOXO negatively regulates *daf-15*/RAPTOR [24], raising the possibility that activation of TORC1 may also contribute to the somatic defects of *daf-16* mutant animals. Indeed, loss of the Rag genes completely suppressed the failure to maintain P and M blast cell quiescence in *daf-16* mutants (Figure 4E).

Our study reveals that amino acids and IIS pathway activation promote release of somatic progenitor cell quiescence via TORC1 activity (Figure 4F). A key finding is that the hypodermis plays a central role in mediating both signaling from insulin-like ligands and sensing of amino acids to regulate nutrient-responsive quiescence in P and M blast cells. Recent work has also implicated the intestine as an important tissue in control of diapause release; monomethyl branched fatty acids promote postembryonic development via TORC1 activity in this tissue [25–27]. Because both the hypodermis and intestine accumulate lipid droplets [28], fatty acid and lipid metabolism might be tightly associated with regulation of progenitor cell quiescence by the IIS and TORC1 pathways. Thus, it will be important in future studies to investigate the relationship between the hypodermis and intestine in monitoring the dietary environments. In mammals, the growth-promoting role of the IIS pathway has long been known [29], but whether and how this pathway affects the behavioral changes in stem and progenitor cells in response to dietary conditions remain to be investigated. Given that many components of the IIS pathway are conserved between *C. elegans* and mammals [30], further dissection of L1 diapause release in the nematode should help guide our understanding of nutritional regulation of mammalian stem and progenitor cell behaviors.

EXPERIMENTAL PROCEDURES

Assessing Viability and Progression of L1 Development

Sterile embryos were prepared as described [21] and incubated in 10 ml sterile M9 containing 0.08% (v/v) ethanol, 5% (w/v) glucose, and/or 1× amino acids as indicated at 20°C with 30 rpm rotation. Larvae were continuously cultured in the same media and assessed for survival or initiation of L1 developmental events. The 5× amino acid stock solution contained 1.179 g L-arginine HCl, 0.283 g L-histidine, 1.283 g L-lysine·HCl, 0.184 g L-tryptophan, 0.389 g L-methionine, 0.717 g L-threonine, 1.439 g L-leucine, 0.861 g L-isoleucine, 1.020 g L-valine, and 0.623 g L-phenylalanine per 100 ml dH_2O. The amino acid ratios were based on *C. elegans* Maintenance Medium [31], but the concentrations were doubled to achieve robust induction of L1 development. Viability was assessed as described [21].

For experiments using temperature-sensitive *daf-2*(*e979*), wild-type or *daf-2* mutant embryos were obtained by growing L4 hermaphrodites into adults on seeded NGM plates at 25.5°C for 24 hr. Embryos were transferred to a fresh plate, maintained at 25.5°C, and scored 24 hr later. A significant percentage (53.7%; n = 201) of *daf-2*(*e979*) embryos failed to hatch as previously described [11]. Hatched L1 larvae were transferred onto a fresh plate and scored for L1 arrest 48 hr later. All arrested L1s examined had unmigrated P cells (n = 117). For rescue experiments (Figures 2E and 2F), *daf-2* mutant L4 hermaphrodites carrying the *daf-2*(+) array were placed on seeded NGM plates and cultured at 25.5°C. After 24 hr, the resulting adults were removed, and their hatched progeny were scored 48 hr later.

Real-Time qPCR Analysis

Starved L1 larvae were prepared from multiple 10-cm plates as described [21], mixed together, and divided equally for culture in each buffer condition. Total RNA was prepared after vortexing worms with acid-washed glass beads (425–600 μm; Sigma) in RNAiso plus as directed (Takara).

For quantitation of relative abundance of *ins* transcripts, cDNA was synthesized using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time qPCR was performed using a sybr Fast qPCR Kit (KapaBiosystems) on a StepOnePlus Real-Time PCR System (Applied Biosystems), according to the manufacturer's instructions except that the total reaction volume was reduced to 10 μl. Each reaction contained cDNAs derived from approximately 30 ng total RNA. Relative transcript levels were determined by the comparative Ct method using *act-1* as an endogenous control. Relative abundance of lin-4 was determined by the relative standard curve method according to the manufacturer's "Guide to Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PCR" (Applied Biosystems).

Statistical Analysis

Statistical analyses were carried out using Microsoft Excel software. All experiments were repeated at least three times, and ≥35 animals were scored per sample in each experiment unless otherwise indicated.

RNAi Experiments

RNAi was conducted by feeding [32], except that the plates contained a 4× peptone concentration. For preparation of L1 larvae, 25 gravid young YB1639 adults were placed on a 10-cm RNAi plate seeded with HT115 *E. coli* harboring the L4440 vector (control) or vectors targeting *let-363* or *daf-15* [21]. After about 4 days, gravid F1 progeny were treated by the Alkali/bleach method to prepare sterilized F2 animals for phenotypic analysis.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and three figures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2015.03.016.

ACKNOWLEDGMENTS

We thank Drs. A. Fire, O. Hobert, T. Inoue, Y. Jin, Y. Kohara, M. Maduro, S. Mitani, and S. Strome for reagents and strains. Some *C. elegans* strains used in this study were provided by the Caenorhabditis Genetics Center, which is supported by the NIH—Office of Research Infrastructure Programs (P40 OD010440), and the Mitani lab, which is funded through the National BioResource Project of the Ministry of Education, Culture, Sports, Science and Technology (MEXT). Supported by Japan Society for the Promotion of Science KAKENHI grant numbers 23229001 (T.K.) and 23370083 (K.K.), MEXT KAKENHI grant numbers 24657081 (T.K.) and 23116703 (M.F.), the National Science Foundation (IOB-0515682; A.E.R.), and American Heart Association Midwest Affiliate (AHA no. 0520074Z; M.F.).

Received: April 8, 2014
Revised: January 12, 2015
Accepted: March 12, 2015
Published: April 16, 2015

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