Starvation Protects Germline Stem Cells and Extends Reproductive Longevity in *C. elegans*

Giana Angelo, *et al.*

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Starvation Protects Germline Stem Cells and Extends Reproductive Longevity in *C. elegans*

Giana Angelo and Marc R. Van Gilst*

The study of starvation-resistant biological programs has elucidated numerous mechanisms influencing aging. Here we present the discovery and characterization of starvation-induced adult reproductive diapause (ARD) in *Caenorhabditis elegans*. ARD differs from the *C. elegans* dauer diapause in that it enables sexually mature adults to delay reproductive onset 15-fold and extend total adult life span at least threefold. The effectiveness of ARD requires apoptotic death of the entire germ line, except for a small population of protected germline stem cells (GSCs). When feeding is resumed, surviving GSCs regenerate a new germ line capable of offspring production near the level of nonstarved animals. The starvation-sensing nuclear receptor NHR-49 is required for ARD entry and recovery. Our findings establish mechanisms for preserving stem cell potency and reproductive potential during prolonged starvation.

Upon severe dietary restriction, many organisms, including some mammals, initiate programs of developmental or reproductive diapause, which are reversible states of dormancy (*1*–*3*). In these states, animals often respond to prolonged starvation by considerably extending their normal life span and reproductive period (*3*–*5*). Consequently, studying starvation-mediated diapause has provided a wealth of insight into nutritional control of development, reproduction, and aging (*6*–*9*).

When starved, the soil nematode *Caenorhabditis elegans* can developmentally arrest at multiple larval stages. The most-studied arrests are the larval stage one (L1) and dauer diapauses. Embryos that hatch in the absence of food can survive at least 2 weeks of starvation by entering an L1 diapause (*5*). Worms entering dauer arrest, an alternative developmental fate specialized for enduring long periods of starvation and stress, can withstand several months of nutrient deprivation (*3*). In both cases, worms delay their developmental progression from larvae to adults, which occurs in only 2 days under favorable conditions, by weeks or months. When food is restored, animals exit the diapause, resume larval development, and mature into adults with full reproductive capacity and a normal adult life span, implying that developmental diapause also halts the aging process in arrested larvae (*3*, *5*, *10*).

Discovery of an adult reproductive diapause (ARD). While studying starvation response in late development, we observed adult reproductive arrest. When a population of wild-type (WT) hermaphrodites was completely removed from its bacterial food source during the fourth and final stage of larval development (L4), many animals transitioned into adults and halted reproductive activities, therefore establishing an ARD (Fig. 1A). ARD initiation was optimal if a developmentally synchronized population of animals was removed from food when the majority of animals were in the mid-L4 stage of development (fig. S1, A to C). In this case, there were three distinct fates: (i) Animals arrested as L4 larvae; (ii) animals arrested as adults harboring no more than one live embryo per gonad arm; or (iii) animals developed into adults with multiple embryos in utero, which hatched and caused death through facultative vivipary, more commonly known as "bagging" (*11*) (Fig. 1, C to E). The relative ratio of these three fates differed depending on when starvation was started. For example, Fig. 1B shows the percentage of each outcome when starvation was initiated at the optimal developmental stage. However, if starvation was initiated late in L4 development, the majority of animals would miss the adult arrest and adopt the bagging fate, whereas food withdrawal early in L4 development primarily resulted in animals that arrested as L4 larvae (fig. S1D). ARD was dependent, in some fashion, on population density, because high densities of worms were required for optimal ARD establishment (see supporting online materials and methods). Additionally, isolation of individual ARD animals from a dense population of starved worms led to premature diapause exit and bagging (table S1).

A distinguishing feature of ARD was inhibition of embryo development and facultative

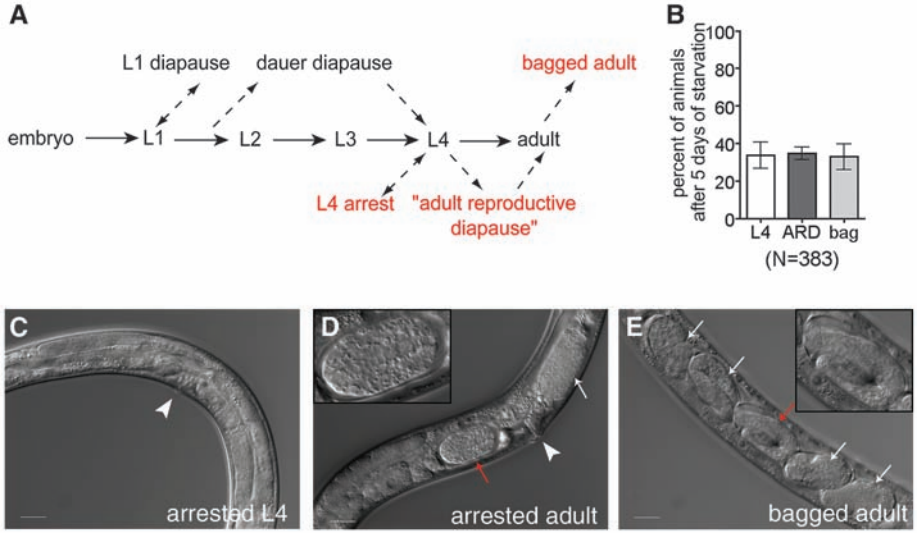

Fig. 1. (**A**) Starvation at receptive periods during early larval development in *C. elegans* leads to the previously characterized L1 and dauer developmental arrests. Acute starvation during the fourth larval stage (L4) leads to the formation of ARD, whereas starvation during late L4 or adulthood results in hermaphrodite bagging. (**B**) When a population of hermaphrodites in the middle of L4 was acutely starved, we observed three potential outcomes: L4 larval arrest, ARD, and bagged adults. The data are presented as the mean (±SEM) percentage of animals that adopted each of the three fates. Data are compiled from nine independent starvations (n = 9). The total number of animals scored in all nine experiments is also shown. (**C**) An arrested WT L4 after 5 days of starvation. The vulva is marked with an arrowhead. (**D**) A typical WT hermaphrodite in the fifth day of ARD. This animal contained two embryos in utero (indicated with arrows). The vulva is marked with an arrowhead. The inset shows an enlargement of one of the two uterine embryos (red arrow); it is in a premorphogenic stage of embryogenesis, when cells have not yet begun to differentiate. (**E**) A WT hermaphrodite in the process of bagging with five embryos in utero (indicated with arrows). The inset shows an enlargement of one of these embryos in the morphogenic stage of embryogenesis, when cells terminally differentiate and tissues and organs begin taking shape (red arrow). Scale bars = 20 μm.

Division of Basic Sciences, Fred Hutchinson Cancer Research Center, 1100 Fairview Avenue North, Seattle, WA 98109, USA.

*To whom correspondence should be addressed. E-mail: vangilst@fhcrc.org

vivipary (Fig. 1D). Under standard laboratory conditions, embryos develop and hatch within 14 hours of fertilization. If adult worms are starved and do not establish ARD, egg laying is inhibited and hatching of multiple embryos will occur in the uterus within 24 to 48 hours (*11*). In contrast, up to two embryos were retained within the uterus of ARD animals for at least 30 days. These embryos were viable for at least the first 5 days of starvation. For example, if ARD animals were isolated 3 or 5 days after starvation, thereby promoting diapause exit, embryos resumed a more normal rate of development and hatched inside the adult within 24 hours (table S1). Differential interference contrast (DIC) images suggested that the viability of uterine embryos, as determined by embryo morphology, started to decrease around day 7 of starvation. Inviable embryo remains then occupied the uterus as "corpses" (*12*) (Fig. 3F). These data argue that the ARD mechanism involves slowing or arresting the development of uterine embryos for at least 5 days of starvation, so that they can resume normal development and hatching upon ARD exit.

The mechanism for preventing embryonic development in ARD animals is still not apparent. Starvation-induced embryonic diapause is common in many animals, and it has recently been shown that hypoxia can cause embryonic diapause in *C. elegans* (*2*, *13*). However, because isolation of individual animals in ARD led to diapause release, we were unable to monitor embryonic development in arrested adults; therefore, it is not clear whether embryos in these animals fail to hatch because they are in an embryonic diapause or development is simply slowed down.

The adult diapause extends life span and reproductive longevity. In theory, the effectiveness of a starvation-induced diapause depends on the ability of arrested worms to survive extended periods of starvation, search for improved nutritional conditions, and recover to produce offspring. To characterize the survivability characteristics of ARD, we measured two parameters in animals rescued from ARD after periods of starvation of up to 30 days. First, we quantified brood size, a parameter indicative of how well reproductive potential is maintained during starvation (Fig. 2A). Second, we determined how well ARD protected the "youth" of somatic tissues by measuring the life span of worms after food restoration (Fig. 2B).

Entry into ARD offered notable survival advantages over worms that underwent the bagging fate. Even at 30 days of starvation, arrested adults recovered when returned to food and produced up to 20 progeny through self-fertilization (Fig. 2A). Much larger broods (>100 progeny when starved up to 15 days and >50 at 30 days) were obtained when animals were reintroduced to food and mated with well-fed males. This result suggests that viable oocytes are produced even after very long periods of starvation; however, the fecundity of recovered hermaphrodites that self-fertilize is limited by the survival of functional sperm. Consistent with this hypothesis, very few sperm are observed in worms starved for extended periods, even after rescue (Fig. 3, D and G). The normal reproductive period of *C. elegans* begins at the onset of adulthood (~2 days after

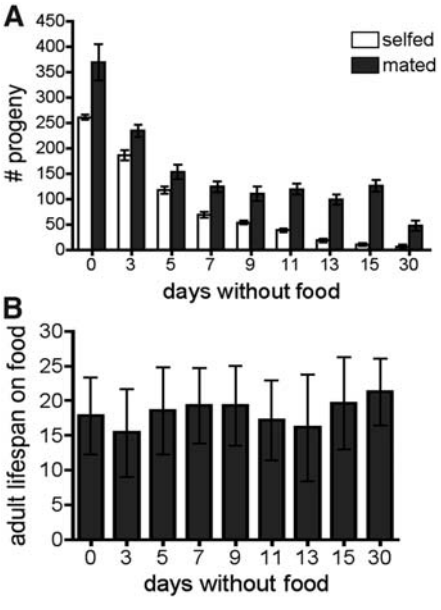

Fig. 2. (**A**) The number of progeny produced by WT animals rescued from ARD after various periods of starvation. Hermaphrodites were allowed to self-fertilize (white bars) or mate with fed males (gray bars). Data represent the mean brood size (±SEM) per animal (n = 19 to 24 animals per time point). Worms were obtained from three independent starvations. (**B**) The mean adult life span (±SD) of animals rescued from ARD after various periods of starvation on nematode growth media plates (n > 23 animals per time point).

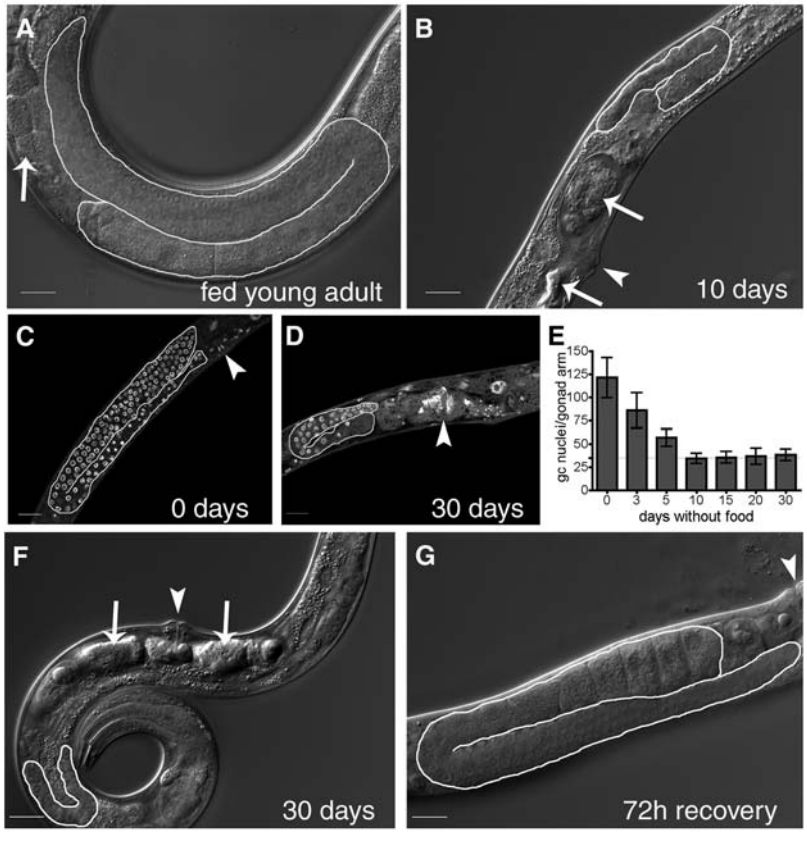

Fig. 3. (**A**) A typical WT hermaphrodite germ line shortly after reaching adulthood. In all images the gonad is outlined in white, the vulva is marked with an arrowhead, and uterine embryos are marked with arrows. The distal tip cell is opposite the vulva in (A), (C), and (G). (**B**) By 10 days of ARD, the gonad was considerably condensed and the embryos appear inviable. (**C**) DAPI (4′,6′-diamidino-2-phenylindole) staining of L4 worms at the time of food withdrawal. (**D**) DAPI staining of arrested adults after 30 days in ARD. (**E**) Quantification of germ cell nuclei by DAPI staining showed a significant reduction in germ cell nuclei over time. Time course data are displayed as the mean (±SD) number of surviving germ cells per gonad arm (n > 16 gonad arms per time point for days 5 to 30, and n = 5 gonad arms per time point for days 0 and 3). Worms were obtained from three independent starvations. The loss of germ cell nuclei plateaus when about 35 germ cells remain. (**F**) After 30 days of starvation, the gonad was considerably condensed, and the embryos remained as corpses (arrows). (**G**) After 72 hours of refeeding, a remarkable recovery was observed, and the germ line had regenerated and appeared similar to that of a well-fed young adult. No sperm are visible in the spermatheca, adjacent to the proximal end of the gonad. Scale bars = 20 μm.

hatching from embryos); thus, the initiation of reproduction can be extended by at least 15-fold when animals enter ARD.

ARD also appears to share the anti-aging properties of other developmental diapauses (Fig. 2B). Animals starved for up to 30 days were able to exit ARD and still live a normal adult life span (~18 days at 20°C) when returned to food. These results are consistent with prior studies showing that starvation of sterilized *C. elegans* adults can dramatically extend total life span (*14*). Our results are distinct from these studies, however, in that we observed the extension of life span and reproductive longevity in fertile WT animals. Nonetheless, it is possible that sterilization of adult nematodes mimics a component of the adult arrest and thereby leads to the extension of life span through a similar mechanism.

Protection of germline stem cells (GSCs) and germline regeneration. Perhaps the most striking feature of ARD was plasticity of the germ line during maintenance and recovery. Shortly after ARD initiation, we observed substantial loss of cellular volume and germ cell number in the gonad (Fig. 3, A to D). After more prolonged starvation (>10 days), the germ line was reduced to a small population of ~35 germ cell nuclei per gonad arm (Fig. 3E). This surviving population of germ cells was maintained at a relatively constant level for another 20 days, implying that these cells were protected from the mechanism that precipitated death of the rest of the germ line (Fig. 3E). Changes in somatic gonad morphology were also marked by retreat of the distal tip cell (Fig. 3B and fig. S3, A to C).

Reintroduction to food resulted in robust recovery, so that the germ line of an animal rescued after 30 days of starvation appeared similar to that of a young adult that had never been starved (Fig. 3G). Consequently, exit from ARD must involve processes that stimulate the proliferation and differentiation of GSCs in order to regenerate a fully mature and functional female germ line. Thus, the population of germ cells protected during extended starvation must contain functional GSCs.

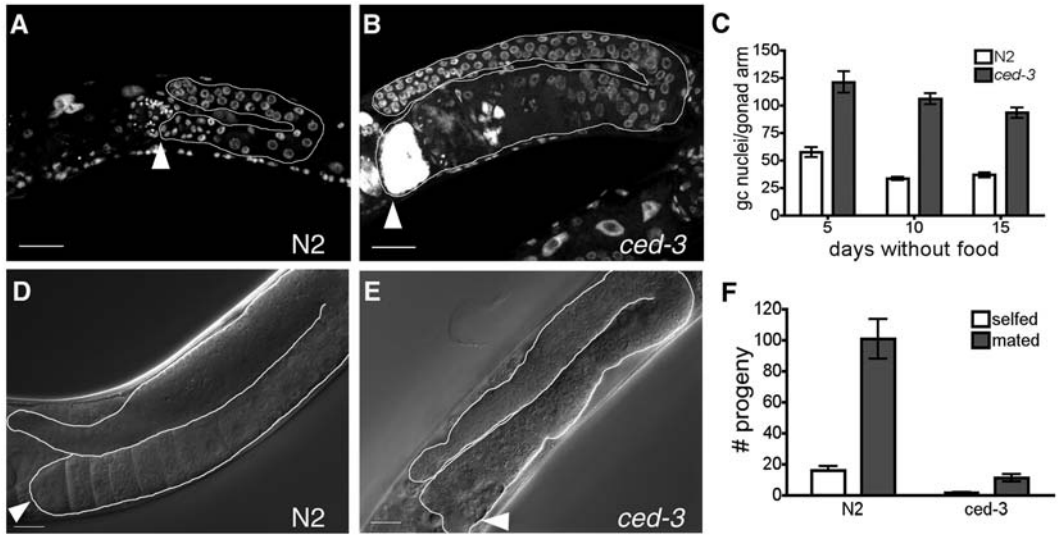

Fig. 4. (**A** and **B**) Whole-animal DAPI staining revealed that *ced-3(n1286)* mutants possess more germ cell nuclei than WT worms after 15 days of starvation in ARD. The proximal end of the gonad is indicated with a white arrowhead. The mass of DAPI staining in the *ced-3(n1286)* animal represents endomitotic nuclei in the proximal gonad. (**C**) The bar graph represents the mean (±SEM) number of germ cell nuclei present in arrested WT (white bars) and *ced-3(n1286)* (gray bars) adults after increasing days of starvation (n = 8 to 10 gonad arms per data point). (**D** and **E**) DIC images of recovered adults that had spent 15 days in ARD were taken after recovery. (**F**) Rescued *ced-3* adults produced fewer progeny by both self-fertilization (white bars) and mating with well-fed WT males (gray bars). Bars represent the mean (±SEM) number of progeny produced per animal (n > 15 animals per data point) upon recovery on food after 15 days of starvation. Scale bar = 20 μm.

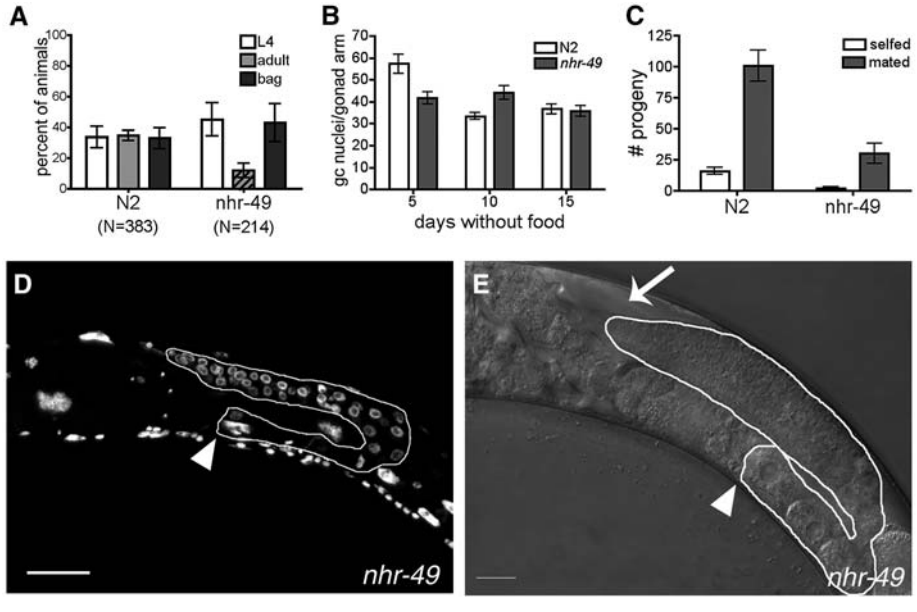

Fig. 5. (**A**) *nhr-49(nr2041)* mutants were severely impaired in their ability to form a normal ARD. Data are presented as the percent of animals (±SEM, n = 5) that arrested as L4s (white bars), bagged (black bars), or remained adults in ARD or some other form (gray bars). Total numbers of worms assayed in all five experiments are indicated on the graph. Of the 12% of *nhr-49(nr2041)* animals that did not arrest as L4s or die by bagging, fewer than 1% showed the primary characteristics of ARD (fig. S5). (**B**) The bar graph represents the mean (±SEM) number of germ cell nuclei per gonad arm (n = 7 to 10 gonad arms per time point) in arrested WT (white bars) and *nhr-49(nr2041)* adults (gray bars) after increasing days of starvation. (**C**) Rescued *nhr-49(nr2041)* mutants produced fewer progeny by both self-fertilization (white bars) and mating with well-fed WT males (gray bars). Bars represent the mean number of progeny (±SEM, n > 14 animals per time point) produced per recovered animal after 15 days of starvation. (**D**) A representative image of a DAPI-stained *nhr-49(nr2041)* adult after 15 days of starvation. The gonad is outlined, and the proximal end of the gonad is indicated with a white arrowhead. (**E**) A representative DIC image of a recovered *nhr-49(nr2041)* adult that had spent 15 days in starvation before rescue. The yolklike substance in the body cavity is indicated with a white arrow. Scale bar = 20 μm.

Programmed cell death (PCD) or apoptosis in *C. elegans* is active during adulthood as a means to control total germ cell number in healthy animals and also to mediate germ cell death in response to pathogens, stress, and short-term starvation (*15*–*18*). To determine whether apoptosis was responsible for the germline atrophy observed during ARD, we examined the apoptosis-deficient *ced-3(n1286)* mutant. The *ced-3* gene encodes a caspase essential for apoptosis. We found that inactivation of *ced-3* did not inhibit ARD entry but did prevent the reduction in germ cell nuclei normally observed during the course of ARD (Fig. 4, A to C). Although *ced-3(n1286)* mutants appeared healthy when starved for 15 days in ARD, these mutants were unable to successfully recover upon refeeding, because germ cell proliferation resumed in the distal gonad, but oocyte formation and embryo production were severely impaired (Fig. 4, D to F, and fig. S4, A to D).

These results demonstrate that the PCD mechanism is a critical factor for extending the reproductive potential of animals rescued from prolonged starvation in ARD. The germ cell loss observed in ARD could potentially be explained by the normal rate of germline apoptosis in combination with a failure to replace dead germ cells with new germ cell proliferation (*15*). However, there are clear differences between starvation-mediated cell death, which results in the death of nearly all differentiated cells, and the apoptosis mechanism that functions in the gonad of well-fed animals, which targets only some germline cells in order to nurse growing oocytes (*15*). Thus, it is likely that the ARD mechanism is specifically regulating germ cell proliferation and apoptosis.

NHR-49 is required for ARD. In mammals, both the peroxisome proliferator–activated receptor α (PPARα) and hepatocyte *nuclear* factor-4α (HNF-4α) nuclear receptors (NRs) mediate metabolic response to food withdrawal, implying that NRs function in starvation physiology (*19*, *20*). The *C. elegans* counterpart of these receptors is the NHR-49 protein, which is an HNF-4α homolog that mediates the induction of fatty acid oxidation and gluconeogenesis genes in response to food deprivation (*21*).

We found that NHR-49 was required for the establishment of ARD. When *nhr-49* loss-of-function animals [*nhr-49(nr2041)*] were starved at the same point in the L4 program as WT animals, approximately 12% did not arrest as L4s or bag as adults (Fig. 5A). However, less than 1% of these animals entered a normal ARD. First, in all *nhr-49(nr2041)* animals that failed to bag, embryos in the uterus were inviable (fig. S5, A to B). This observation raises the question of whether *nhr-49* mutants are able to arrest embryonic development, and consequently only survive extended starvation if dead embryos are produced, thus preventing death via bagging. Second, in surviving *nhr-49(nr2041)* adults, sperm were strongly attracted to the proximal oocyte, whereas in WT animals in the reproductive diapause, sperm were primarily confined to the spermatheca (fig. S5, C to E). Finally, *nhr-49(nr2041)* animals were severely impaired in their ability to recover from starvation and produce offspring (Fig. 5C and table S2). Two days after rescue of starved *nhr-49(nr2041)* adults, the uterus of these mutants was filled with cellular debris, and yolk-like material was present throughout the body cavity (Fig. 5E and fig. S6C). The rate of cell death in *nhr-49(nr2041)* mutants was comparable to that of WT animals, however, implying that the accumulation of cellular debris is likely to be a consequence of impaired autophagy or phagocytic mechanisms normally required for maintaining a clean proximal gonad (Fig. 5, B and D, and fig. S6D).

The fact that *nhr-49(nr2041)* mutants could not properly mediate ARD entry brought up the question of whether the loss of *nhr-49* function and poor ARD establishment would also interfere with the protection of GSCs. However, we found that *nhr-49* and normal ARD establishment were not required for the survival of GSCs and for their ability to proliferate upon recovery from starvation (Fig. 5, D and E, and figs. S5G and S6C), implying that these components of the ARD are separable from the NHR-49–mediated mechanism that facilitates ARD establishment.

It is clear that NHR-49 mediates numerous changes in glucose and fat metabolism upon starvation; how NHR-49 senses these changes and responds is not yet understood (*21*, *22*). The *Drosophila* homolog of NHR-49, HNF-4α, has been shown to interact with free fatty acids released from lipases immediately after the initiation of starvation (*23*). Given the strong homology of these two NRs both in structure and in function, it is likely that a similar mechanism occurs in *C. elegans*. Consequently, we expect that lipases and the upstream signals that activate lipases in response to starvation will also be key features in this overall signaling network. Recent studies have shown that GSC arrest leads to the activation of lipid hydrolysis in *C. elegans* (*24*).

Discussion. We have shown that the initiation of ARD enables the formation of a starvation-resistant adult that can extend reproductive potential for at least 30 days. Based on our findings, we propose a "disposable germline" hypothesis: As ARD progresses, nutrient deprivation leads to a loss of oocytes and meiotic germ cell nuclei. Meanwhile, a small population of mitotic germ cells is maintained in the stem cell niche in quiescence or self-renewal. Mitotic germ cells in general are protected from apoptosis in the *C. elegans* germ line (*15*). Because these cells are generally held in mitosis by Notch signaling from the stem cell niche, we predict a role for Notch signaling in ARD (*15*, *16*). In support of this hypothesis, a $P_{lag\text{-}2}$::GFP (P, promoter; GFP, green fluorescent protein) promoter shows that expression of the *lag-2* gene, which encodes the Notch ligand necessary for inhibiting exit from mitosis, is expressed at high levels even after 90 days of starvation (fig. S3). We also predict that autophagy and/or phagocytosis of apoptotic germ cells provides fuel necessary for supporting viability during starvation. As a whole, this model implies that worms have adapted a mechanism to exploit differentiated germ cells for fuel while preserving pluripotent stem cells for tissue regeneration when conditions improve. Although the ecological advantage of the ARD is not yet clear, we suggest that this alternative diapause may exist because it is specially adapted to survive starvation in environmental contexts where the dauer diapause is not as effective, or it functions simply as an added layer of protection against harsh environmental conditions. Like the *C. elegans* dauer diapause, our results show that ARD is dependent on population density, implying that pheromones and/or other signals may also be an important part of this process. However, we cannot yet rule out the possibility that a crowded population facilitates ARD by depleting important nutrients from the growth media.

The effect of ARD on the reproductive system is quite distinct from the prevailing models of the impact of caloric restriction on somatic cells, which posit that reduced calorie intake leads to slowing of somatic cell aging (*9*). We have observed here that starvation of adult *C. elegans* leads to a "reversal" of the aging process in the reproductive system, because a new differentiated germ line is regenerated when food is restored. Our characterization of this reproductive diapause presents *C. elegans* as a model for this process and for understanding how stem cells and their host tissues survive and recover from prolonged periods of nutrient deprivation. Studies in fruit flies show that this phenomenon may be conserved across metazoan species, because nutrient deprivation leads to changes in germline proliferation and apoptosis in *Drosophila* (*25*, *26*). Vitamin A levels can control GSC death and proliferation in rodent testes in a process that may mirror that of germline plasticity in *C. elegans* ARD (*27*). Finally, starvation of cultured cancer cell lines results in the death of differentiated cancer cells, whereas side populations of cancer stem cells survive (*28*). Altogether these findings suggest a provocative mechanism for how calorically restricted animals in general, including mammals, may extend their reproductive period (*29*), and we expect that studies of the *C. elegans* ARD will reveal essential factors involved in somatic cell and GSC aging, as well as factors involved in controlling germline resurrection in response to nutritional signals. Our implication of NHR-49 reveals a NR important for robust ARD establishment, and our characterization of a *ced-3* mutant reveals an important role for apoptosis in ARD maintenance and recovery.

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Supporting Online Material

www.sciencemag.org/cgi/content/full/1178343/DC1
Materials and Methods
Figs. S1 to S6
Tables S1 and S2

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