Supporting Online Material for

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

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Published 27 August 2009 on *Science* Express
DOI: 10.1126/science.1178343

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SUPPLEMENTAL METHODS

C.ELEGANS STRAINS

Worm maintenance and experiments were carried out at 20°C. Worms were grown on the *E. coli* (OP50) strain for all experiments. Most nematode strains used were provided by the Caenorhabditis Genetics Center (CGC), which is funded by the NIH National Center for Research Resources. CGC strains included: wild-type N2 strain (Bristol), MT3002 *ced-3(n1286),* and JK2868 qIs56 [*lag-2::gfp*; *unc-119(+)*]. *nhr-49(nr2041)* was a gift from Carl Johnson at Axys Pharmaceuticals.

PLATE COMPOSITION

For optimal diapause formation, we found that plate preparation was critical. We used the following ingredients for nematode growth media (NGM) plates (per 1L of media): 3 g NaCl, 25 g agar (Sigma-Aldrich), 2.5g peptone (Fisher Scientific), 1 ml 1M $CaCl_2$, 1 ml 1M $MgSO_4$, 25 ml 1M Potassium Phosphate Buffer (ph 6.0), and 1.0 ml cholesterol stock (Sigma-Aldrich; 8 mg/ml in 100% ethanol). Since maintaining sterile plates for the duration of starvation was critical, plates were poured in a laminar flow hood. A higher agar concentration than normal was used to minimize the burrowing of starved animals.

Some starvations were performed on agarose plates. Wild-type worms recovered similarly from both agarose and NGM plates. We used the following ingredients for agarose plates (per 1L of media): 3 g NaCl, 15 g agarose (Invitrogen), 1 ml 1M $CaCl_2$, 1 ml 1M $MgSO_4$, 25 ml 1M Potassium Phosphate Buffer (pH 6.0), and 1.0 ml cholesterol stock (8 mg/ml).

The addition of antibiotics in starvation assays is often used as a means to reduce growth of contaminants. We tested the recovery of wild-type arrested adults starved on NGM plates containing carbenicillin (50 μg/ml final concentration) and found that antibiotic had a significant detrimental impact on several aspects of the adult reproductive diapause (ARD), including early death of uterine embryos, decreased lifespan during starvation and following recovery on food, and diminished progeny production following recovery. Therefore, antibiotics were not included in any of our starvation assays.

ENTRY OF ANIMALS INTO THE ADULT DIAPAUSE

Worms were synchronized by hypochlorite treatment and L1 release. A population of animals in the mid-L4 stage of development, as assessed by DIC microscopy (fig. S1, A to C), was collected from feeding plates in M9 buffer and washed six times with M9 buffer. Animals were then plated onto 10 cm NGM or agarose plates with no food at a density of approximately 10,000 worms per plate. When worms were treated in this manner we observed three potential outcomes. Starved worms either: (i) arrested as L4 larvae, (ii) entered adult reproductive diapause, or (iii) died by bagging, the hatching of fertilized embryos in the uterus which leads to the death of the parent. Only those adults containing one or two viable embryos *in utero* that did not hatch and cause bagging were considered to be in adult reproductive diapause and were selected for further analysis. We found that any animals containing more than two embryos were not in ARD and would die shortly due to bagging. In our study, it was not possible to more precisely define the developmental point of diapause commitment for two reasons: First, even synchronized larval populations contained some heterogeneity, and diapause entry could not be established with single animals. Second, because of the presence of residual gut bacteria, it was not possible to determine how long after food withdrawal that starvation actually initiated, making it impossible to determine if animals developed from the mid-L4 stage into arrested adults before or after starvation signals were activated.

BROOD SIZE ASSAYS

For brood size measurements, we counted the number of progeny produced once arrested adults were returned to food following an increasing number of days spent in adult reproductive diapause. Individual arrested adults were placed onto 6 cm NGM-lite plates seeded with OP50 and transferred daily until no more embryos were laid. For mating broods, arrested adults were given time to recover on food (24 or 48 hours depending on the amount of time necessary for the appearance of full germline regeneration) before being combined with well-fed males (3 males to one recovered adult hermaphrodite). After 24 hours of mating, males were removed in order to minimize mating-induced trauma.

LIFESPAN ASSAYS

Lifespans were determined by counting the number of days arrested adults survived on OP50 after being rescued from adult reproductive diapause. Approximately 35 arrested adults for each time point were transferred to 6 cm NGM-lite plates seeded with OP50. Day 0 represents the first day back on food and adults were transferred to new plates daily until progeny production had ceased. Animals were considered dead when they no longer responded to a gentle tap with a worm pick.

WHOLE ANIMAL DAPI STAINING AND GERM CELL COUNTS

Arrested adults were tube fixed in ice cold, 100% methanol (5 min, -20^{o}C) and washed one time with PBS. Fixed animals were incubated (30 minutes, room temperature, protected from light) in a 4',6'-diamidino-2-phenylindole hydrochloride (DAPI) solution at a final concentration of 500 ng/ml. Stained animals were washed one time in PBS and transferred to a glass slide. 1% 1,4-diazabicyclo[2.2.2]octane (DABCO) was added to preserve fluorescence signal. Stained animals were imaged on a Zeiss LSM 510 META spectral confocal and two photon microscope. Germ cell nuclei were visualized using Zeiss LSM Image Browser v 4.0 and manually marked and counted throughout a series of z-stacks.

PREMATURE EXIT FROM ADULT REPRODUCTIVE DIAPAUSE

We found that the presence of contaminants on or inside starvation plates, including superficial carcass of dead animals, surface bacteria, mold, and yeast could contribute to partial release from adult reproductive diapause (fig. S2, A to C). Very small amounts of these contaminants were enough to affect both the embryonic and the reproductive components of adult diapause. Release from the embryonic arrest led to the slow development of the one or two embryos initially present in utero, which eventually hatched inside the adult, and sometimes additional ovulation and fertilization events. Oftentimes, contamination was localized to small regions and not immediately visible on the surface of the starvation plates. Additionally, contaminants could occasionally be seen growing inside the intestinal lumen of released animals. Scale bar = 20 μm.

VISUALIZING DIAPAUSE EXIT UPON SINGLE WORM ISOLATION

Individual arrested adult wild-type worms (5 days in diapause) were picked to 6 cm NGM-lite plates containing a ring of palmitic acid (10 μg/μl in ethanol), which precipitates out of solution and forms a physical barrier that helps prevent worms from escaping. Arrested adults were examined 24 hours later.

Supplementary figure 1

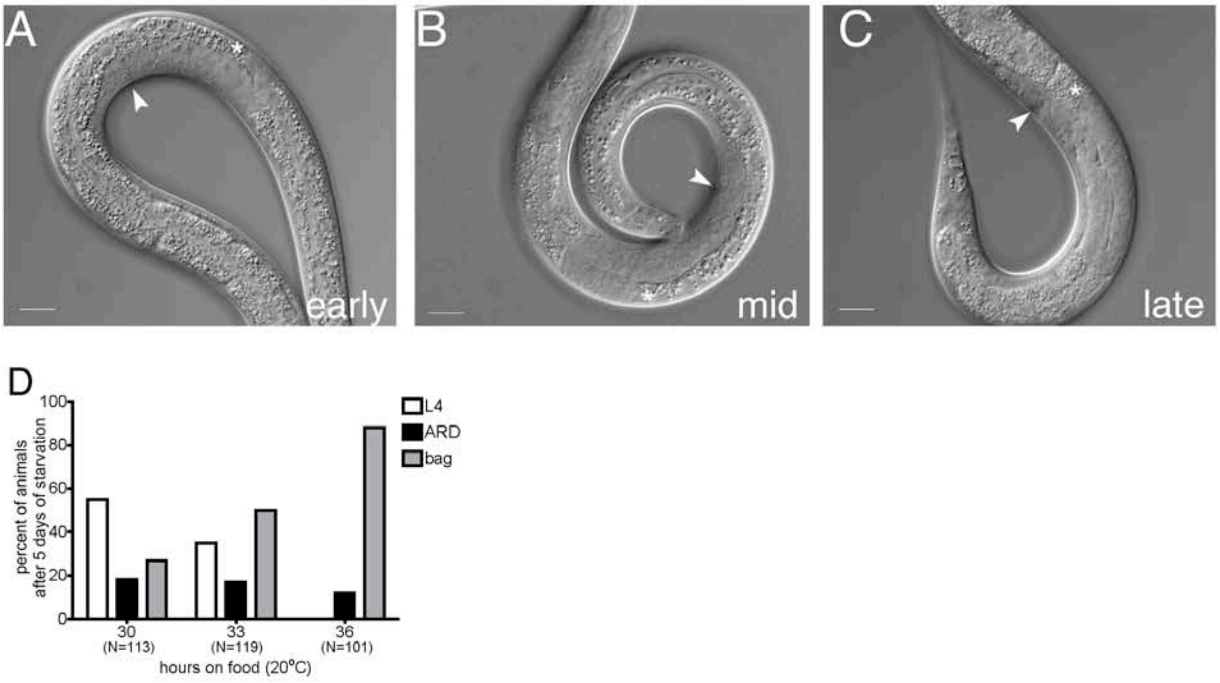

Fig. S1. (A-C) Representative images of worms that were categorized as in the early, mid, and late phases of L4 development based on gonad migration. The vulva is marked with an arrowhead and the distal end of the gonad is marked with an arrow. **(D)** After 5 days of starvation, a sample of the starved population was scored for the number of animals in each of three starvation outcomes: L4 arrest, ARD, and bagged adult. Figure D shows a representative example of one time course experiment. Averaged data from 9 experiments are shown in the text in **Figure 1B**. Removing worms from food when the majority of the population was in the mid-L4 stage was found to be optimal and routinely used for ARD induction. Under our conditions, it took approximately 32-33 hours on food at 20^{o}C for L1s to reach this mid-L4 phase for food withdrawal. Starvation earlier (30 hours) resulted in a higher proportion of L4 arrested animals, and starvation later (36 hours) resulted in a higher proportion of bagged animals. Scale bar = 20 μm.

Supplementary figure 2

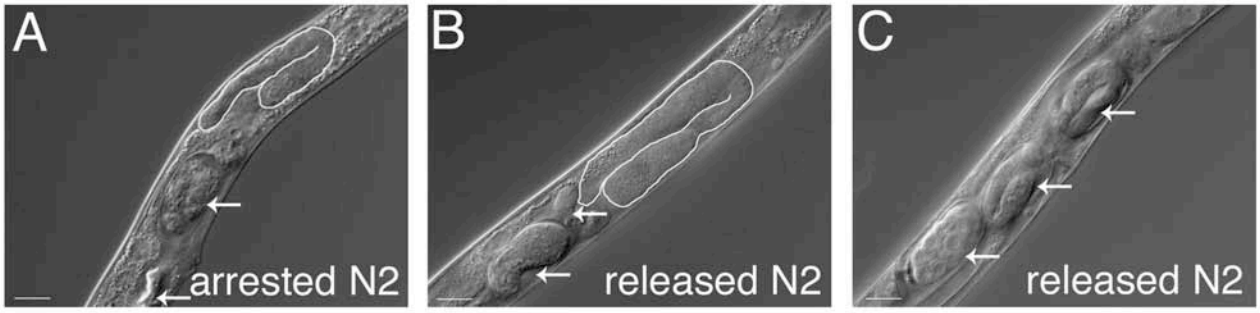

Fig. S2. (A) An arrested wild-type worm in adult reproductive diapause after 10 days of starvation. In this case, the arrested embryos (marked by arrows) are no longer viable and the gonad (outlined) is significantly condensed. **(B)** A wild-type worm that has been released from adult reproductive diapause due to plate contamination after 12 days of starvation. One embryo has developed to the comma stage while the second embryo has already hatched inside the adult (both marked by arrows). The gonad (outlined) is less condensed than that of an N2 adult in ARD for a comparable amount of time **(**see panel A**)** and one large oocyte has formed in the proximal gonad. **(C)** Another example of a wild-type worm that has prematurely released from adult diapause due to plate contamination after 12 days of starvation. In addition to progression through embryonic development, a new ovulation and fertilization event has occurred (marked by arrows). The presence of viable embryos in animals released after 10 days of starvation may indicate that these embryos were still viable upon release or, alternatively, that the dead embryos were expelled and replaced by new embryos.

Supplementary figure 3

Fig. S3. Significant changes in gonad morphology were observed in arrested adults, but not in starved L4 animals. Shown are representative DIC and fluorescent microscopy images of a WT strain harboring a *lag-2::gfp* reporter that marks the distal tip cell. **(A)** In L4 animals starved for 5 days, both distal tip cells are located opposite the vulva (arrowhead), demonstrating that starvation has no impact on distal tip cell localization. **(B)** In contrast, starvation leads to a significant atrophy of the germline and somatic gonad in arrested adult animals at day 5 of starvation, including retreat of the distal tip cell. **(C)** *lag-2::gfp* expression in an arrested adult 90 days after starvation, fluorescent signal in the uterus is due to autofluorescence.

Supplementary figure 4

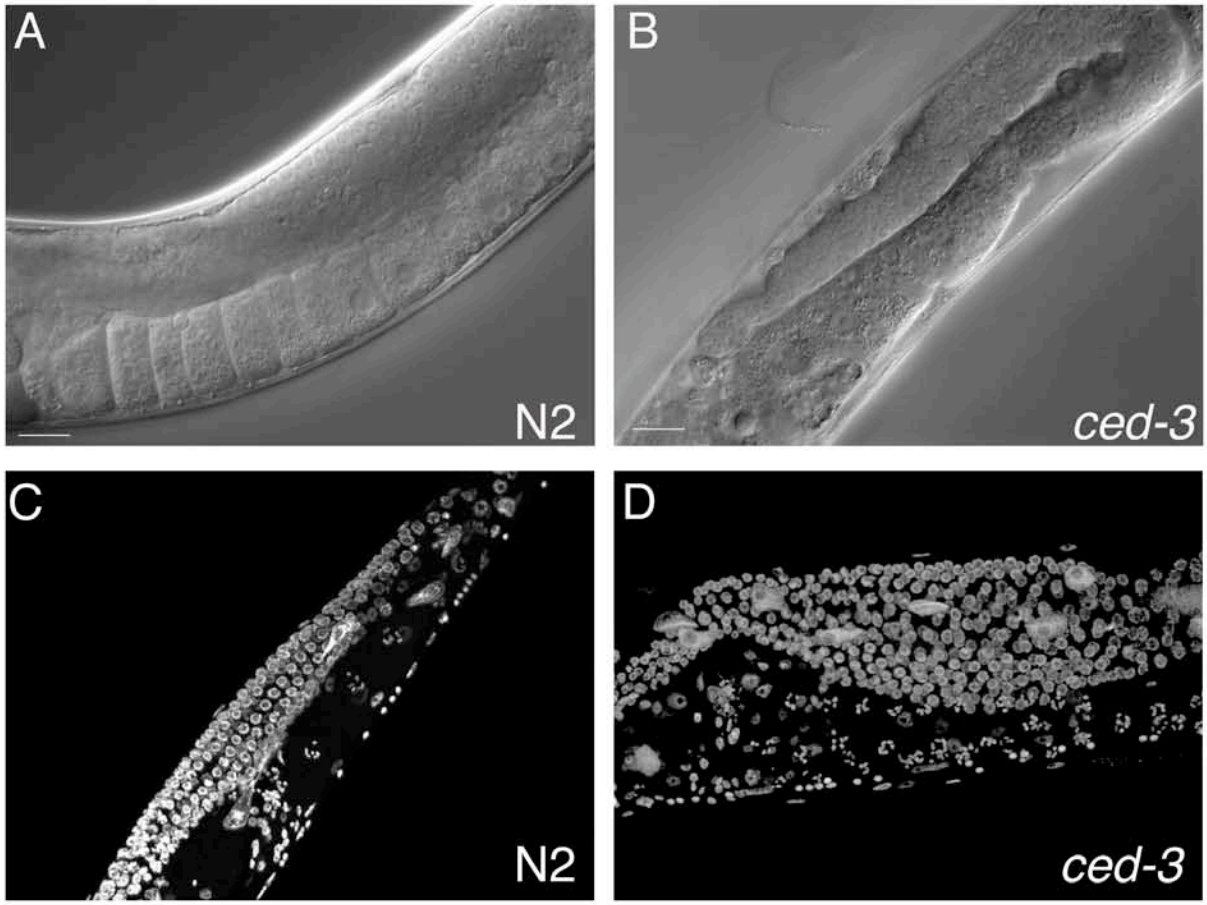

Fig. S4. (A & B) DIC images of recovered adults that had spent 15 days in adult diapause. N2 worms were able to recover a fully functional germline with normal morphology and organization. Although *ced-3(n1286)* adults could reproliferate their germline, abnormal oocytes accumulated in the proximal gonad. **(C & D)** Whole animal DAPI staining confirmed that upon recovery on food after 15 days of starvation, *ced-3* mutants resumed germ cell proliferation but accumulated differentiated germ cells in the diakinesis stage in their proximal gonad.

Supplementary figure 5

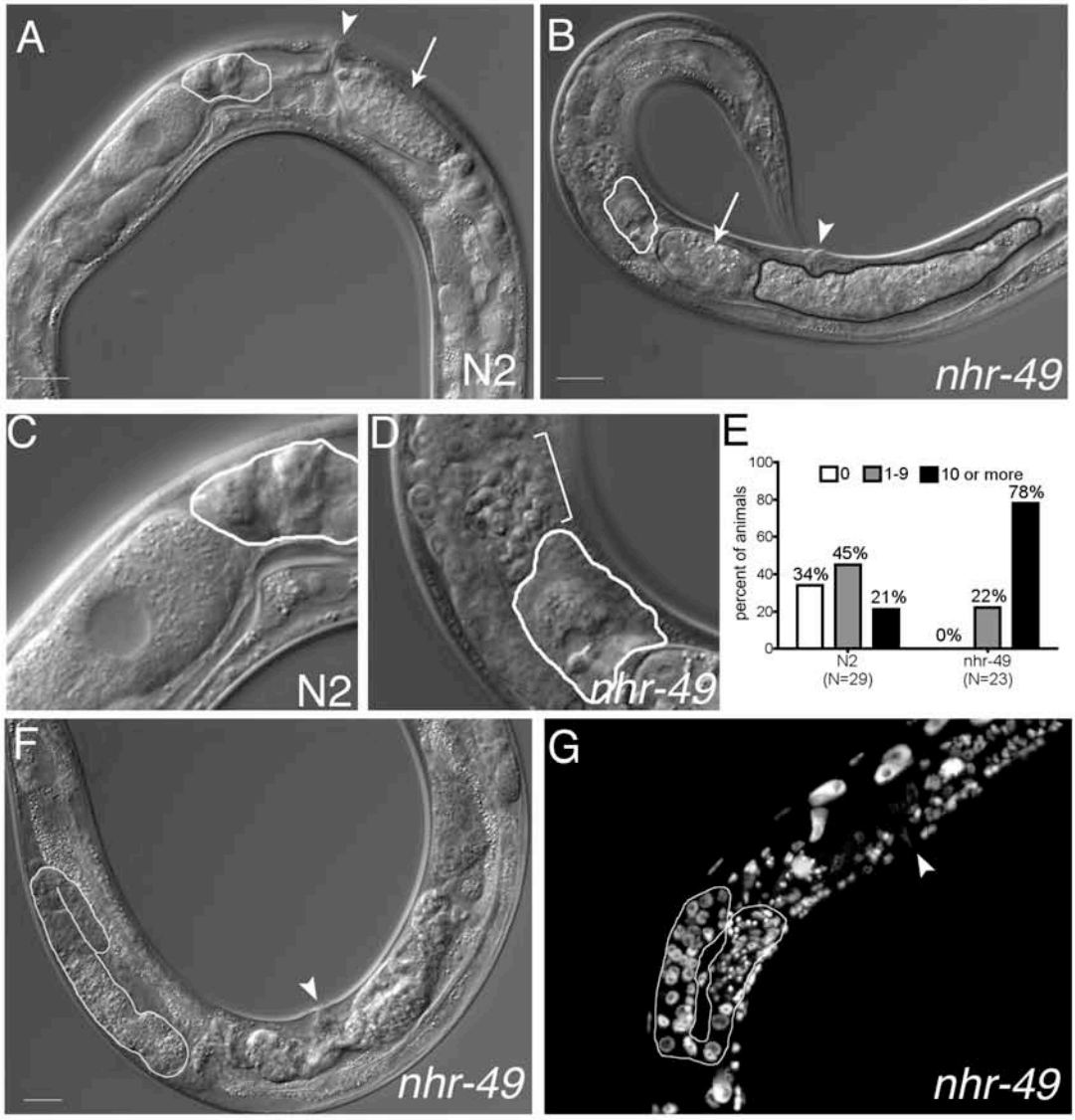

Fig. S5. **(A)** A WT animal in ARD held one arrested embryo (white arrow) in utero and had a visibly condensed germline and retracted DTC, the spermatheca is outlined in white. **(B)** A typical arrested *nhr-49(nr2041)* animal after 5 days of starvation, the embryo is inviable and there is an accumulation of material in the uterus (outlined in black) **(C)** Sperm are confined to the spermatheca (outlined in white) in WT animals in ARD. **(D)** An arrested *nhr-49(nr2041)* animal with sperm (white bracket) outside of the spermatheca and localized on the proximal oocyte. **(E)** The presence of sperm outside of the spermatheca and near the proximal oocyte in animals starved up to 7 days was scored for N2 and *nhr-49(nr2041)* adults. Shown is the percentage of animals with no sperm (white bars), 1-9 sperm (gray bars), or 10 or more sperm (black bars) present on the proximal oocyte (N=29 for WT animals and 23 for *nhr-49(nr2041)* mutants) **(F)** A DIC image of an *nhr-49(nr2041)* worm after 10 days of starvation, significant debris is seen in the proximal region of the gonad (outlined) (G) A DAPI image of an *nhr-49(nr2041)* worm after 10 days of starvation shows that a significant reduction in germ cells has occurred. In all images, the vulva is marked with an arrowhead. Scale bar = 20 μm.

Supplementary figure 6

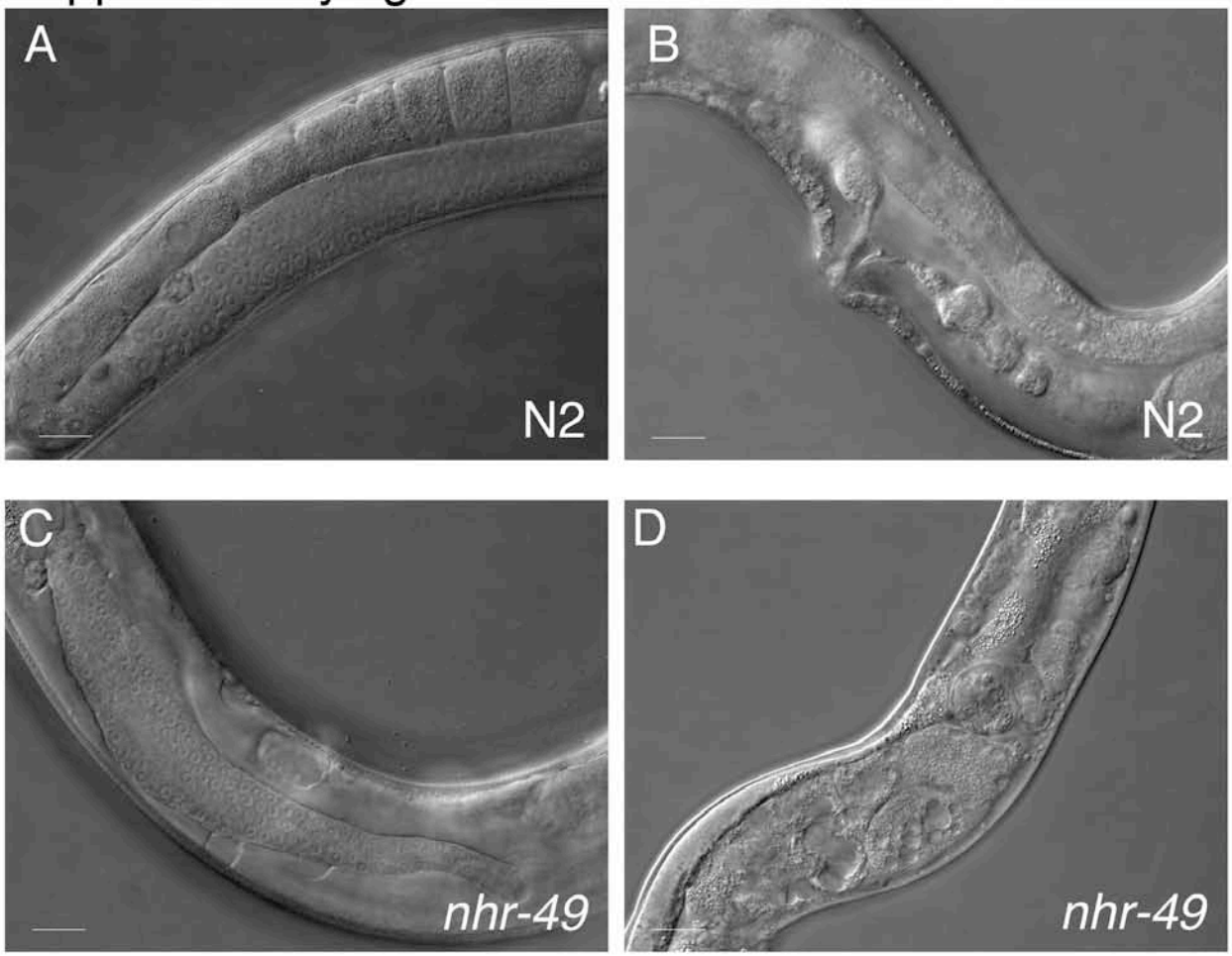

Fig S6. An N2 before rescue by food restoration is shown in Panel B, and after rescue in Panel A. After 15 days of starvation, N2 worms were able to recover from ARD to produce a functional germline with normal morphology and organization, differing only from the germline of a young adult in the reduction of viable sperm. Importantly, in N2 animals, the uterus was free of debris before recovery. An *nhr-49(-/-)* mutant is shown before rescue in Panel D, and after rescue in Panel C. DIC images of *nhr-49(nr2041)* animals recovered after 15 days of starvation. Although these animals could, at least partially, regenerate the germline, debris accumulated in the uterus before recovery and yolk and debris were present throughout the body cavity after rescue.

	# of days spent in ARD before isolation	# of arrested adults isolated	escape or visible contamination	# exiting ARD within 1 day of isolation
1	1	16	0	16
2	5	36	16	20
3	3	19	0	18
4	3	10	4	6
5	3	8	0	8
TOTALS		89	20	68

Table S1. Exit from ARD upon isolation from the population. Five separate experiments are shown and identified in the furthermost left column. ARD animals were selected 1,3, and 5 days after starvation and isolated in a fatty acid ring. Of these, nearly all assayed animals exited ARD within one day of starvation resulting in the development of uterine embryos and bagging. This experiment shows that the embryos contained in the uterus of these animals when in ARD were viable and could resume normal development upon isolation and diapause exit.

	Incomplete germline recovery	Yolk accumulation in body cavity	Debris in uterus	Debris in gonad	Abnormal recovery of any kind
WT	0/13	2/13	2/13	1/13	2/13
nhr-49 (nr2041)	3/13	9/13	9/13	2/13	12/13

Table S2. The phenotypes of recovered WT and *nhr-49(nr2041)* animals after 15 days of starvation. Recovery abnormalities often seen in *nhr-49(nr2041)* worms included excessive accumulation of a yolk like substance in somatic tissues, and a significant amount of debris in the uterus. Less frequently, we observed a failure to completely regenerate the germline. Although WT worms occasionally displayed an abnormal recovery, the severity of this abnormality was always less than that observed in *nhr-49(nr2041)* animals.