sequence of calcium oscillations in the cell cycle is to trigger centrosome duplication, and in particular we suggest that IP_3-dependent calcium oscillations regulate the initiation of centrosome duplication through CaMKII activation. Consistent with the requirement for CaMKII to trigger centrosome duplication, chelation of calcium in cycling *Xenopus* egg extracts blocks the cell cycle before S phase entry, when centrosome duplication starts (*13*). CaMKII is localized on centrosomes (*15, 16*) and phosphorylates several centrosomal proteins in vitro (*16*). These proteins may be candidate substrate(s) for initiation of centrosome duplication by CaMKII.

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34. After incubation of the S phase–arrested extracts (*4*) for 6 hours, centrosomes and microtubules were centrifuged onto cover slips at room temperature without depolymerization of microtubules. The cover slip was fixed with 99% methanol at –20°C for 5 min and then stained with a rabbit polyclonal antibody to γ-tubulin (Sigma) and α-tubulin mAb (Sigma).
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24 August 2001; accepted 21 November 2001

Regulation of Life-Span by Germ-Line Stem Cells in *Caenorhabditis elegans*

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The germ line of the nematode *Caenorhabditis elegans* influences life-span; when the germ-line precursor cells are removed, life-span is increased dramatically. We find that neither sperm, nor oocytes, nor meiotic precursor cells are responsible for this effect. Rather life-span is influenced by the proliferating germ-line stem cells. These cells, as well as a downstream transcriptional regulator, act in the adult to influence aging, indicating that the aging process remains plastic during adulthood. We propose that the germ-line stem cells affect life-span by influencing the production of, or the response to, a steroid hormone that promotes longevity.

Killing the germ-line precursor cells, Z2 and Z3, extends the life-span of *C. elegans* by ~60% (*1*). This longevity is not a result of sterility, because removing the entire reproductive system (germ line and somatic gonad) has no effect on life-span. In order for germ line–ablated animals to live longer than normal, they require DAF-12, a nuclear hormone receptor, and DAF-16, a forkhead-family transcription factor. We found that this effect could be reproduced genetically: *mes-1(bn7)* mutants, which lack germ cells, were long lived (Fig. 1A), as were *glp-1(q158)* mutants (Fig. 1B) (*2, 3*). *glp-1* encodes the receptor for a germ-line proliferation signal that is produced by the distal tip cells of the somatic gonad (*4–7*). In *glp-1(q158)* mutants, Z2 and Z3 generate only a few germ cells, which then enter meiosis and differentiate as sperm (*7*). In both mutants, life-span extension was suppressed by a *daf-16* null mutation and by ablation of the somatic gonad precursor cells (Fig. 1) (*8, 9*). Many other mutants with defective germ-line proliferation were also long lived [Web table 1, experiments A and B (*10*); (*9*)].

The germ-line precursors are stem cells that divide continuously during development.

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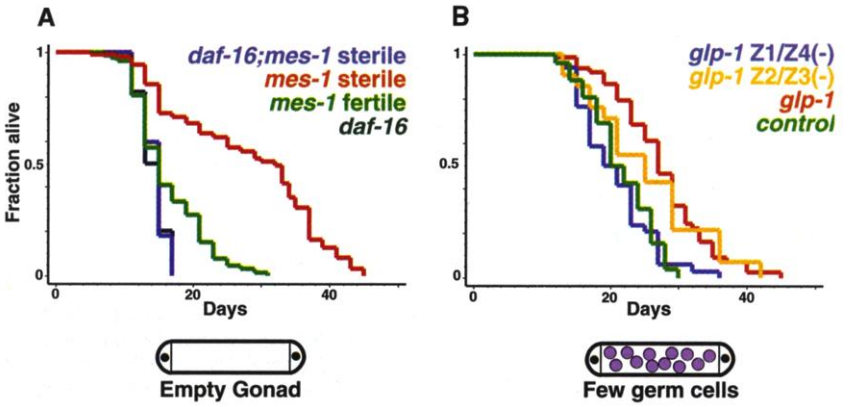

Fig. 1. Life-spans of germ-line mutants. In each graph, experimental and control animals were grown in parallel. *n*, total number of animals observed in each experiment/total number of uncensored animals. *m*, mean adult life-span (days). (–), ablated. *P* values refer to experimental and control animals in a single experiment. (**A**) Life-spans of germ-line(−) mutants. At 20°C, *mes-1*(*bn7*) is 50% penetrant; half of the animals have normal germ lines (*3*). *mes-1*(*bn7*) fertile, n = 48/38, m = 15.8; sterile, n = 48/31, m = 29.8; Z1/Z4(−), n = 52/51, m = 17.2, $P \leq$ 0.0001. *daf-16*(*mu86*), n = 54/26, m = 14.1; *daf-16*(*mu86*); *mes-1*(*bn7*) sterile, n = 62/22, m = 14.2, P = 0.90. Note that *daf-16* mutants are short-lived (*25*). (**B**) Life-spans of *glp-1*(*q158*) mutants. All of these strains contained the *dpy-19*(*e1259*) mutation. *dpy-19*(*e1259*) (control), n = 48/26, m = 21.5; *dpy-19*(*e1259*) *glp-1*(*q158*), intact, n = 95/52, m = 27.4, $P \leq$ 0.0001; *dpy-19*(*e1259*) *glp-1*(*q158*), Z2/Z3(–), n = 30/17, m = 25.3, P = 0.04; *dpy-19*(*e1259*) *glp-1*(*q158*), Z1/Z4(−), n = 35/34, m = 20.7, P = 0.72.

As development progresses, germ cells located farthest from the distal tip cells enter meiosis and then differentiate into sperm (during the L4 stage) or oocytes (during adulthood) (*6*), but a pool of proliferating stem cells is maintained well into adulthood (Fig. 2).

We found that neither sperm nor oocytes are required for the germ line to shorten life-span. Previously, *fem-3(e1996)* mutants, which do not produce sperm and develop as females, were found to have normal life-spans (*11*). We found that three additional female mutants, *fog-1(q180)*, *fog-2(q71)*, and *fog-3(q470)* (*12*–*14*), also had normal life-spans [Web table 1, experiments C and D (*10*)]. In *daz-1(tj3)* mutants, oocyte precursor cells arrest development at meiotic prophase and subsequently undergo apoptosis (*15*). We found that the life-spans of *daz-1* mutants were similar to those of the wild type (Table 1, experiment A). In addition, germ-line ablation extended the life-span of males, which do not produce oocytes, to the same extent as for control hermaphrodites (Table 1, experiment B).

To determine whether germ-line stem cells might influence life-span, we forced these cells to exit mitosis and enter meiosis at different times using a temperature-sensitive allele of *glp-1, e2141* (*16*). Surprisingly, we found that inducing this switch by shifting *glp-1(ts)* mutants to the nonpermissive temperature either during development or in early adulthood (when the animals were already producing progeny) extended life-span (Fig. 2). This demonstrates that germ-line stem cells do influence life-span, and that they can exert their influence in the adult. The life-span increase we observed after shifting L4 larvae or young adults was smaller than we observed after shifting younger animals. Thus, germ-line proliferation in the larva may also influence life-span.

We also asked whether excessive germ-line proliferation might shorten life-span. We tested two mutants in which germ-line stem cells fail

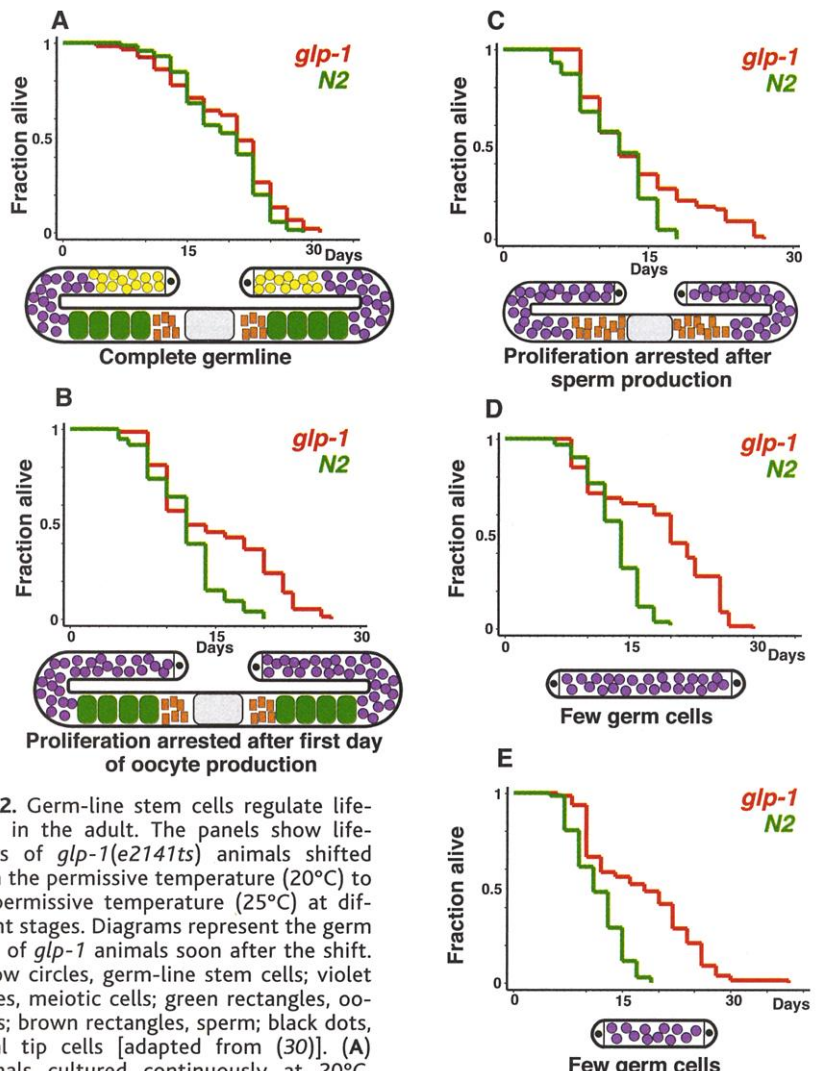

Fig. 2. Germ-line stem cells regulate life-span in the adult. The panels show life-spans of *glp-1*(*e2141ts*) animals shifted from the permissive temperature (20°C) to nonpermissive temperature (25°C) at different stages. Diagrams represent the germ lines of *glp-1* animals soon after the shift. Yellow circles, germ-line stem cells; violet circles, meiotic cells; green rectangles, oocytes; brown rectangles, sperm; black dots, distal tip cells [adapted from (*30*)]. (**A**) Animals cultured continuously at 20°C. Wild type (N2), n = 80/71, m = 19.4; *glp-1*(*e2141ts*), n = 60/46, m = 19.9, P = 0.24. (**B**) Animals cultured at 20°C until day 1 of adulthood, and then shifted to 25°C. N2, n = 80/57, m = 11.9; *glp-1*(*e2141ts*), n = 80/79, m = 15.0, $P \leq$ 0.0001. (**C**) Animals cultured at 20°C until L4 and then shifted to 25°C. N2, n = 80/64, m = 11.6; *glp-1*(*e2141ts*), n = 65/64, m = 13.9, P = 0.008. (**D**) Animals shifted from 20°C to 25°C at L2. N2, n = 78/59, m = 13.3; *glp-1*(*e2141ts*), n = 80/80, m = 18.6, $P \leq$ 0.0001. (**E**) Animals cultured continuously at 25°C. N2, n = 80/70, m = 11.6; *glp-1*(*e2141ts*), n = 80/77, m = 17.7, $P \leq$ 0.0001. This experiment was repeated twice, yielding similar results (*9*).

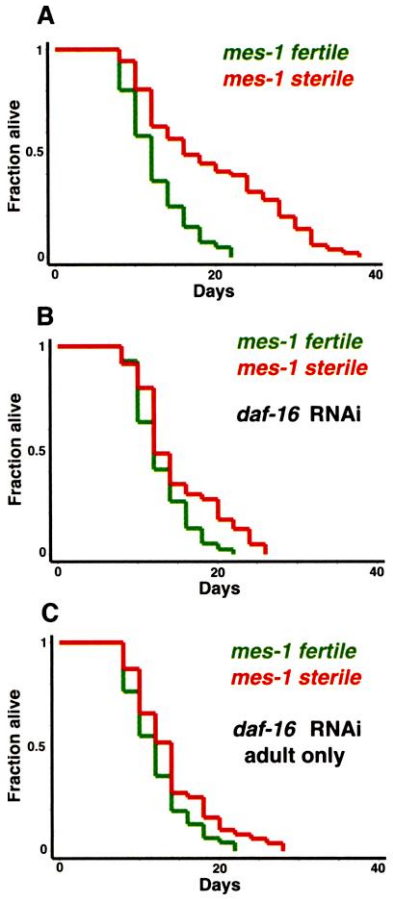

Fig. 3. Time of action of DAF-16 in germ-line regulation of life-span. Panels show the response of *mes-1(bn7)* fertile and sterile animals to a shift from control bacteria (vector only) onto bacteria expressing *daf-16* dsRNA. *daf-16* RNAi bacteria were grown on plates containing ampicillin, and carried an ampicillin-resistance gene. Bacteria carrying the plasmid vector alone were not ampicillin resistant. The sterile Mes-1 phenotype was assayed using a dissecting microscope. (**A**) *mes-1(bn7)* mutants grown on bacteria carrying the plasmid vector. Fertile animals, n = 70/41, m = 12.5; sterile animals, n = 66/51, m = 19.5, $P \leq 0.0001$. (**B**) *mes-1(bn7)* grown on *daf-16* RNAi bacteria. Fertile animals, n = 67/40, m = 12.9; sterile animals, n = 66/42, m = 14.9, P = 0.022. (**C**) *mes-1(bn7)* shifted to *daf-16* RNAi bacteria at the onset of adulthood. Fertile animals, n = 69/47, m = 12.2; sterile animals, n = 79/51, m = 14.1, P = 0.036.

to differentiate and instead overproliferate, *glp-1(oz112gf)* and *gld-1(q485)* (*17, 18*). Both had short life-spans [Web table 1, experiments E and F (*10*)], although, in principle, this could also be caused by an independent, deleterious effect of germ-line overproliferation.

Because the germ line acts in the adult to influence life-span, we also investigated the timing of DAF-16 transcription factor function. To do this, we reduced *daf-16* activity at specific times using RNA interference (RNAi) (*19, 20*). We cultured *mes-1* mutants on bacteria expressing *daf-16* double-stranded RNA either throughout life, or only during adulthood, and found that both treatments completely suppressed their longevity (Fig. 3, B and C). Thus, DAF-16 appears to be required specifically in the adult to effect germ-line modulation of life-span.

Table 1. Life-spans of *daz-1(tj3)* mutants and germ line–ablated males. The animals described in each experiment (A and B) were grown in parallel (*28, 29*). *P* values are relative to the control animals grown in parallel. Life-spans were measured at 20°C.

Strain	Mean life-span (75th percentile) in days	No. of animals observed/no. of uncensored animals	*P* Value
	Experiment A		
Wild type	19.3 (23)	80/71	–
daz-1(tj3)	21.0 (26)	65/41	0.080
	Experiment B		
unc-50(e306); him-5(e1490) males	16.4 (23)	52/20	–
unc-50(e306); him-5(e1490) males Z2/Z3 (–)	23.9 (33)	42/27	0.008
unc-50(e306); him-5(e1490) hermaphrodites	15.9 (21)	58/39	–
unc-50(e306); him-5(e1490) hermaphrodites Z2/Z3 (–)	24.0 (30)	65/43	0.0001

Many long-lived mutants are resistant to heat and oxidative stress (*21–24*). We found that this was also true of germ line–ablated animals [Web table 2 (*10*)]. It is possible that resistance to oxidative damage causes longevity; alternatively, the germ line could influence both stress resistance and longevity independently of one another.

In summary, we have found that the aging process of *C. elegans* is modulated, during adulthood, by the activity of germ-line stem cells. How might these cells affect aging? One possibility is that germ-line proliferation shortens life-span by increasing energy expenditure, channeling resources that could otherwise be used for maintaining cellular integrity toward growth and reproduction. However, there does not appear to be a simple trade-off between reproduction (or energy expenditure) and aging in this system; for example, animals that lack the entire reproductive system are not long-lived. Another possibility is that the role of germ-line stem cells is simply to produce more germ-line tissue, which then influences life-span regardless of its state of differentiation. However, the gonads of *daz-1* mutants have a much smaller mass than those of *glp-1* mutants shifted to high temperature as adults, because oocyte precursors die instead of becoming large, mature oocytes (*15*). Yet *daz-1* animals are not long-lived. Therefore the germ-line stem cells may be uniquely capable of influencing life-span. We propose that stem-cell proliferation influences life-span by affecting either the production of, or the response to, a steroid hormone ligand for DAF-12, which, in turn, promotes longevity. In addition, a signal dependent on stem-cell proliferation must regulate the nuclear localization of DAF-16 in somatic nongonadal tissues (*25*).

Surprisingly, we found that the aging process is subject to modulation by the germ line in the adult. It was particularly striking that DAF-16 acts exclusively in the adult to mediate germ-line modulation of aging. Consistent with this, ablating the germ line at hatching causes DAF-16 to accumulate in nuclei only during adulthood (*25*). In contrast, when life-span is extended by mutations in the *daf-16*–dependent insulin/IGF-1 system, DAF-16 accumulates in nuclei throughout development and into adulthood (*25*).

In conclusion, our findings demonstrate that germ-line stem cells preside over two fundamental processes in the life cycle of *C. elegans*: reproduction and aging. These cells initiate the cascade of germ line development, thereby generating the pool of mature gametes, and they also regulate a steroid-dependent system that accelerates aging. By governing both processes, germ-line stem cells may help to coordinate the rate of aging with reproduction. Because killing germ-line precursor cells in *Drosophila melanogaster* also extends life-span (*26*), it is possible that this system is evolutionarily conserved.

Note added in proof: While this paper was in press, Gerisch *et al.* (*31*) reported that the germ line may regulate the activity of *daf-9*, which encodes a cytochrome p450 and may produce a steroid ligand for *daf-12*.

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29. We also ablated the germ line of wild-type males and found that the ablation increased life-span significantly (36% mean increase, $P \leq 0.0001$). In this experiment, a large number of animals (23/73 intact, and 40/69 ablated) crawled off the plates. Many of the ablated animals crawled off the plates at old ages, suggesting that the animals that would have lived the longest were lost.
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27 August 2001; accepted 15 November 2001

Abnormal Vascular Function and Hypertension in Mice Deficient in Estrogen Receptor β

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Blood vessels express estrogen receptors, but their role in cardiovascular physiology is not well understood. We show that vascular smooth muscle cells and blood vessels from estrogen receptor β (ERβ)–deficient mice exhibit multiple functional abnormalities. In wild-type mouse blood vessels, estrogen attenuates vasoconstriction by an ERβ-mediated increase in inducible nitric oxide synthase expression. In contrast, estrogen augments vasoconstriction in blood vessels from ERβ-deficient mice. Vascular smooth muscle cells isolated from ERβ-deficient mice show multiple abnormalities of ion channel function. Furthermore, ERβ-deficient mice develop sustained systolic and diastolic hypertension as they age. These data support an essential role for ERβ in the regulation of vascular function and blood pressure.

Steroid hormones regulate a wide range of cellular events by activating a receptor family of transcription factors (*1*). Estrogens influence gene expression, growth, and cellular differentiation in target tissues by activating one or both of two estrogen receptors, ERα and ERβ (*2, 3*). Estrogen receptors have been studied intensely in female reproductive physiology, but functional estrogen receptors are also present and physiologically important in other tissues of both sexes, including the liver, brain, bone, and the cardiovascular system (*4*).

ERα and ERβ both are expressed in vascular endothelial and smooth muscle cells, and in myocardial cells (*5*). ERβ expression is induced in vascular cells following injury (*6*). Both estrogen receptors are necessary and sufficient for estrogen-mediated protection against measures of vascular injury in mice (*7, 8*). Estrogen receptors also regulate the expression of a number of vasodilator and vasoconstrictor proteins, including multiple components of the renin-angiotensin system (*5*). An association between an ERβ gene polymorphism and systemic blood pressure in postmenopausal Japanese women (*9*) is the only genetic data to implicate estrogen receptors in blood pressure regulation.

Vascular tone is regulated by a complex set of variables that determine the contractile state of vascular smooth muscle (*10, 11*). Inherited forms of hypertension have been shown to involve mutations in genes regulating renal salt reabsorption (*12*), but the pathogenesis in most individuals with hypertension remains unknown. Both acute and longer term changes in vascular tone due to estrogen are mediated principally by nitric oxide (*5, 13–15*). Endothelial-dependent vascular relaxation is mediated by production of nitric oxide (NO) by endothelial nitric oxide synthase (eNOS) (*16*). Estrogen enhances production of NO by endothelial cells by increasing eNOS activity or expression of the eNOS gene, or both (*17, 18*). In vivo, estrogen enhances vasodilatation in both primates and humans with normal and abnormal endothelial function (*5, 19, 20*). Endothelial cell-independent vascular contraction is mediated by the direct actions of contractile agonists on vessel wall smooth muscle cells (*11*). Estrogen reduces vasoconstriction in vessels from which the endothelium has been removed from both humans and wild-type (WT) animals (*21, 22*), an effect that is blocked by pharmacologic inhibition of inducible nitric oxide synthase (iNOS) (*22*). To examine the role of estrogen receptors in vascular physiology, the existence of an estrogen effect on vasoconstriction was explored in vessels from WT mice.

We studied endothelial cell-independent vascular contraction (*11, 22*) in endothelium-denuded vascular rings from WT mice. Treatment of endothelial-denuded aortic rings from WT mice with 17β-estradiol (E2) for 18 to 20 hours attenuated constriction to the alpha (1B)-adrenergic receptor agonist phenylephrine (PE) [decrease of 45% and 69%, respectively, $P < 0.05$ for both, (Fig. 1, A and B) (*5, 21, 22*)]. The attenuating effect of E2 on PE-induced constriction was partially reversed by treatment with the general inhibitor of nitric oxide synthases, N(G)-nitro-L-arginine (L-NNA) (Fig. 1A), and with the

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