

levels of *Gremlin* but not *Fgf4* transcripts are detected in limb buds of *Shh*^{-/-} embryos lacking one *Gli3* allele (Fig. 3). In double-homozygous embryos, *Gremlin* and *Fgf4* expression in limb buds is comparable to that in *Gli3*^{-/-} embryos. The differential restoration of *Fgf4* indicates that its induction may depend on a threshold of BMP antagonism.

Hoxa11 and *Hoxd11* are essential to pattern the zeugopod, whereas *Hoxa13* and *Hoxd13* are essential for autopod patterning (25). In *Shh*^{-/-} limb buds, *Hoxa11* remains expressed (23), whereas *Hoxd11* is rapidly down-regulated (Fig. 4A). The disruption of one *Gli3* allele in *Shh*^{-/-} embryos partially restores *Hoxd11* expression such that its anterior boundary in limb buds is located at a position similar to that in wild type (arrowheads, Fig. 4A). This restoration provides a likely molecular explanation for improved zeugopod development in *Shh*^{-/-}, *Gli3*^{+/-} embryos (Fig. 1B). The expressions of both *Hoxa13* and *Hoxd13* are low in limb buds of *Shh*^{-/-} embryos (Fig. 4, B and C) (4–6). The additional inactivation of one *Gli3* allele restores *Hoxa13* expression to intermediate levels (Fig. 4B), whereas *Hoxd13* transcripts remain low (arrowhead, Fig. 4C). In limb buds of double homozygous embryos, all three 5'*Hox* genes are expressed at levels similar to those expressed in *Gli3*^{-/-} embryos (Fig. 4, A to C). These results indicate that the progressive restoration of distal limb development (Fig. 1B) is likely due to *Gli3* dose-dependent restoration of the distal 5'*Hoxa* and distal 5'*Hoxd* expression domains in limb buds of *Shh*^{-/-} embryos.

Our study provides genetic evidence for an important sequential interaction of GLI3 with dHAND and SHH. The SHH-independent nature of the digit polydactyly in *Gli3*^{-/-} limb buds is most likely a direct consequence of the early anterior expansion of the expression of “posterior” genes such as 5'*Hoxd* genes (7, 8). The scapula and stylopod (not affected in *Shh*^{-/-} embryos) (5, 6) may be patterned by the genetic interaction of *GLI3* with *dHAND* before the activation of SHH signaling (Fig. 1A, panel 1) (7). In limb buds of *Shh*^{-/-} embryos, *Gli3* expression expands posteriorly, concurrent with the down-regulation of “posterior” genes and the onset of apoptosis (5, 6, 12), which cause the “shut-down” of distal limb development and antero-posterior patterning. The *Gli3*-dose-dependent restoration of cell survival and limb bud development in *Shh*^{-/-} embryos indicates that, in wild-type limb buds, SHH counteracts GLI3 to enable progression of outgrowth and patterning. The polarizing region is propagated distally, and SHH signaling is up-regulated via the SHH-FGF feedback loop (Fig. 1A, panel 2) (11, 12). Such up-regulation of SHH signaling should increasingly inhibit GLI3 processing and should result in a graded GLI3-83 repressor distribution as shown biochemically (15). The resulting full-length GLI3 protein may also function in positive transcriptional regulation

of SHH targets (18, 19). Although the SHH-GLI3 interactions enable distal limb development and formation of a digit arch, additional signals such as BMPs participate in specification of digit identities (26). Lastly, aspects of the dorso-ventral neural tube patterning (disrupted in *Shh*^{-/-} embryos) are also restored in mouse embryos mutant for both *Shh* and *Gli3* (27). This shows that regulation of GLI3 by SHH signaling (and vice versa) is of general functional importance during embryonic development.

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Timing Requirements for Insulin/IGF-1 Signaling in *C. elegans*

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The insulin/IGF-1 (where IGF-1 is insulin-like growth factor–1) signaling pathway influences longevity, reproduction, and diapause in many organisms. Because of the fundamental importance of this system in animal physiology, we asked when during the animal's life it is required to regulate these different processes. We find that in *Caenorhabditis elegans*, the pathway acts during adulthood, to relatively advanced ages, to influence aging. In contrast, it regulates diapause during development. In addition, the pathway controls longevity and reproduction independently of one another. Together our findings show that life-span regulation can be dissociated temporally from phenotypes that might seem to decrease the quality of life.

In *C. elegans*, mutations that decrease the activity of DAF-2, an insulin/IGF-1-like receptor, or downstream phosphatidylinositol 3-kinase,

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phosphoinositide-dependent kinase, or AKT (also known as protein kinase B) signaling components prolong youthfulness and double the life-span of the animal. The DAF-2 pathway influences other processes as well. All *daf-2* examined mutations increase resistance to oxidative stress and delay reproduction (some alleles also reduce fertility). Strong *daf-2* mutations cause juvenile animals to enter a state of diapause, called dauer, instead of growing to adulthood. All of these mutant phenotypes re-

REPORTS

quire the activity of DAF-16, a forkhead family transcription factor (1–3).

Mutations in components of the insulin/IGF-1 pathway also extend life-span in flies (4, 5), and mutations that inhibit pituitary development or growth hormone receptor signaling, which in turn decreases IGF-1 signaling, extend the life-span of mice (1, 6). Components of insulin/IGF-1 signaling pathways also influence reproduction, stress resistance, and entry into diapause-like states in a wide range of organisms (1–3).

To investigate when the *C. elegans* insulin/IGF-1 pathway acts to regulate longevity, diapause, reproduction, and stress resistance, we used RNAi (RNA interference), which decreases mRNA levels (7), to decrease *daf-2* and *daf-16* activity at different times during the life cycle. Reverse transcriptase polymerase chain reaction (RT-PCR) analysis confirmed that feeding animals bacteria that are expressing *daf-2* or *daf-16* double-stranded RNA (dsRNA) decreased mRNA levels (Fig. 1) (8). We found that culturing wild-type animals on bacteria expressing *daf-2* dsRNA from the time of hatching produced *Daf-2*(–) phenotypes similar to those produced by partial loss-of-function *daf-2* mutations (9–12). At 20°C the animals grew to adulthood and became long-lived, stress-resistant adults with normal brood sizes but protracted reproductive schedules (Fig. 2A, Table 1, and table S1) (8). High temperature is known to induce some dauer formation in the wild type (13). Our *daf-2* RNAi treatment increased the frequency of dauer formation at high temperatures [27°C; 45% dauers ($n = 72$, where n is the number of animals) for treated animals versus 10% dauers ($n = 81$) for control animals] (8). Strong *daf-2* mutations delay growth to adulthood and cause uncoordinated movement and some embryonic lethality (9, 14, 15). We did not observe these phenotypes (not shown), suggesting that they result from a reduction of *daf-2* activity that is greater than the reduction from RNAi treatment. Conversely, feeding *daf-2*(1370) mutants bacteria expressing *daf-16* dsRNA completely suppressed their delayed development (16), longevity, and reproductive phenotypes (Fig. 3A). This *daf-2* mutation is temperature sensitive. At 25.5°C, the animals become dauers; this phenotype was partially suppressed by *daf-16* RNAi (17).

To study when *daf-2* influences adult longevity, we shifted wild-type animals to bacteria expressing *daf-2* dsRNA at different ages and determined their life-spans. We found that initiating *daf-2* RNAi treatment in young adults extended life-span to the same extent as did initiating RNAi at hatching (Fig. 2, A versus G; $P = 0.38$; table S1). Thus, *daf-2* is required during adulthood to regulate adult life-span.

To determine whether *daf-2* could also function during development to influence adult life-span, we lowered insulin/IGF-1 signaling specifically during development. First, we initiated *daf-2* RNAi during development and then attempted to turn off the RNAi process during adulthood (8). We reasoned that if animals were exposed to the dsRNA of a gene required for RNAi to function, such as *dcr-1* (dicer) (18), then RNAi activity should remain low. To this end, we shifted animals exposed to *daf-2* RNAi at hatching onto bacteria expressing *dcr-1* dsRNA when they reached adulthood. These animals did not have long life-spans (Fig. 4 and table S1).

We also turned off the effects of a *daf-2* mutation at different stages by shifting *daf-2*(*e1370*) mutants to bacteria expressing *daf-16* dsRNA. We found that initiating *daf-16* RNAi treatment during young adulthood completely suppressed the long life-spans of *daf-2*(*e1370*) mutants (Fig. 3, A versus G; $P = 0.19$; and table S1). Thus, the presence of DAF-16 activity during development was irrelevant. Therefore, the insulin/IGF-1 pathway appears to operate exclusively during adulthood to influence adult life-span.

We found that initiating *daf-2* RNAi treatment later in adulthood, throughout the reproductive period, also extended life-span (Fig. 2, G to J; table S1). The magnitude of this extension declined steadily, and became insignificant after about 6 days of adulthood (Fig. 2M and table S1), though there was a sharp decline in *daf-2* mRNA at

this time (Fig. 1A). Treating *daf-2*(*e1370*) mutants with *daf-16* RNAi during the reproductive period also affected life-span (Fig. 3, H to L; table S1). Again, the magnitude of the effect declined with age, although we continued to observe effects on life-span until about day 15 of adulthood (Fig. 3, M to P; table S1). In these experiments, *daf-2* and *daf-16* appeared to function for different durations during adulthood; however, wild-type animals (used in the *daf-2* RNAi experiments) age more quickly than *daf-2* mutants (used in the *daf-16* RNAi experiments). In fact, as assayed by tissue morphology, day 6 wild-type animals are about the same age as day 15 *daf-2*(*e1370*) mutants (19). Thus, both genes may function at the same time, throughout the reproductive period, to influence aging. We note that the pathway could conceivably function later as well (if, for example, the rate of protein turnover falls in old animals).

We next investigated when *daf-2* and *daf-16* RNAi could affect the timing of reproduction. We found that treating adults with *daf-2* RNAi had no effect on reproduction (Fig. 2, F to J; table S1). Likewise, treating *daf-2* mutants with *daf-16* RNAi during adulthood failed to suppress the *daf-2* reproductive phenotype (Fig. 3, G to L; table S1). In contrast, initiating either RNAi treatment at hatching did affect the timing of reproduction (Figs. 2A and 3A). Thus, *daf-2* appears to control reproduction and longevity independently of one another.

Fig. 1. *daf-2* and *daf-16* bacterial RNAi lower mRNA levels. (A) RT-PCR analysis of *daf-2* mRNA after RNAi treatment. Shown are RT-PCR products from serial dilutions of total RNA isolated from control animals grown on bacteria containing vector only (lanes 1 to 5) or on bacteria expressing *daf-2* dsRNA (lanes 6 to 10). (a) RNAi was initiated at hatching, and RNA was harvested at L4. (b) RNAi was initiated on day 8 of adulthood and harvested on day 10 of adulthood. (B) RT-PCR analysis of *daf-16* mRNA after RNAi treatment. Conditions were the same as in (A), except animals were treated with bacteria expressing *daf-16* dsRNA.

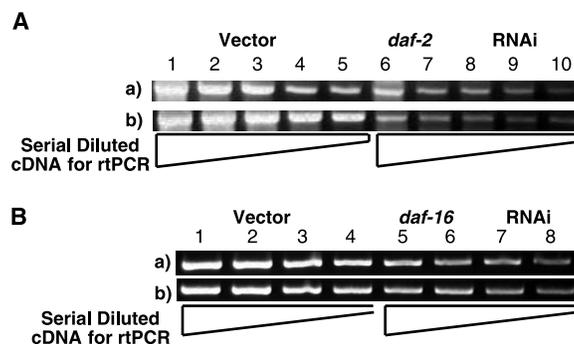


Table 1. Effect of *daf-2* function on stress resistance. The results of two independent trials are shown. Trial 1 is the first row of numbers ($n \geq 25$); trial 2 is the second row of numbers ($n \geq 40$).

	% alive in 0.4 M paraquat after					
	1 hour	2 hours	3 hours	4 hours	5 hours	6 hours
Control (RNAi vector bacteria)	52	48	32	20	4	0
	56	50	34	22	12	4
<i>daf-2</i> RNAi initiated at hatching	78	76	74	47	44	41
	87	83	70	52	40	38
<i>daf-2</i> RNAi initiated as day-1 adult	94	94	72	56	53	50
	90	85	70	58	44	34

REPORTS

To determine when *daf-2* and *daf-16* might function in reproduction, we subjected the animals to RNAi during development. We found that initiating *daf-2* RNAi treatment of wild-type animals at any time before the last larval stage, L4, delayed reproduction (Fig. 2, A to D) and that initiating *daf-16* RNAi treatment of *daf-2(e1370)* mutants at any time before L4 restored normal reproductive timing (Fig. 3, A to E). Treatment during or after L4 did not affect reproduction (Fig. 2, E to J, and Fig. 3, F to L). These findings show that reproductive timing can be specified independently of the dauer decision (which occurs before L3) (13), and they suggest that the *daf-2* pathway may function late in development to affect the timing of reproduction. However, we cannot rule out the possibility that the DAF-2 pathway controls

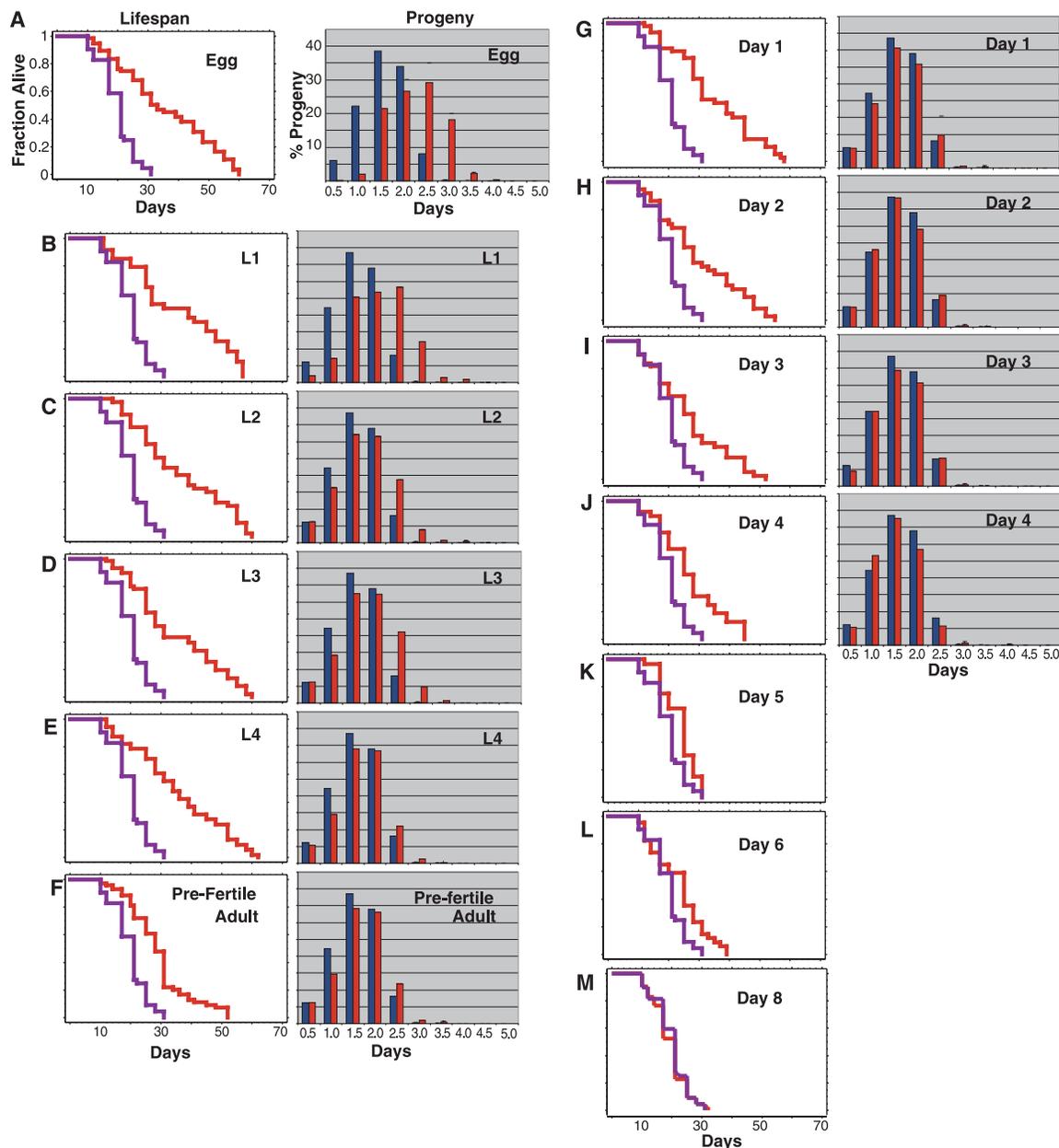
reproduction during adulthood, but that initiating RNAi during or after L4 does not lower signaling activity below threshold until after DAF-2 and DAF-16 have completed their roles in reproduction. This seems less likely, because message levels fall sharply within 48 hours of RNAi treatment (Fig. 1), whereas reproduction continues for many days.

The DAF-2 pathway also regulates stress resistance (9, 10, 20). Because *daf-2* larvae (21) and dauers (13) are stress resistant, *daf-2* must regulate stress resistance in the larvae. We found that animals treated with *daf-2* dsRNA as adults were resistant to the oxidative-damaging agent paraquat (Table 1). Thus, *daf-2* also acts in the adult to regulate stress resistance. Because the DAF-2 pathway regulates life-span during adulthood as well, this finding supports the

hypothesis that increased resistance to oxidative stress contributes to longevity (9, 10, 20). Consistent with this, overexpression of the antioxidant superoxide dismutase gene during adulthood can extend the life-span of *Drosophila* (22, 23), as can administering the antioxidant Euk134 to adult worms (24).

In this study, we have investigated the temporal requirements for DAF-2 and DAF-16 action by using RNAi to reduce gene activity at specific times during the life cycle. As with any conditional expression system, we cannot be certain when any residual *daf-2* or *daf-16* activity not removed by RNAi might function. However, the fact that *daf-16* RNAi completely suppressed the strong longevity and reproductive phenotypes of the *daf-2(e1370)* mutant suggests that the level of

Fig. 2. (A to M) *daf-2* RNAi affects life-span and reproduction at different times. The time at which animals were transferred onto bacteria expressing *daf-2* dsRNA is shown in the upper right corner of each panel. Blue lines, the life-spans of animals grown on control bacteria carrying the RNAi vector alone; red lines, life-spans of animals grown on bacteria expressing *daf-2* dsRNA. Reproductive profiles of animals in the concurrent life-span assays are depicted to the right of each life-span profile. The percent of total progeny produced at each 12-hour time interval is shown. Scales are identical for all graphs. Reproductive profiles of animals treated with bacteria expressing *daf-2* dsRNA after day 4 of adulthood are not shown because these animals were post-reproductive. For statistics, see table S1.



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residual pathway activity is likely to be minimal, as does the fact that inferences from reciprocal *daf-2* and *daf-16* RNAi experiments were always in agreement.

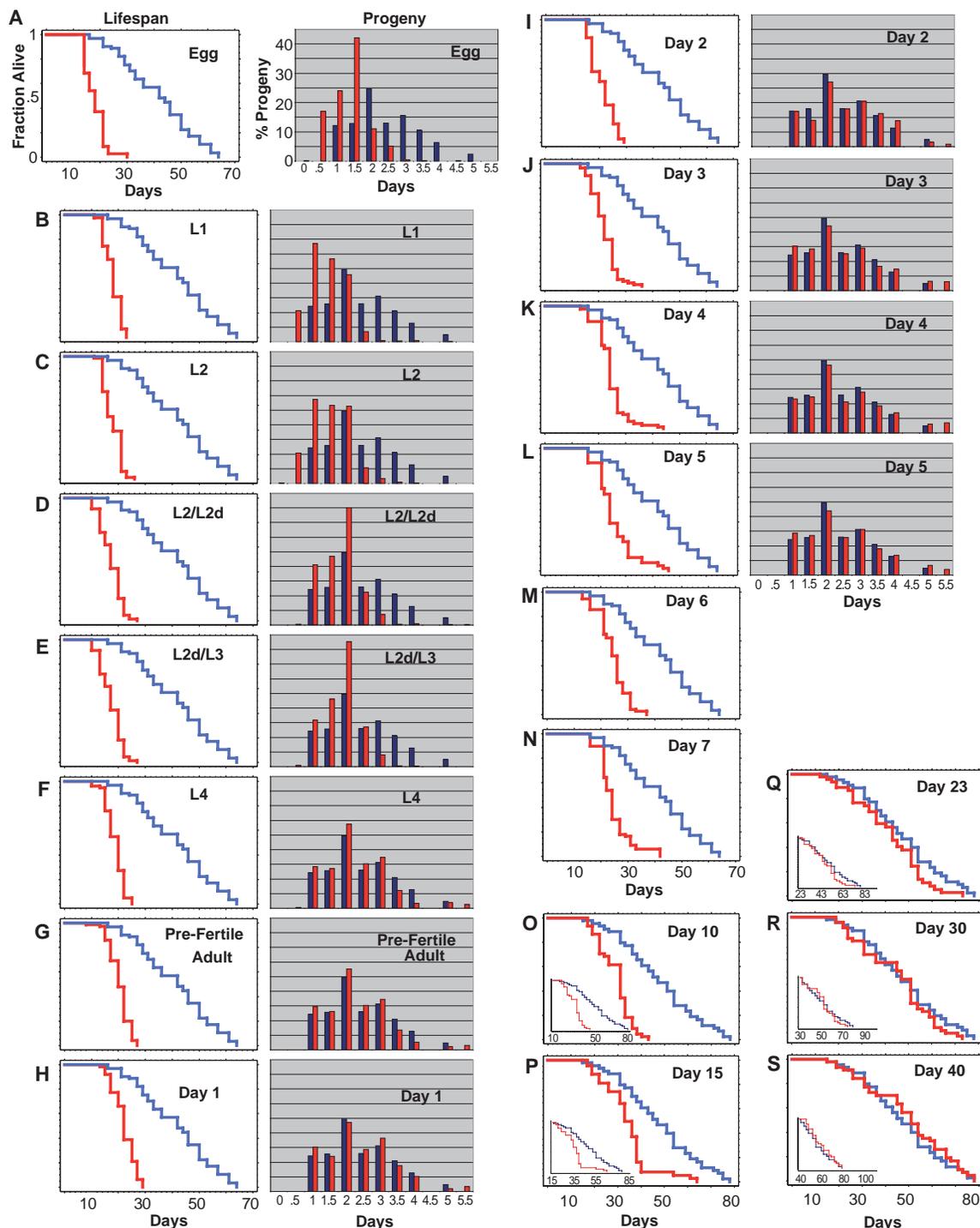
Our findings suggest that the DAF-2 pathway functions exclusively during adulthood, throughout the reproductive period, to influence adult life-span. This conclusion suggests that the pathway controls downstream gene expression in an ongoing fashion through much of adulthood. Because the dauer is a juvenile form (25), the DAF-2 pathway must act in separate

regulatory events to control dauer formation and adult life-span. Previously we proposed that *daf-2* regulates a longevity process that determines the life-span of adults and that can also be expressed in conjunction with dauer-specific traits to give dauers their long life-spans (12). It will be interesting to learn whether the pathway regulates the same genes at two different life stages to influence the life-spans of adults and dauers.

Our findings indicate that the DAF-2 pathway participates in multiple, independent

regulatory events to influence aging, reproduction, and diapause. In this regard, the pathway is similar to many growth factor signaling pathways (such as the epidermal growth factor or transforming growth factor- β pathways), which regulate different aspects of cell growth and differentiation independently of one another. The pleiotropy of insulin/IGF-1 pathway mutations in many organisms, particularly the linkage with diapause-like states, has raised the possibility that longevity achieved

Fig. 3. (A to S) *daf-16* RNAi affects the life-span and reproduction of *daf-2(e1370)* mutants at different times during the life cycle. The time that *daf-2(e1370)* animals were transferred onto *daf-16* RNAi bacteria is shown in the upper right corner of each panel. Blue, life-spans of *daf-2(e1370)* animals grown on bacteria carrying the RNAi vector alone; red, life-spans of *daf-2(e1370)* animals grown on *daf-16* RNAi bacteria. Scales of graphs (A) to (N) are identical and (O) to (S) are identical. Insets: T_0 is set to the day at which RNAi treatment was initiated in the experimental population. For statistics, see table S1. Reproductive profiles of animals in the concurrent life-span assays are depicted to the right of each life-span profile. The percent of total progeny produced at each 12-hour time interval is shown. Blue, *daf-2(e1370)* mutant animals grown on control bacteria; red, *daf-2(e1370)* mutant animals grown on bacteria expressing *daf-16* dsRNA. Reproductive profiles of animals cultured on *daf-16* RNAi bacteria after day 5 of adulthood are not shown because the animals were postreproductive.



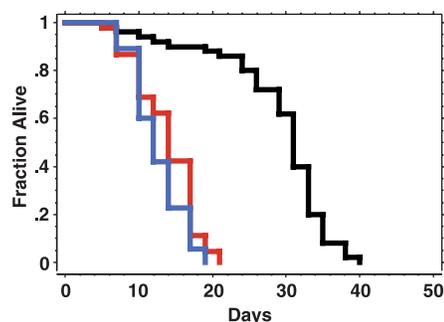


Fig. 4. Loss of *daf-2* function during development does not increase life-span. Wild-type animals were grown on bacteria expressing *daf-2* dsRNA from hatching until the first day of adulthood and then transferred to bacteria expressing dsRNA of *dcr-1*. Red line, life-spans of wild-type animals grown on *daf-2* RNAi bacteria during development and then shifted during day 1 of adulthood to bacteria expressing *dcr-1* dsRNA. Blue line, life-span of wild-type animals grown on the control RNAi bacteria during development and then shifted during day 1 of adulthood to *dcr-1* RNAi bacteria. Black line, life-span of wild-type animals grown on *daf-2* RNAi bacteria during development and adulthood. Life-span studies were conducted at 25°C. For statistics, see table S1.

through this pathway would invariably be associated with impaired growth or reproduction. Instead, our findings suggest that, in other organisms as well, it may be possible to manipulate insulin/IGF-1 signaling during adulthood so as to extend youthfulness and life-span without affecting growth or reproduction.

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 Materials and Methods
 Table S1

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Targeting of Cyclic AMP Degradation to β_2 -Adrenergic Receptors by β -Arrestins

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Catecholamines signal through the β_2 -adrenergic receptor by promoting production of the second messenger adenosine 3',5'-monophosphate (cAMP). The magnitude of this signal is restricted by desensitization of the receptors through their binding to β -arrestins and by cAMP degradation by phosphodiesterase (PDE) enzymes. We show that β -arrestins coordinate both processes by recruiting PDEs to activated β_2 -adrenergic receptors in the plasma membrane of mammalian cells. In doing so, the β -arrestins limit activation of membrane-associated cAMP-activated protein kinase by simultaneously slowing the rate of cAMP production through receptor desensitization and increasing the rate of its degradation at the membrane.

Many hormones elicit their effects on cells by binding to and activating cell-surface guanine nucleotide binding protein (G protein)-coupled receptors (GPCRs) (1). Once activated, GPCRs couple to and activate specific G protein isoforms that promote the production of intracellular second messengers such as cAMP, thus initiating signaling cascades that result in diverse cellular responses. To limit the magnitude of GPCR signals, and to return the cell to its unstimulated state, further receptor-G protein coupling must be prevented and the already synthesized second messenger molecules must be degraded. Receptor uncoupling occurs through desensitization (2, 3), whereby activated receptors become phosphorylated and bind to β -arrestin proteins, inhibiting further interaction with G proteins. Cyclic AMP is degraded by the phosphodiesterase (PDE) family of en-

zymes (4, 5). Because many PDE isoforms are targeted to subcellular structures through association with signaling and scaffolding proteins (4-11), the rate of cAMP degradation likely depends on the type and amount of PDEs present at a specific subcellular location (12, 13). In this manner, free diffusion of cAMP within the cell is impeded and microdomains of cAMP signaling are created where generation of cAMP by adenylyl cyclase is highest and PDE activity is lowest (13, 14).

In addition to receptor desensitization, the β -arrestins also function as multivalent adaptor proteins that recruit a variety of cytosolic proteins to their sites of action at the plasma membrane (15). In doing so, β -arrestins allow diverse plasma membrane-associated signals to be targeted to and regulated by GPCRs. We investigated whether PDE targeting to the plasma membrane is also regulated by GPCR stimulation. In transfected human embryonic kidney (HEK293) cells overexpressing recombinant stimulatory G protein (G_s) and adenylyl cyclase-coupled β_2 adrenergic receptors, stimulation with the β agonist isoproterenol resulted in time-dependent targeting of endogenous PDE4D3 and PDE4D5 to cell membranes (Fig. 1, A and B), the major PDE4 isoforms detected in these cells (16). The increase was detected 2 min after stimulation, continued to increase until 5 min after stimulation, and then declined. This transient nature of the recruitment was similar to that observed for β -arrestins (Fig. 1, A and B)

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