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C. elegans ortholog of mammalian Ku70 interacts with insulin-like signaling to modulate stress resistance and life span

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Abstract

The mammalian Ku heterodimer has important roles in DNA double strand break repair, telomere maintenance, cell cycle checkpoint-arrest, tumor suppression, and cellular stress resistance. To investigate the evolutionarily conserved functions of Ku, we knocked down expression by RNA interference (RNAi) of Ku genes in *C. elegans*. We found that *C. elegans* Ku70 (CKU-70) is required for resistance to genotoxic stress, regulates cytotoxic stress responses, and influences aging. The latter effects are dependent on an IGF-1/insulin-like signaling pathway previously shown to affect life span. Reduction of CKU-70 activity amplifies the aging phenotype of long-lived insulin receptor *daf-2* mutations in a *daf-16*-dependent manner. These observations support the view that organismal stress resistance determines life span and Ku70 modulates these effects.

Keywords

cku-70; cku-80; daf-2; daf-16; aging; stress response; DNA repair

Cytotoxic stress resistance and aging rate are strongly influenced by an insulin-like/IGF-1 (IIGF) signaling pathway in *C. elegans*. Mutation of an IIGF receptor encoded by *daf-2* increases mean life span by 100% (1,2). The life span extension of *daf-2* mutants are suppressed by mutations in *daf-16* (1,3), which has been identified as a forkhead/winged helix family transcription factor (4,5). This pathway influences the expression of stress and metabolic genes and thereby affects life span (6-13). The IIGF pathway phenotypically interacts with heat shock factor (HSF), the regulator of heat shock protein (HSP) expression genes (14-16). The up-regulation of heat shock proteins appears a key component to the life span extension observed in these mutations (9,14,15). Almost all long-lived mutants show increased resistance to stresses, including heat (17-19), UV (20), oxidative stress (6,7), and heavy metals (10), which is likely in part to be due to elevated levels of HSPs (9,14) and other stress induced proteins (13). For example, HSP-16, a small HSP member of the α-crystallin family of molecular chaperones (21), over-accumulates in heat shocked *age-1* mutants (9) and *daf-2* mutants have a constitutively elevated *hsp-16* mRNA abundance (14). Introduction of extra-copies of the *hsp-16* gene results in an increased *daf-16*-dependent, thermotolerance, and life span extension (22). Overexpression of either the gene encoding HSP-70F (23) or HSF (14,15) also extends life span in *C. elegans*.

These results are consistent with the proposal that life extension results, at least in part, from elevated maintenance of protein conformation (24).

In eukaryotic cells, a complex comprising Ku and DNA-dependent protein kinase (DNA-PK) is required for DNA double stranded break (DSB) repair. Ku is a heterodimer consisting of a

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70 and 80 kDa subunit (Ku70 and Ku80) that binds in a DNA sequence-independent manner to ends, nicks, gaps, and hairpins and in a sequence-dependent manner to internal sites (25). In addition to its role in DSB repair, Ku functions in telomere maintenance, cell cycle checkpoint arrest, and tumor suppression. Ku70 and Ku80 deficient mice are viable and fertile but have reduced life span and marked immunodeficient, neuronal, and growth retardation phenotypes (26,27). The individual loss of *ku70* and *ku80* also reveals unique phenotypes, suggesting functions specific to each gene (26-28).

There is a growing body of evidence linking HSPs to DNA repair activity. Mouse Hsp70.1 and Hsp70.3 are required for maintenance of genomic stability in stress conditions (29). Exposure to ρ-rays induces HSP70 (30), while prior heat shock induction of HSP70 increases radioresistance (31,32) and decreases radiation induced cell death (33). Furthermore, human HSP70 can bind and enhance the specific endonuclease activity of human apurinic/apyrimidinic endonuclease (HAP1), a key component of the base excision repair pathway (34). In parallel, Ku70 regulates stress response genes (25,35,36). Overexpression of Ku70 in fibroblasts results in the specific repression of HSP70 upon heat shock, while induction of the remaining suite of HSPs remain normal (36). Under normal conditions HSF is a cytoplasmic monomer. Upon heat shock, it enters the nucleus, forms homo-trimers, is differentially phosphorylated, and binds to specific promoter sites (heat shock elements, HSEs) (37). In vitro translated Ku70 interacts with HSF by binding to and displacing it from HSEs in the promoter of HSP70, suggesting that Ku70 may negatively regulate HSF binding activity in vivo (25).

We were prompted by the role of Ku70 in mammals as a negative regulator of HSF to study the conserved features in *C. elegans*. The *C. elegans* orthologs of mammalian Ku70 and Ku80 have been identified by the *C. elegans* Genome Sequencing Consortium (CAB55094 and CAA83623), and their encoding genes have been designated *cku-70* and *cku-80*, respectively. We propose a similar mechanism of action of Ku70 in *C. elegans* whereby CKU-70 down-regulation will decrease resistance to genotoxic stress yet increase heat resistance. The net effect on life span is then examined.

MATERIALS AND METHODS

Strains

Worms strains N2[wild type], NL2099[*rrf-3*(*pk1426*)], CB1370[*daf-2*(*e1370*)], DR1564[*daf-2*(*m41*)], DR26[*daf-16*(*m26*)], DR1309[*daf-16*(*m26*); *daf-2*(*e1370*)], and SS104[*glp-4*(*bn2*)] were obtained from the *Caenorhabditis* Genetics Center. All strains were cultured on nematode growth plates (NGM) plates at 20°C unless stated otherwise.

cku-70 and cku-80 RNA interference (RNAi)

For *cku-70*(RNAi), an 828-bp fragment of cDNA-spanning exons 2-8 was amplified (primers 5′-ATAGCACTCTGCTTGTGCC-3′ to 5′-CCAAAATTATCTTCTCTCCACC-3′), subcloned into the L4440 vector (a gift from A. Fire, Stanford), and transformed in to HT115 (DE3) cells. For *cku-80*(RNAi), an 853-bp fragment of cDNA-spanning exons 2-10 was similarly cloned (primers 5′-ATTAATGCGTCAGGCTAC-3′ to 5′-CACCATCATCTACCTCATC-3′). NGM plates for RNAi were prepared as described previously (38). For all experiments L4 larvae were initially transferred onto RNAi plates, kept at 20°C overnight and allowed to lay eggs for 3-6 h. These synchronous eggs were left to develop at 20°C until reaching either L4 or adulthood and were then transferred onto fresh RNAi plates for all subsequent assays.

Methyl methane sulfonate sensitivity assay

Approximately 100 adult hermaphrodites were transferred from RNAi plates into liquid culture at 20°C, 1×10^{10} cells/ml OP50 in S-basal (39). Several hundred eggs laid over 3 h were isolated using 40 μM nylon mesh filters (Falcon®). Eggs were then exposed to various concentrations of methyl methane sulfonate (MMS), in 1 ml of S-basal, in 24-well microtiter plates for 1 h at 25°C with orbital shaking at 100 rpm. Eggs were then washed twice to remove excess MMS and plated on NGM plates. Plates were kept at 25°C for 3 days, and the number of adults was then scored.

Self-fertility assay

Approximately 15-30 hermaphrodite L4 larvae were transferred onto individual RNAi plates and allowed to develop to adulthood. These individuals were transferred to new plates every day, and their progeny was counted after development at 20°C for 2-3 days.

Thermotolerance assay

For each population, two plates of 25-30 4-day-old adult hermaphrodites were transferred onto fresh RNAi plates then shifted to 35°C. Thereafter, individuals were scored for death, lack of touch-provoked movement as described previously (18).

Life span assay

For each treatment, four populations of 25-30 L4 hermaphrodites were transferred onto fresh RNAi plates and left overnight 20°C. Day one of the life span assay commenced when these individuals were shifted to 25°C. Populations were transferred onto fresh RNAi plates daily until cessation of egg laying, and then individual worms were scored as alive or dead every 1-3 days. The day of death was determined by a lack of touch-provoked movement as described previously (17) and recorded for all individuals.

Dauer formation assay

Synchronous adults were left to lay eggs at 20°C for 3 h on RNAi plates. For wild-type and *daf-2*(*e1370*) strains, these plates were then shifted to 27°C for 3 days and then the number of adults and dauer larvae were counted. Dauer larvae were additionally scored by their resistance to 1% SDS exposure for 30 min. For *daf-2*(*m41*) populations, plates were moved to 20°C for 4 days and similarly scored.

Statistical tests

Differences in survival during life span or heat shock assays were assessed using the nonparametric Log rank test as implemented in the Prism™ software package. Differences in total fertility and mean survival after MMS exposure were examined using Student's *t*-test (2-tailed).

RESULTS

cku-70 and cku-80 effects on MMS sensitivity

We hypothesized that knocking down Ku functions in *C. elegans* would render worms sensitive to DNA damage. MMS is a DNA-alkylating agent and known mutagen (40). Exposure to MMS resulted in an increasing proportion of eggs that failed to develop in a dose dependent manner (Fig. 1). Furthermore, reduction of either CKU-70 or CKU-80 activity via RNAi significantly increased this sensitivity, consistent with their roles in DNA repair processes. Comparison of wild type and *cku-80*(*ok861*), a *C. elegans* Gene Knockout Project strain carrying an ~1300 bp deletion, also revealed a significant increase in sensitivity (data not shown). Similar

experiments after exposure of eggs to various doses of UV found no effect of either *cku-70* (RNAi) or *cku-80*(RNAi) (data not shown). These results are consistent with CKU-70 and -80 having no role in repair of UV-induced DNA lesions.

cku-70 and cku-80 effects on fertility

A moderate reduction of DNA repair processes may be expected to lead to impaired genomic stability. Somewhat surprising is that overall fertility was not significantly reduced after either *cku-70*(RNAi) or *cku-80*(RNAi) (Table 1). Commencement of egg laying was also not affected by *cku-70*(RNAi), suggesting no change in developmental rate (data not shown).

cku-70 and cku-80 effects on thermotolerance

Altered thermotolerance would be expected if CKU-70 were a negative regulator of stress protein induction. Wild-type thermotolerance was significantly increased after *cku-70*(RNAi) (Table 2). However, this increase was found to be *daf-16* dependent, such that *daf-2*(*e1370*) thermotolerance was also significantly increased but not in either *daf-16*(*m26*) or *daf-16* (*m26*);*daf-2*(*e1370*) genetic backgrounds. No effect on thermotolerance was observed after *cku-80*(RNAi) (Table 2) or between wild-type and *cku-80*(*ok861*) genotypes (data not shown). This demonstrates that CKU-70 and CKU-80 also have distinct functions in *C. elegans*.

cku-70 and cku-80 effects on life span

Reduction of *cku-70* activity did not alter wild-type life span (Fig. 2). However, using *rrf-a 3* (*pk1426*) worms that show increased sensitivity to RNAi (41) when compared with wild type, we observed a slight but significant increase (mean increase ~14%) in life span after *cku-70* (RNAi). Further examination of *cku-70*(RNAi) revealed that life span of *daf-2*(*e1370*) was also significantly increased (mean increase ~35%), while *daf-16*(*m26*) life span was marginally but significantly decreased (mean decrease ~11%). In the double mutant, *daf-16*(*m26*);*daf-2* (*e1370*) no life span difference was detected. In contrast, *cku-80*(RNAi) did not alter wild-type, *rrf-3*(*pk1426*), or *daf-2*(*e1370*) life span. Similarly, wild type and *cku-80*(*ok861*) did not differ in life span (data not shown).

To determine if life span effects of *cku-70*(RNAi) were due to deleterious effects on the germline, we used *glp-4*(*bn4*);*daf-2*(*e1370*) double mutants that lack germ cells and have previously been shown to have extended life span beyond that of *daf-2*(*e1370*) alone (42). Here we observed a mild but reproducible increase in life span (mean increase ~9%) after *cku-70* (RNAi) but no effect from *cku-80*(RNAi). Life span assays were conducted as either duplicate or triplicates experiments with equivalent results. Representative data are shown (Fig. 2).

cku-70 effects on Dauer formation

Dauer development phenotypes are common to genes regulating stress resistance and life span. We examined the role of CKU-70 in dauer formation. Based on the finding that *cku-70* (*RNAi*) enhanced the increased life span of *daf-2*, we examined the possibility that dauer formation may have also been affected. However, reduction in CKU-70 activity has no effect on dauer formation (Table 3). *cku-70*(RNAi) did not increase dauer formation in wild-type worms nor alter the frequency of dauer formation in *daf-2*(*e1370*) or *daf-2*(*m41*) worms. Although *cku-70* functions to regulate stress response, it appears not to be involved in dauer formation.

DISCUSSION

We investigated the functions of *C. elegans cku-70* and *cku-80*, orthologs to mammalian *ku-70* and *ku-80*, and have discovered that CKU-70 and CKU-80 are required for viability after DNA damage. We show a dose-dependent effect of the radiomimetic MMS in wild-type worms

leading to decreasing egg viability, similar to previous reports (43). Reduced activity of either *cku-70* or *cku-80* further reduces egg viability, consistent with an impaired ability to repair DNA breaks. Thus, the *C. elegans* ortholog of mammalian *ku-70* and *ku-80* have phenotypes consistent with roles in DNA repair. Increased sensitivity to MMS has been observed for several other *C. elegans* DNA radiation-repair (*rad*) mutants (43). We found that neither *cku-70* nor *cku-80*(RNAi) altered UV sensitivity. UV light-induced DNA damage is repaired via excision repair pathways, in which the Ku70/80 heterodimer is thought not to be involved. Surprisingly, reduction of *cku-70* or *cku-80* activity did not lower overall fertility and led to no obvious change to development rate. RNAi knockdown of *cku-70* or *cku-80* over multiple generations may be required before effects on fertility become apparent.

Reduced nematode CKU-70 activity increases thermotolerance, consistent with CKU-70 being a negative regulator of HSF-1 (25). Intrinsic thermotolerance was increased after *cku-70* (RNAi) in both wild-type and *daf-2*(*e1370*) backgrounds. This gain was found to be dependent on wild-type DAF-16, such that no increase was observed in *daf-16*(*m26*) or *daf-16(m26*);*daf-2* (*e1370*) backgrounds. DAF-16 activity is required for elevated stress resistance and life span phenotypes seen in *daf-2* mutants (3,20).

At normal growth temperature, no life span effect was observed for *cku-70*(RNAi) in wild-type worms. In a *rrf-3*(*pk1426*) we observe a slight increase in life span (mean increase ~14%). Mutation in the gene *rrf-3*, encoding a RNA-directed RNA polymerase, has increased sensitivity to RNAi, while remaining viable and fertile (41). This suggests that the reduction of CKU-70 in the wild-type populations was suboptimal but ultimately may only produce marginal increases in mean life span. However, reduction of *cku-70* activity appears to amplify the aging phenotype of IIGF mutants. Long-lived *daf-2* mutations become even longer lived (mean increase ~35%) and short-lived *daf-16* mutants slightly shorter lived (mean decrease ~11%).

An as yet unidentified germ line signal has been observed to modulate life span adult (44). Ablation of the germ line precursor cells or mutation causing absence of germ cells in adults also increased *daf-2* mutant life span (42,44). We propose that the increase in *daf-2*(*e1370*) life span by *cku-70*(RNAi) may be due in part to processes independent of any germ line signals. First, we observed no gross fertility deficit from RNAi knockdown of *cku-70*. Second, in a *glp-4(bn2)*;*daf-2*(*e1370*) mutants, which essentially lack germ cells (45), a life span increase after *cku-70*(RNAi) is still reproducibly detected (mean increase ~9%).

Reduction of *cku-80* activity also resulted in increased MMS sensitivity but without thermotolerance or life span phenotypes, regardless of the genetic background. The discordance between the *cku-70* and *cku-80*(RNAi) results suggest we have uncovered thermotolerance and life span phenotypes specific to CKU-70, rather than phenotypes derived from a CKU-70/80 heterodimer. In contrast, *ku86* mutant mice (where Ku86 is the murine ortholog of human Ku80) exhibit characteristics of premature senescence and reduced life span (27). A possible explanation for this disparity is that unlike in the mouse no somatic cell proliferation takes place during nematode adulthood.

The underlying molecular nature of any CKU-70 and DAF-16 interaction remains unclear. The coordinate regulation of DNA repair/genomic stability processes and stress response has been observed previously (29,31,32) consistent with a direct interaction. The cumulative effect of these processes may alter rates of aging. In a screen for proteins interacting with DNA damage response proteins CKU-70 and CKU-80 exhibited reciprocal interactions (46). However, neither DAF-16 nor HSF-1 was identified as an interactor; further investigation will determine if either protein interacts with CKU-70.

Despite *cku-70*(RNAi) increasing sensitivity to DNA damage, it can also increase life span, suggesting that DNA damage does not play a major role in determination of *C. elegans* life span. However, loss of the *C. elegans* RecQ5 ortholog also causes sensitivity to DNA damage and results in a shortened in life span (47). Thus, understanding of the subtleties genomic stability may play in determining *C. elegans* aging requires further work. Interestingly, recent work shows checkpoint functions can also regulate stress responses and modulate life span (Olsen and Lithgow, unpublished data).

If CKU-70 is involved in a mechanism that coordinately regulates stress responses, DNA repair, genomic stability, and ultimately aging, what then is the endogenous role of CKU-70 in stress responses? The Ku heterodimer binds with high affinity to broken DNA ends, preventing degradation and facilitating repair (48). Breaking of DNA after a genotoxic stress may also involve chromatin damage. The HSPs would then assist in repair of the protein component of damaged chromatin. Under benign conditions, CKU-70 will have limited DNA ends to bind and so may associate, for example, with HSP70 promoters to suppress expression. After DNA breakage, the high affinity of CKU-70 for binding of DNA ends could result in titration of CKU-70 away from HSP70 promoters, which in turn would relax suppression and lead to increased HSP70 expression. Such a model is consistent with observations in cell culture, where ρ-ray exposure induces Hsp70 expression (32) and the overexpression of Ku70 followed by heat shock results in the specific lack of Hsp70 induction (35,36).

Increased DAF-16 activity (e.g., *daf-2* mutants) is thought to increase expression of stress genes, including HSPs (9,14). Overexpression of HSF-1 itself increases life span, although in a DAF-16-dependent manner (14,15). The combined loss of both DAF-16 and HSF-1 function does not further decrease life span compared with loss of each singularly. Thus, DAF-16 and HSF-1 function in a common pathway to control life span in the nematode. Reduction of *cku-70* activity may further increase HSF-1 binding on HSP70 promoters, which in turn may increase expression of HSP70 through stress and life span. Decreased DAF-16 activity (e.g., *daf-16* mutants) results in impaired stress and HSP gene induction, lowered thermotolerance, and decreased life span. The combined dysregulation of HSF-1 activity by both lowered DAF-16 and CKU-70 activity throughout an entire life span may be further detrimental.

This study describes an interesting interplay between DNA repair and aging. Processes intended to reduce the risks of tumor formation may, as a by-product, shorten maximal life span consistent with the notion that aging results from late-acting detrimental actions of genes selected for early life beneficial effects (49,50).

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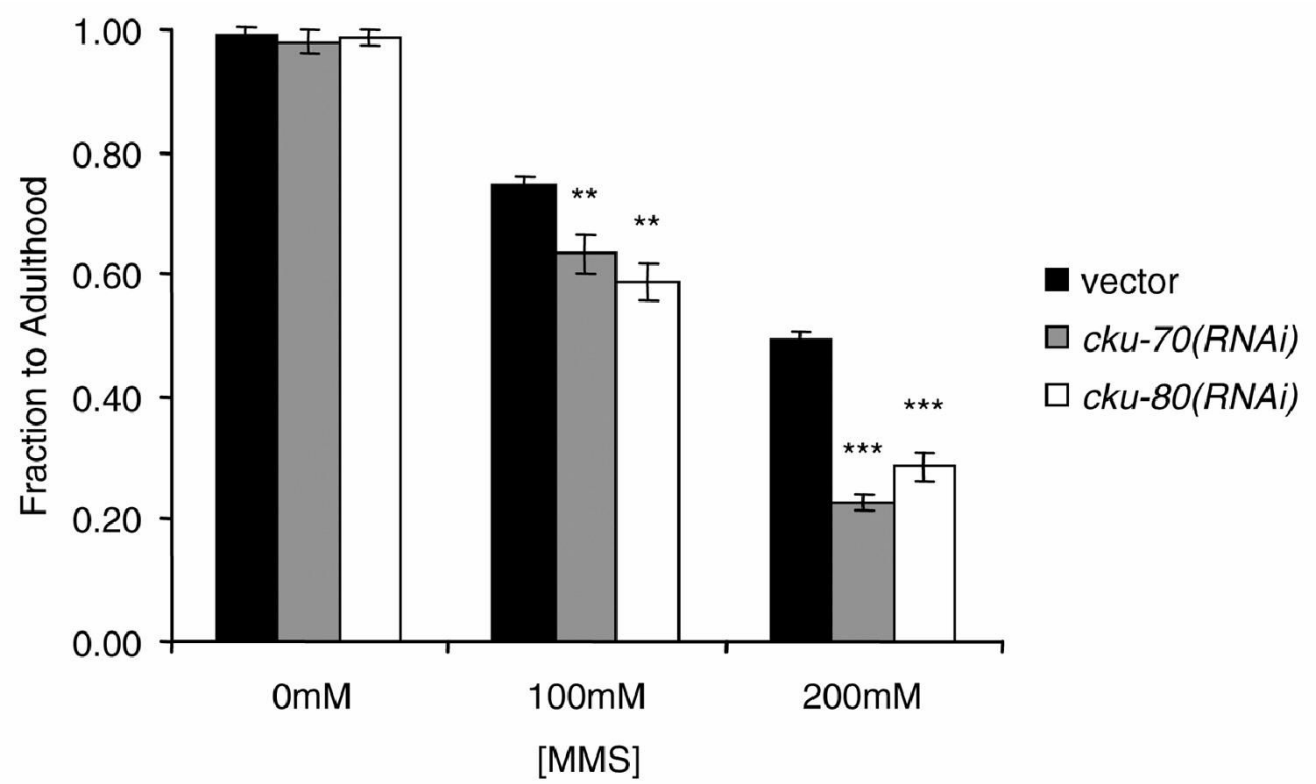
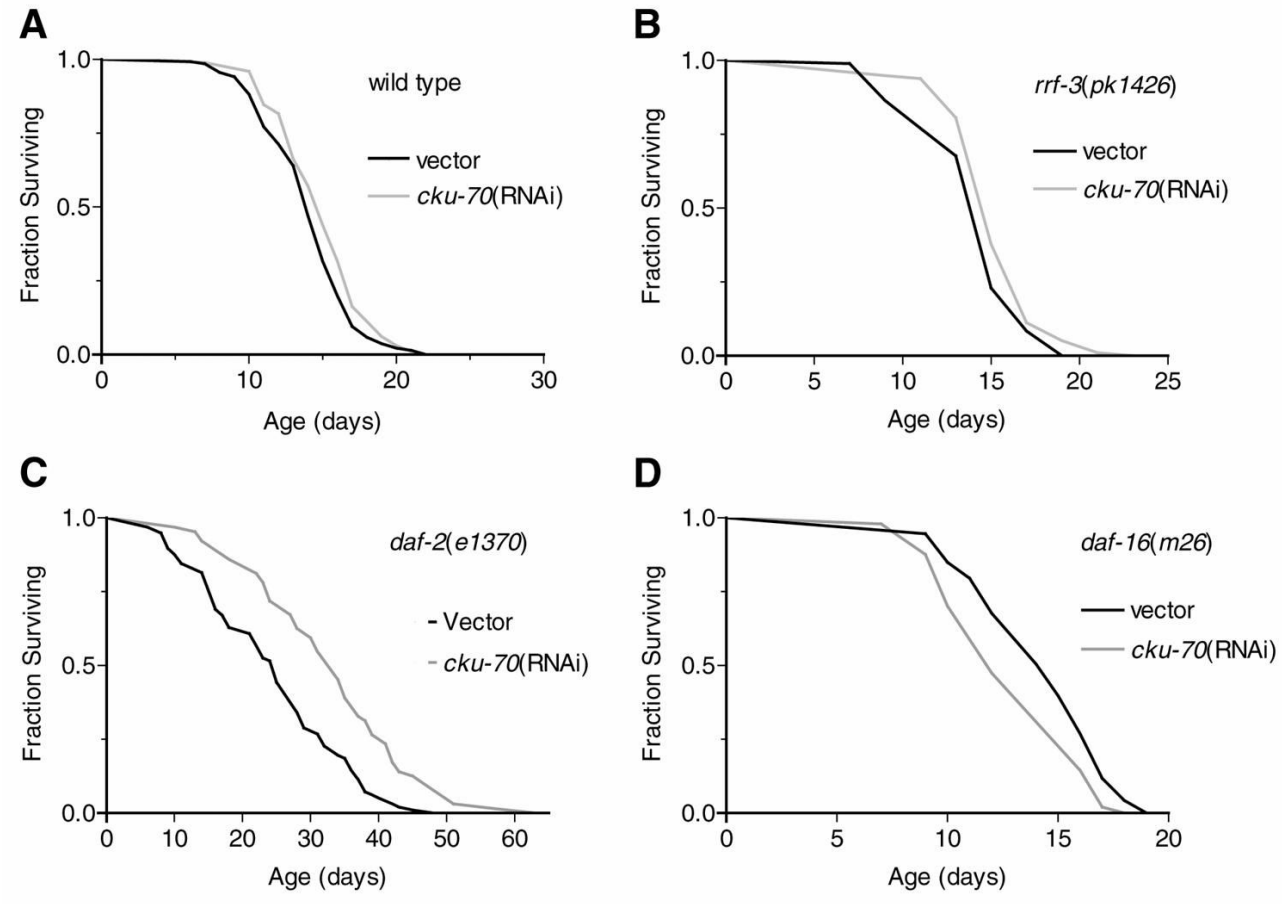


Figure 1.
Effects of *cku-70* and *cku-80*(RNAi) on MMS sensitivity of progeny from parents treated with either *cku-70*(RNAi) or *cku-80*(RNAi). $^{**}P < 0.01$, $^{***}P < 0.001$.

Fig. 2



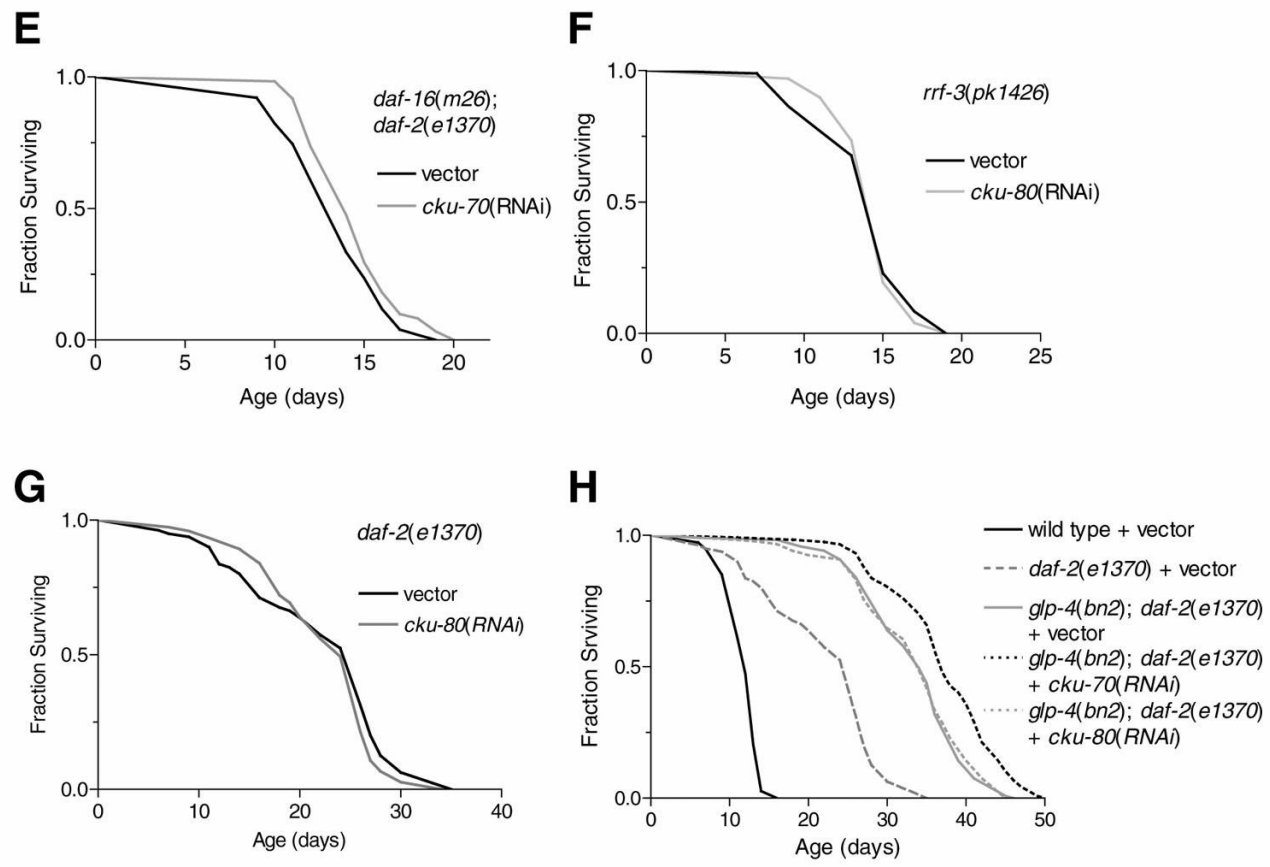


Figure 2.

Effects of *cku-70* and *cku-80* on adult hermaphrodite life span (days) at 25°C. ***A***) For wild-type worms *cku-70*(RNAi) did not significantly change life span; mean life span ± SE was 14.1 ± 0.3 days (vector: *n*=136) and 15.0 ±0.3 [*cku-70*(RNAi): *n*=98], replicate experiments *r* = 2. ***B***) *rrf-3*(*pk1426*) mean life span was slightly increased (*P*<0.01), 14.2 ± 0.3 (vector: *n*=96) and 15.5 ± 0.3 [*cku-70*(RNAi): *n*=98], *r* = 2. ***C***) *daf-2*(*e1370*) mean life span was significantly increased (*P*<0.0001), 24.2 ± 1.1 (vector: *n*=97) and 32.6 ± 1.4 [*cku-70*(RNAi): *n*=64], *r* = 3. ***D***) *daf-16*(*m26*) mean life span was significantly decreased (*P*<0.0001), 14.3 ± 0.3 (vector: *n*=93) and 12.8 ± 0.3 [*cku-70*(RNAi): *n*=97], *r* = 3.***E***) *daf-16*(*m26*);*daf-2*(*e1370*) mean life span was not significantly changed 13.5 ±0.4 (vector: *n*=51) and 14.5 ± 0.3 [*cku-70*(RNAi): *n*=61], *r* =2. ***F***) In *rrf-3*(*pk1426*) *cku-80*(RNAi) had no effect on life span 14.2 ± 0.3 (vector: *n*=96) and 14.7 ±0.2 (*cku-80*(RNAi): *n*=98), *r* = 2. ***G***) *daf-2*(*e1370*) mean life span was not affected by *cku-80*(RNAi), 22.1 ± 0.8 (vector: *n*=80) and 22.5 ± 10.7 [*cku-80*(RNAi): *n*=75], *r* = 2. ***H***) *glp-4*(*bn2*);*daf-2*(*e1370*) life span was significantly increased (*P*<0.0001) compared with *daf-2* (*e1370*) alone, 33.2 ± 0.6 (vector: *n*=119) vs. 22.1 ± 0.8 (vector: *n*=80). *glp-4*(*bn2*);*daf-2* (*e1370*) life span was slightly increased (*P*<0.0001) by *cku-70*(RNAi), 37 ± 0.7 [*cku-70* (RNAi): *n*=117] but unchanged by *cku-80*(RNAi), 33.1 ± 0.7 [*cku-80*(RNAi): *n*=119], *r* = 2.

Table 1

Fertility effect of *cku-70* and *cku-80*

Treatment	Mean total offspring ± SE	n
vector	294 ±6	28
cku-70(RNAi)	282 ±6 ns	27
cku-80(RNAi)	287 ±13 ns	13

ns: not significant.

Table 2

Effects of *cku-70* and *cku-80* on adult thermotoleranc

Genotype	Vector	Mean survival in minutes ± SE at 35°C n	*cku-70*(RNAi)	n
Wild type	489 ±13	56	531 ±18*	54
daf-2(e1370)	820 ±8	48	916 ±9**	46
daf-16(m26)	496 ±10	50	469 ±11ns	50
daf-16(m26); daf-2(e1370)	543 ±14	51	522 ±14ns	52

Genotype	Vector	Mean survival in minutes ±SE at 35°C n	*cku-80*(RNAi)	n
wildtype	413 ±13	52	401 ±18 ns	54

* $P < 0.05$

** $P < 0.01$.

Table 3

Effects of *cku-70* on dauer formation

Genotype		Temperature °C	n	%Dauers*
Wild type	vector	25	411	0%
	cku-70(RNAi)	25	402	0%
daf-2(e1370)	vector	25	220	100%
	cku-70(RNAi)	25	193	100%
daf-2(m41)	vector	20	435	77%
	cku-70(RNAi)	20	425	73%

*Resistant to 1% SDS.