Decreased Energy Metabolism Extends Life Span in *Caenorhabditis elegans* Without Reducing Oxidative Damage

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ABSTRACT

On the basis of the free radical and rate of living theories of aging, it has been proposed that decreased metabolism leads to increased longevity through a decreased production of reactive oxygen species (ROS). In this article, we examine the relationship between mitochondrial energy metabolism and life span by using the *Clk* mutants in *Caenorhabditis elegans*. *Clk* mutants are characterized by slow physiologic rates, delayed development, and increased life span. This phenotype suggests that increased life span may be achieved by decreasing energy expenditure. To test this hypothesis, we identified six novel *Clk* mutants in a screen for worms that have slow defecation and slow development and that can be maternally rescued. Interestingly, all 11 *Clk* mutants have increased life span despite the fact that slow physiologic rates were used as the only screening criterion. Although mitochondrial function is decreased in the *Clk* mutants, ATP levels are normal or increased, suggesting decreased energy utilization. To determine whether the longevity of the *Clk* mutants results from decreased production of ROS, we examined sensitivity to oxidative stress and oxidative damage. We found no evidence for systematically increased resistance to oxidative stress or decreased oxidative damage in the *Clk* mutants despite normal or elevated levels of superoxide dismutases. Overall, our findings suggest that decreased energy metabolism can lead to increased life span without decreased production of ROS.

MUTATIONS in *clk-1* have been shown to increase longevity in both worms and mice, suggesting that these mutations affect an evolutionarily conserved mechanism of life span extension (LAKOWSKI and HEKIMI 1996; LIU *et al.* 2005; LAPOINTE *et al.* 2009). The *CLK-1* protein encodes a hydroxylase involved in the synthesis of ubiquinone (EWBANK *et al.* 1997), a multifunctional, lipid-like molecule that transfers electrons in the electron transport chain and may also act as an intracellular antioxidant (MAROZ *et al.* 2009). *clk-1* was originally identified in worms in a screen for maternally rescued mutations that result in abnormal development and behavior. In addition to slow development and slow defecation, *clk-1* mutants show decreased brood size, a decreased rate of thrashing, and a decreased rate of pharyngeal pumping (WONG *et al.* 1995). It was a surprise, however, that *clk-1* worms also displayed extended longevity, because, at the time that it was discovered, only two other mutants, *age-1* and *daf-2*, with very different phenotypes, had been found

to extend longevity (FRIEDMAN and JOHNSON 1988; KENYON *et al.* 1993).

It is currently uncertain how mutations in *clk-1* result in the overall slowing of development and physiologic rates as well as an extended life span. One classic theory of aging, called the rate of living theory, postulates the existence of a link between energy metabolism and aging (PEARL 1922; SPEAKMAN 2005). This theory proposes that what determines the life span of an organism is the rate at which it produces and uses energy at the cellular level. Thus, the fact that *clk-1* worms exhibit slow physiologic rates and development suggests a decrease in the rate that these worms utilize energy, and, by the rate of living theory, this could account for their long life span.

In support of the rate of living theory, the loss of *clk-1* has been shown to result in decreased whole-worm oxygen consumption (FELKAI *et al.* 1999; YANG *et al.* 2007) and decreased electron transfer from complex I to complex III in the electron transport chain (KAYSER *et al.* 2004b), although this has not been observed by all investigators (MIYADERA *et al.* 2001). While some reports have suggested that energy consumption is not reduced in *clk-1* worms, at least under liquid culture conditions (BRAECKMAN *et al.* 2002), the observation that *clk-1* worms have higher levels of ATP than wild-type worms (BRAECKMAN *et al.* 1999) suggests a decreased use

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of energy in *clk-1* worms regardless of whether energy production is normal or decreased. It has also been found that *clk-1* double-mutant combinations that exhibit slower development than *clk-1* worms live even longer than *clk-1* worms (LAKOWSKI and HEKIMI 1996). In addition, overexpression of *clk-1* prevents the slowing of the defecation rate with age, increases mitochondrial function, and decreases life span (FELKAI *et al.* 1999).

Drawing on ideas from the free radical theory of aging (HARMAN 1956), it has been suggested that a possible mechanism underlying the rate of living theory is that decreased metabolism results in a lower rate of production of reactive oxygen species (ROS). As the free radical theory of aging proposes that aging results from the accumulation of molecular damage caused by ROS, then lower ROS production should result in slower aging. In *clk-1* worms, it has not been possible to directly measure levels of ROS *in vivo*; however, measurement of hydrogen peroxide production from submitochondrial particles has demonstrated increased ROS generation in *clk-1* mitochondria compared to wild type (YANG *et al.* 2009). In addition, the superoxide production potential is increased in *clk-1* worms compared to wild-type N2 worms (BRAECKMAN *et al.* 2002). Despite showing increased levels of ROS production, *clk-1* worms have been found to have normal or decreased levels of oxidative damage (KAYSER *et al.* 2004a; YANG *et al.* 2007, 2009) and decreased accumulation of lipofuscin (BRAECKMAN *et al.* 2002). The decrease in oxidative damage that occurs in spite of increased ROS production likely results from increased antioxidant defenses. In support of this conclusion, *sod-2* and *sod-3* mRNA are increased in *clk-1* worms compared to wild type (YANG *et al.* 2007).

Clearly, the levels of ROS production and antioxidant defense are altered in *clk-1* worms and likely contribute to the physiology and life span of these worms. Evidence supporting a role for altered ROS levels in determining the *clk-1* phenotype comes from the demonstration that increasing the levels of ROS through decreasing superoxide dismutase expression has been shown to modulate a variety of phenotypes in *clk-1* worms (SHIBATA *et al.* 2003; YANG *et al.* 2007). It is important to note, however, that the decrease in oxidative damage in *clk-1* worms appears not to contribute to their long life as it is possible to experimentally increase oxidative damage in *clk-1* worms beyond wild-type levels without reducing life span (YANG *et al.* 2007).

In addition to *clk-1*, four other genes have been identified that yield a *clk-1*-like phenotype (Clk phenotype), which includes slow development, slow defecation, slow pharyngeal pumping, decreased brood size and long life span coupled with maternal rescue (homozygous mutants from heterozygous mothers are phenotypically normal) (HEKIMI *et al.* 1995; LEMIEUX *et al.* 2001). The Clk phenotype has been studied in most detail in *clk-1* worms (WONG *et al.* 1995) and, subsequently, with *gro-1* (LEMIEUX *et al.* 2001), *clk-2* (BENARD

et al. 2001), and *tpk-1* worms (DE JONG *et al.* 2004), while *clk-3* worms have not been extensively studied [although *clk-3* worm energy metabolism and oxygen consumption have been examined (BRAECKMAN *et al.* 2002; SHOYAMA *et al.* 2009)]. Despite the phenotypic similarity of these mutants, the mutations that have been identified thus far have been shown to occur in genes encoding proteins with a wide range of functions with no obvious relationship to one another. *gro-1* encodes a tRNA-modifying enzyme (LEMIEUX *et al.* 2001), *clk-2* encodes a homolog of yeast Tel2p and a regulator of several PI3K-related protein kinases (AHMED *et al.* 2001; BENARD *et al.* 2001; JIANG *et al.* 2003; TAKAI *et al.* 2007), and *tpk-1* encodes thiamine pyrophosphokinase, which is necessary for the assimilation of thiamine (vitamin B1) (DE JONG *et al.* 2004).

All of the Clk mutants that have been identified exhibit slow physiologic rates and increased life span, suggesting that one may be sufficient for the other. To test this hypothesis, we identified six novel Clk mutants and demonstrate that these strains bear all of the characteristic features of the Clk phenotype, including extended longevity. We further show that mitochondrial function is decreased in the Clk mutants but that this decrease does not result in increased resistance to oxidative stress or decreased oxidative damage. Our results provide a plausible explanation for the extended life span observed in the Clk mutants and support aspects of the rate of living theory of aging while casting further doubt on the free radical theory of aging.

MATERIALS AND METHODS

General methods and strains: *Caenorhabditis elegans* strains were cultured as described (BRENNER 1974). Animals were maintained on nematode growth medium (NGM) agar plates, which were seeded with OP50, a slow-growing mutant of *Escherichia coli*. All strains were maintained at 20°. Wild-type animals were N2 Bristol strain. Mutations used were in the Bristol background unless specified. The following mutations, mutant combinations, and strains were used for mapping: LGI—*unc-11(e47)*, *unc-54(e190)*, *unc-35(e259)*; LGII—*dpy-10(e128)*, *clk-3(qm38)*, *unc-52(e444)*, *dpy-10(e128)* *unc-4(e120)*, *unc-4(e120)* *bli-1(e769)*, *unc-85(e1414)* *dpy-10(e128)*, *unc-4(e120)* *daf-19(m86)*, *unc-85(e1414)*; LGIII—*dpy-17(e164)*, *unc-32(e189)*, *unc-25(e156)*, *unc-45(e286)*, *dpy-1(e1)*, *clk-1(qm30)*, *dpy-17(e164)* *unc-32(e189)*, *unc-93(e1500sd)* *dpy-17(e164)*, *dpy-17(e164)* *unc-79(e1068)*, *clk-2(qm37)*, *gro-1(e2400)*; DA438—*bli-4(e937)*; *rol-6(e187)II*; *daf-2(e1368)* *vab-7(e1562)III*; *unc-31(e928)IV*; *dpy-11(e224)V*; *lon-2(e678)X*; RW7000—Bergerac strain that contains ~500 Tc1 loci, used for STS mapping (WILLIAMS 1995). During these experiments we also generated the following double-mutant strains: *daf-2;clk-2*, *daf-2;clk-3*, *daf-2;clk-4*, *daf-2;clk-5*, *daf-2;clk-7*, *daf-2;clk-8*, *daf-2;clk-9*, *daf-2;clk-10*, and *daf-2;gro-1*.

Isolation of mutants: Screening for Clk mutants was carried out as previously described (WONG *et al.* 1995). Briefly, wild-type animals (P0) at the L4 or young adult stage were mutagenized with ethyl methanesulfonate. Groups of 15 mutagenized relatively young adult hermaphrodites (P0) were plated on 9-cm petri dishes and allowed to self-fertilize. They

were removed after having laid ~300 eggs (20 eggs each). The resulting F₁ animals were left on the plate until they had laid a total of ~6000–10,000 F₂ eggs and were then removed from the plates. When most resulting F₂ animals had grown to adulthood, 30 adults with no detectable morphological or behavioral mutant phenotype were transferred onto individual small plates. Strains issued from those F₂ animals that produced an entire brood of slow growing, but anatomically wild-type, worms that also had a slow defecation cycle were analyzed further.

To determine further the heritability of these mutations, hermaphrodites from candidate mutant strains (*m/m*) were mated to N2 males and three or four of the resulting cross-progeny (F₁) were selected. The self-progeny (F₂) of these individuals were then examined for the presence of phenotypically wild-type animals. When ~25% of the F₂'s showed the mutant phenotype, the strain was discarded because this suggested that the animal originally picked was an escaper, that is, an animal carrying a fully zygotic mutation that was not strongly expressed. When substantially <25% of the F₂ animals displayed the mutant phenotype, 24 phenotypically wild-type F₂'s were picked onto individual plates and their F₃ progeny were examined. In most cases, ~25% of the F₃ broods were composed almost entirely of animal displaying a mutant phenotype. When none of the F₃ broods displayed a fully mutant phenotype, the procedure was repeated. When the repeated procedure was still unsuccessful at obtaining fully mutant F₃ broods, we considered that the phenotype of the original strain was synthetic in origin, that is, due to more than one independently segregating mutation, and the strain was discarded.

A total of 10,600 F₂ animals and their progeny were screened in the way described above, and six new *clk* mutants were isolated. All of the mutants were outcrossed with the wild type at least three times. One of the mutants described here [(*clk-10(qm169)*)] was isolated serendipitously in a screen for slow-growing mutants without maternal effect.

Mapping of mutations: Mutations were linked to chromosomes in one of three ways. Initially, linkage analysis was performed by using the DA438 strain, which carries at least one marker for each chromosome (AVERY 1993). When this did not yield a significant result, linkage to single-marker mutations was performed, or linkage to STSs by PCR analysis (for *qm151*) was performed as described (WILLIAMS 1995). Once linkage to a chromosome was established, some mutations were mapped more precisely using standard 2- and 3-point mapping strategies.

Assays for developmental and behavioral phenotypes: All analyses were carried out at 20°.

Embryonic lethality: Ten young, egg-laying adult hermaphrodites that had reached adulthood before they were picked were placed on fresh plates and allowed to lay eggs for 4–6 hr. A number of eggs were then picked from this plate onto a fresh plate, and the number of unhatched eggs was counted after 48 hr.

Embryonic development: Adult hermaphrodites were placed in a drop of M9 buffer and cut open with a razor blade to release the eggs. Two-celled eggs were chosen and placed onto fresh plates. The embryos were monitored every hour until hatching.

Self-brood size: L4 worms were placed onto fresh plates and incubated until they had matured and had laid the first few eggs. The hermaphrodites were then transferred onto fresh plates daily to prevent overcrowding until egg laying ceased. The total number of progeny produced were counted once the offspring had matured to adulthood.

Life span: Life-span studies were completed as previously described (YANG *et al.* 2007; VAN RAAMSDONK and HEKIMI

2009) on either normal NGM plates or plates supplemented with 100 μ M fluorodeoxyuridine (FUDR). Animals were scored as dead when they no longer responded with movement to light prodding on the head.

Defecation: Young adult hermaphrodites were placed singly onto fresh plates, and the length of the defecation cycle of the animals was measured as the time between consecutive pBoc contractions (the pBoc contraction is the contraction of the posterior body muscle that initiates defecation).

Mobility span: Mobility-span assays were completed on NGM plates supplemented with 100 μ M FUDR. To assess mobility, a circle was drawn around each worm and the plate was left for ~5 min. Worms that were visibly mobile or had moved outside of the circle were scored as mobile. Worms still present in the circle were scored as immobile. Worms that were able to move only their head were scored as immobile. Twenty worms per plate were examined with three trials per strain.

Scoring of maternal rescue: *Defecation:* Wild-type males were mated to homozygous mutant hermaphrodites, and 10 of the resulting wild-type F₁ hermaphrodites (*clk/+*) were pooled and allowed to lay self-fertilized eggs for a 4-hr period. F₂ animals were scored and singled. Subsequent analysis of the phenotype of the F₃ broods, which issued from each F₂ animal, allowed us to determine which F₂'s were maternally rescued homozygotes, as indicated by the slow growth of the entire F₃ brood.

Post-embryonic development: Wild-type males were mated to homozygous mutant hermaphrodites, and 10 of the resulting wild-type F₁ hermaphrodites (*clk/+*) were pooled and allowed to lay F₂ eggs for a 4-hr period. About 200 eggs were picked and left for 3 hr to hatch. All larvae that had hatched during that period were placed onto individual fresh plates and monitored once every 3 hr until the final development of the vulva. Their genotype was determined subsequently as for defecation.

Oxygen consumption and ATP levels: Oxygen consumption was measured as previously described with minor modifications (SUDA *et al.* 2005). Briefly, five first-day adult worms were picked into 0.25 μ l M9 buffer of a 0.5- μ l sealed tiny chamber at 22°. A fiber optical oxygen sensor (AL300 FOXy probe from Ocean Optics) was inserted into this chamber, and oxygen partial pressure was monitored for 15–30 min. The change in oxygen levels during this period was calculated as oxygen consumption. Oxygen consumption is presented per milligram protein. To quantify protein levels, worms were frozen and stored at –80°. Subsequently, frozen worm pellets were broken to powder in liquid nitrogen with mortar and pestle. The resulting powder was dissolved in 200 μ l NET buffer (50 mM Tris-HCl, pH 7.5, 150mM NaCl, 0.1% NP-40, 1 mM EDTA). After brief centrifugation, the supernatant was transferred to a new tube, and protein concentration was determined by the Bradford method using a Bio-Rad protein assay kit.

For ATP measurement, ~200 synchronized young adult worms were collected in M9 buffer and washed three times. Worm pellets were treated with three freeze/thaw cycles and boiled for 15 min to release ATP and destroy ATPase activity and then spun at 4° and 11,000 $\times g$ for 10 min. ATP contents were measured with an ATP detection kit according to the manufacturer's instructions (Invitrogen, Carlsbad, CA).

Western blotting for SOD-1 and SOD-2 protein: One hundred young adult worms of each genotype were picked, lysed in 2 $\times$ loading buffer, electrophoresed on 12% SDS-polyacrylamide gels, and then blotted onto nitrocellulose membrane (Bio-Rad). After applying primary antibody (1:1000, anti-SOD1 or anti-SOD2) and secondary antibody (1:10,000 mouse anti-rabbit IgG; Invitrogen), the membranes were incubated with the ECL plus detection reagent (Amersham Biosciences) and scanned using a Typhoon trio plus scanner. The anti-SOD1 and anti-SOD2 antibodies were generated in our laboratory (YANG *et al.* 2007).

Generation of *daf-2* double mutants: To isolate Clk mutants on a *daf-2* background, *daf-2* males were crossed with Clk mutant hermaphrodites. The resulting F₁ progeny were left to self-fertilize, and the F₂ worms were singled out and grown at 25°. The F₂ worms producing slow-growing dauer [*daf-2(e1370)* are dauer constitutive at 25°] progeny were considered to be *clk*; *daf-2* double mutants. For Clk genes linked to the same chromosome as *daf-2* mutants, dauer non-Unc recombinants of *daf-2 unc-32/clk* hermaphrodites were picked at 25° and then transferred to 15° to recover from the dauer stage. The recombinants were then singled out on plates at 20°. The plates with slow F₂ worms were considered to contain a *clk*; *daf-2* double mutant. The genotype of all double mutants was checked through a complementation test.

Sensitivity to oxidative stress and oxidative damage: Juglone sensitivity was assessed in 1-day-old adult worms on plates containing 240 µM juglone (Sigma). For this assay, plates were made fresh on the day of the assay as the toxicity of juglone decreases rapidly over time. Survival was monitored for 4 hr. To assess the ability of worms to develop under oxidative stress, a minimum of 40 eggs were placed on plates containing either 0.2 or 0.3 mM paraquat and seeded with OP50 bacteria.

Oxidative damage was assessed using an Oxyblot assay kit (Millipore) to detect carbonylated proteins. In this assay, carbonyl groups are derivatized to 2,4-dinitrophenylhydrazones (DNP-hydrazones), which can then be detected by Western blotting with a DNP-specific antibody. The Oxyblot assay was completed according to the manufacturer's protocol. Protein (7 µg) was used in each lane. After detection, membranes were stripped and reprobed with an anti-tubulin antibody to control for loading.

Statistical analysis: Survival plots were compared using the log-rank test. Significance between strains for juglone sensitivity assays were assessed by ANOVA. The effect of SOD RNA interference (RNAi) on development was assessed using a Student's *t*-test.

RESULTS

Clk mutants exhibit slow development and slow physiologic rates: The Clk phenotype in *C. elegans* is characterized by slow development, slow defecation, decreased brood size, slow pharyngeal pumping, and increased life span (slow aging), all of which are maternally rescued. Thus far, five Clk mutants have been identified (*clk-1*, *clk-2*, *clk-3*, *gro-1*, and *clk-8/tpk-1*). All five Clk mutants have extended longevity despite the fact that they were identified based on their slow development and physiology. This suggests that slowing development and physiology may be sufficient to extend life span in *C. elegans*. To further investigate this hypothesis, we sought to determine whether we could identify additional long-lived mutants by screening for worms with slow development and slow defecation that are maternally rescued. Accordingly, we repeated the screen used to identify *clk-1*, *clk-2*, and *clk-3* worms (HEKIMI *et al.* 1995; WONG *et al.* 1995). In total, we screened the progeny of 10,600 F₂'s and identified six novel morphologically normal mutants with slow development and defecation [we also identified *clk-8* in this screen, which we have partially described elsewhere

(DE JONG *et al.* 2004); this strain will be referred to as *clk-8/tpk-1*].

Having identified the six new Clk mutants in the screen, we next sought to characterize their phenotype in detail in comparison to the wild type (N2) and *clk-1(qm30)* worms. As with *clk-1* worms, all of the newly identified mutants exhibited slow embryonic development, slow post-embryonic development (PED), slow defecation, and a reduced brood size (Figure 1, A–D; Figure S1; Table S1). Post-embryonic phenotypes (PED and defecation) were wild type in heterozygous animals issued from homozygous mothers, thereby indicating that the mutations are all recessive (data not shown). In addition, we confirmed that these phenotypes are maternally rescued by assessing embryonic development and defecation in homozygous mutant offspring of heterozygous mothers (Figure 1, B and D; Table S1). Overall, all 11 Clk mutants exhibit slow development and physiologic rates, which is consistent with decreased energy utilization.

Clk mutants show extended life span: To determine whether the slow development and physiology of the Clk mutants is sufficient for increased longevity, we examined the life span of the Clk mutants. The life span of *clk-1*, *clk-2*, *clk-3*, and *gro-1* have previously been shown to be increased (LAKOWSKI and HEKIMI 1996). Despite the fact that long life was not one of the screening criteria at any time, all 11 Clk mutants exhibited significantly increased life span (Table S1, Figure S2A). The fact that the mutants identified in the screen bear all of the characteristics of the Clk phenotype suggests that we have identified six novel Clk mutants, thereby bringing the total number of Clk mutants to 11. Complementation tests were carried out between all 11 mutants and no allelism was found. In addition, all 11 genes were found to map to distinct locations (HEKIMI *et al.* 1995) (mapping data are summarized in Table S2).

In addition to living longer, we sought to determine whether the physiologic fitness (also referred to as health span) of the Clk mutants was increased compared to wild type. For this purpose, we measured the mobility span of the Clk mutants. Mobility span is a measure of how long the worms remain active, as death in worms is normally preceded by a period of immobility of variable length (HUANG *et al.* 2004). Wild-type worms remained mobile for most of their life span and died within one and a half days of becoming immobile (Figure 2). With the exception of *clk-2* mutant worms, which remained mobile as long as wild-type worms, all of the Clk mutants had a longer period of mobility than wild-type worms (Figure 2). In addition, after the point in time when worms became immobile, all of the Clk mutants survived longer than wild-type worms. Thus, Clk mutants exhibit a longer life span and health span compared to wild-type worms.

Clk mutants have decreased energy utilization: To gain further support for decreased energy utilization in

FIGURE 1.—Clk mutants exhibit an overall slowing of development and physiologic rates. Embryonic development time (A), post-embryonic development time (B), and defecation cycle length (D) are increased in all Clk mutants compared to wild-type N2 worms. Post-embryonic development time (B) and defecation cycle length (D) show almost complete maternal rescue. Self-brood size (C) is also decreased in all Clk mutants compared to wild-type worms. All of these phenotypes suggest decreased energy expenditure in the Clk mutants. *** $P < 0.001$.

the Clk mutants, we measured mitochondrial function as an estimate of energy production, and steady-state levels of ATP. It has previously been shown that *clk-1* mutants have decreased mitochondrial function (FELKAI *et al.* 1999; YANG *et al.* 2007) and increased levels of ATP (BRAECKMAN *et al.* 1999). Measurement of whole-worm oxygen consumption in day 1 adult worms revealed that all of the Clk mutants except for *clk-3* showed decreased oxygen consumption compared to wild-type worms, indicating decreased mitochondrial function (Figure 3A, Figure S2B). As others have observed decreased oxygen consumption in *clk-3* mutants (SHOYAMA *et al.* 2009), this indicates that under certain conditions all 11 Clk mutants have decreased oxygen consumption despite the fact that they were identified in a screen for slow development and defecation. This decrease in mitochondrial function provides a plausible mechanism for the long life of the Clk mutants as decreased expression of mitochondrial genes has been shown to result in increased life span (DILLIN *et al.* 2002; LEE *et al.* 2003).

Next, we examined ATP levels to determine if the decreased mitochondrial function resulted in decreased levels of ATP. Examination of ATP levels revealed that none of the Clk mutants have decreased levels of ATP (Figure 3B). In fact, *clk-6* mutants have significantly increased ATP levels, and there is a trend toward increase in *clk-1* and *clk-10* mutant worms. The

fact that ATP levels are normal or increased despite decreased mitochondrial function suggests that the Clk mutants have decreased energy expenditure compared to wild-type worms.

To determine whether the mitochondrial function of the Clk mutants remains low throughout their life span, we examined oxygen consumption in day 7 adults (a comparison of oxygen consumption at day 1 and day 7 is shown in Figure S3). In N2 worms, oxygen consumption declines with age such that at day 7 the oxygen consumption is ~60% of its day 1 oxygen consumption (Figure 3C). In contrast, all of the Clk mutants except the *clk-8* worms do not show any decrease in oxygen consumption from day 1 to day 7 (Figure 3C). Thus, while mitochondrial function declines with age in wild-type worms, it remains relatively constant during the first week of adulthood in the Clk mutants. A more gradual decline in oxygen consumption over time in long-lived mutants has also been observed in *isp-1* and *daf-2* worms (SHOYAMA *et al.* 2009) and in mouse mutants with only one copy of *Mcl1*, the mouse homolog of *clk-1* (LAPOINTE *et al.* 2009).

Clk mutants do not show increased resistance to oxidative stress: The decreased mitochondrial function and slow development and physiology of the Clk mutants suggest a slow rate of living, which may result in decreased production of ROS. According to the free radical theory of aging, decreased production of ROS

FIGURE 2.—*Clk* mutants have increased life span and mobility span. To determine whether the slowing of physiologic rates and development resulted in early immobility and thus a reduced health span, we examined the mobility span of *Clk* mutants. The mobility span was defined as the period of time where a worm exhibited voluntary, directional movement. Wild-type N2 worms remain mobile for most of their life span and become immobile only 1–2 days before they die. With the exception of *clk-2* worms, the *Clk* mutants exhibited increased mobility spans compared to wild-type worms (dark shading). The *Clk* mutants also exhibited increased survival in a state of immobility compared to N2 worms (light shading). In the bar graph, the height of the darkly shaded bar indicates the mobility span, the height of the lightly shaded bar indicates the period of immobility, and the total bar height indicates the life span.

FIGURE 3.—Clk mutants have decreased energy production but normal levels of ATP. To determine whether energy utilization is decreased in Clk mutants, we examined mitochondrial function and ATP levels. (A) Whole-worm oxygen consumption was measured to assess mitochondrial function in day 1 adult worms. With the exception of *clk-3*, all of the Clk mutants showed decreased oxygen consumption compared to wild-type N2 worms. This suggests that Clk mutants produce less energy than wild-type worms. (B) Measurement of ATP levels in day 1 adult worms revealed that Clk mutants do not have decreased ATP. In combination with their decreased mitochondrial function, this finding suggests decreased energy utilization. (C) We also examined the decline in mitochondrial function among the Clk mutants. With the exception of *clk-8/tpk-1* worms, we found that oxygen consumption declined more gradually in the Clk mutants than in wild type, suggesting a more balanced production of energy throughout their life span than in wild-type worms. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

could account for the extended life span of the Clk mutants. As the direct measurement of ROS levels *in vivo* can be unreliable (LAMBERT and BRAND 2009; MURPHY 2009), we chose instead to examine sensitivity to oxidative stress, which assesses the combination of

ROS production and antioxidant defenses. To test whether the Clk mutants had increased resistance to oxidative stress, we exposed the Clk mutants to juglone, a compound known to raise intracellular concentrations of superoxide. If ROS levels were decreased in the Clk mutants, we would expect that these worms would be more resistant to increasing levels of ROS caused by exposure to juglone than wild-type N2 worms. However, we find no evidence of increased resistance to oxidative stress in the Clk mutants.

On exposure to 240 μ M juglone, wild-type N2 worms begin to die after 2 hr and approximately half are dead after 4 hr on juglone plates (Figure 4A). Examination of the sensitivity to oxidative stress among the Clk mutants reveals that only *clk-2* worms are more resistant to oxidative stress than wild-type N2 worms (Figure 4, A and B). The resistance of *clk-2* worms to juglone-induced oxidative stress has been previously reported (JOHNSON *et al.* 2001). In contrast, *clk-1*, *clk-3*, *clk-4*, *clk-7*, *clk-8/tpk-1*, and *clk-10* worms are more sensitive to oxidative stress than wild-type worms (Figure 4, A and B). Thus, the long life observed in the Clk mutants does not result from increased resistance to oxidative stress. It is also important to note that the increased sensitivity to oxidative stress observed in the Clk mutants does not result in a reduced life span.

Next, we examined the development of Clk mutants on plates containing paraquat, another compound that increases intracellular levels of superoxide, to determine whether Clk mutants might be more resistant to oxidative stress during development. For this experiment, we transferred eggs onto NGM plates supplemented with 0.2 or 0.3 mM paraquat and determined the latest developmental stage achieved. We utilized a semiquantitative approach whereby each strain was given a score according to the furthest developmental stage attained (1 = L1/L2, 2 = L3/L4, 4 = adult, 5 = fertile adult). As with adult worms, we found no evidence that Clk mutants were more resistant to oxidative stress during development than wild-type worms and found in many cases that the Clk mutants were more sensitive than wild-type worms.

At 0.2 mM paraquat, *clk-2*, *clk-8/tpk-1*, and *clk-9* worms developed as well as wild-type worms while *clk-1*, *clk-3*, *clk-4*, *clk-5*, *clk-6*, *clk-7*, and *clk-10* worms showed early developmental arrest in response to oxidative stress (Figure 4C). At a higher concentration of 0.3 mM paraquat, we found that only *clk-2* worms grew to a further developmental stage than wild-type worms (Figure 4D). In contrast, *clk-1*, *clk-3*, *clk-4*, *clk-5*, *clk-6*, *clk-7*, *clk-9*, and *clk-10* worms all showed developmental deficits compared to N2 worms (Figure 4D). Thus, aside from *clk-2* and *clk-8/tpk-1*, all of the Clk mutants appear to be more sensitive to oxidative stress during development than wild-type worms, indicating that, as in adulthood, most of the Clk mutants do not have increased resistance to oxidative stress.

FIGURE 4.—Clk mutants are not resistant to oxidative stress. (A and B) Sensitivity to oxidative stress of the Clk mutants was assessed by exposing adult worms to 240 μ M juglone. Of the 10 Clk mutants assessed, only *clk-2* worms showed increased resistance to oxidative stress compared to wild-type N2 worms. In contrast, *clk-1*, *clk-3*, *clk-4*, *clk-7*, *clk-8*, and *clk-10* worms were more sensitive to oxidative stress than N2 worms. Sensitivity to oxidative stress during development was assessed by transferring eggs to plates containing either 0.2 mM (C) or 0.3 mM (D) paraquat. Under these conditions, only *clk-2* worms were able to develop to a developmental stage further than wild-type worms. In contrast, *clk-1*, *clk-3*, *clk-4*, *clk-5*, *clk-6*, *clk-7*, and *clk-10* worms all arrested at an earlier stage in development

than N2 worms. Overall, Clk mutants are not more resistant to oxidative stress than wild-type worms either during development or during adulthood. In most cases, Clk mutants were more sensitive to oxidative stress than wild-type worms.

While increased sensitivity to oxidative stress suggests increased levels of ROS, this may also result from a decrease in antioxidant defense mechanisms. To investigate this possibility, we examined the levels of SOD-1 and SOD-2 protein in each of the Clk mutants. Examination of SOD-1 levels by Western blotting revealed a significant increase in SOD-1 expression in *clk-4* and *gro-1* worms and a significant decrease in SOD-1 protein in *clk-2* worms (Figure 5A, Figure S2C). In the remainder of the Clk mutants, SOD-1 levels were unchanged from wild-type levels (Figure 5A). In contrast, SOD-2 levels appeared to be increased in most of the Clk mutants, although the increase was significant only in the case of *clk-1*, *clk-4*, *clk-5*, *clk-6*, *clk-10*, and *gro-1* worms (Figure 5B, Figure S2C). Only two Clk mutants, *clk-8/tpk-1* and *clk-9*, failed to show any increase in SOD-2. Overall, SOD protein levels appear to be unchanged or increased among the Clk mutants. The increase in SOD-2 protein levels suggests that ROS generation may be increased in the mitochondria, which is consistent with defects in mitochondrial function.

Since SOD levels were significantly increased in some of the Clk mutants, we sought to determine whether this increase was sufficient to result in decreased oxidative damage. If this were the case, then the extended longevity could potentially be explained by an increase in antioxidant defenses. To examine oxidative damage, we performed a Western blot for carbonylated proteins. Quantification of the resulting blots revealed no evidence for decreased oxidative damage in the Clk mutants (Figure 5C). In most cases, we observed a trend toward increased oxidative damage in the Clk mutants. Thus, it appears that the Clk mutants are more sus-

ceptible to oxidative damage than wild-type worms and that any compensatory upregulation of antioxidant defense is not sufficient to reduce oxidative damage.

Synergistic effects of Clk mutants and *daf-2* life span:

Deletion of *clk-1* has been shown to markedly extend the life span of *daf-2* worms such that *clk-1; daf-2* worms live much longer than if the already extended life spans of *clk-1* and *daf-2* were added together (LAKOWSKI and HEKIMI 1996; HEKIMI *et al.* 2001; BURGESS *et al.* 2003). If the synergism between *clk-1* and *daf-2* life spans occurs at the phenotypic level, we would expect the other Clk mutants to similarly extend the life span of *daf-2* worms, while if the mechanism occurs at a molecular level, this marked extension of *daf-2* life span would be expected to be specific to *clk-1*. To test these predictions, we constructed all double-mutant combinations of Clk mutations and *daf-2(e1370)* and scored their life spans (Figure 6, Figure S2D). We found three main types of interactions with *daf-2*:

1. Strongly synergistic interaction is shown by *clk-1* and *clk-2*. This interaction is characterized by life-span extension well beyond the addition of the two single-mutant life spans. This is especially notable in the case of *clk-2*, which by itself lived only marginally longer than wild-type N2 worms in these experiments.
2. Fully or partially additive interactions with *daf-2* are exhibited by *clk-3*, *clk-7*, *clk-8*, *clk-9*, *clk-10*, and *gro-1*.
3. No additivity was observed for *clk-4* and *clk-5*.

Correlations between phenotypes: Having completed the phenotypic characterization of the Clk mutants, we examined the data set for correlations between the different phenotypes with a particular focus

FIGURE 5.—Clk mutants have increased expression of SOD2 and no decrease in oxidative damage. On the basis of their increased sensitivity to oxidative stress, we examined the levels of SOD1 and SOD2 protein to determine whether the increased sensitivity resulted from a down-regulation of anti-oxidant defense proteins. (A) Examination of SOD-1 protein levels revealed increased SOD-1 in *clk-4* worms but decreased levels in *clk-2* worms. (B) SOD-2 levels were increased in most of the Clk mutants with the difference reaching significance in *clk-1*, *clk-4*, *clk-5*, *clk-6*, and *clk-10* worms. The fact that SOD protein levels were not decreased in Clk mutants suggests that the increase in sensitivity to oxidative stress results from increased levels of ROS production and not from decreased anti-oxidant defense. (C) Next we sought to determine whether the increase in SOD2 expression was sufficient to limit or reduce oxidative damage in the Clk mutants. By Western blotting for carbonylated proteins, we found no evidence for decreased oxidative damage in the Clk mutants. Thus, Clk mutants appear to have increased ROS production, which results in upregulation of SOD2 protein, but this increase in anti-oxidant capacity is not enough to reduce levels of oxidative damage.

on life span (Table S3). The strongest phenotypes correlating with life span were embryonic development time ($R^2 = 0.42$) and mobility span ($R^2 = 0.41$). Mobility has previously been suggested as a good predictor of life span (HUANG *et al.* 2004). Oxygen consumption on day 1 appeared to be correlated with post-embryonic development, brood size, and especially defecation cycle length ($R^2 = 0.43, 0.41, 0.79$). Interestingly, SOD1 and SOD2 protein levels correlated with mobility span and with the ratio of oxygen consumption on day 7 to that on day 1.

DISCUSSION

In this article, we describe the identification of 6 novel Clk mutants and further characterize the 5 previously described Clk mutants. We find that all 11 Clk mutants exhibit slow development, slow defecation, decreased brood size, and long life span. The Clk mutants also have decreased mitochondrial function (this study and SHOYAMA *et al.* 2009), which does not lead to increased resistance to oxidative stress or decreased oxidative damage. Our findings support the rate of living theory of aging and suggest that the mechanism by which decreased energy utilization leads to long life is not the result of increased resistance to oxidative stress.

Mutations in diverse and molecularly unrelated genes lead to a Clk phenotype: In the screen described here, we identified six previously undescribed mutants that possess all of the characteristic phenotypes of Clk mutants, including maternal rescue. On the basis of the number of Clk mutants that have been identified thus far, it is clear that many genes can mutate to yield the Clk phenotype. Furthermore, the group of genes that can mutate to yield the Clk phenotype appears to be quite diverse as the genes with currently defined functions appear to be unrelated (see the Introduction for a description of gene functions). The observation of maternal rescue in all of these strains suggests a critical role of normal gene function during development in preventing the Clk phenotype because the maternally donated gene product in a single egg is sufficient to normalize the phenotype of a 1000-cell adult. The fact that all of the Clk phenotypes occur together as linked phenotypes suggests the possibility of a common process, or response, as the basis of the Clk phenotype.

Clk mutants have decreased mitochondrial function: Examination of mitochondrial function revealed that oxygen consumption is decreased in all of the Clk mutants, except for *clk-3*. It has been previously noted that *clk-3* worms show certain abnormalities such as a large body size (BRAECKMAN *et al.* 2002), which may contribute to our inability to detect a decrease in oxygen consumption in these worms. It is also possible that these worms have altered mitochondrial function but not an overall decrease in oxygen consumption and that these changes in mitochondrial function have a similar

FIGURE 6.—Interaction of *clk* mutations with the insulin/IGF-signaling pathway of life-span extension. On the basis of the dramatic extension of *daf-2* life span by *clk-1*, we crossed the other *Clk* mutants with *daf-2* worms to determine whether these mutations also exhibited synergistic effects on life-span extension. *clk-2* (A) mutation was found to dramatically increase the life span of *daf-2* worms despite only marginally increasing life span in a wild-type background. *clk-3* (C), *clk-7* (E), *clk-8* (F), *clk-9* (G), and *clk-10* (H) mutations exhibited additive effects on *daf-2* life span in that they extend *daf-2* life span and wild-type life span by similar amounts. In contrast, *clk-4* (D) and *clk-5* (E) mutations did not increase the life span of *daf-2* worms and thus are non-additive.

effect on the alterations in the other *Clk* mutants. The fact that other researchers have observed decreased mitochondrial function in *clk-3* worms under different conditions and at different time points than we used here suggests that at least under certain conditions and at certain time points *clk-3* worms have decreased oxygen consumption (SHOYAMA *et al.* 2009). Overall, decreased mitochondrial function appears to be a common feature among the *Clk* mutants.

Interestingly, it has previously been shown in *C. elegans* that decreases in mitochondrial function generally result in increased life span. *isp-1* encodes an iron sulfur protein in mitochondrial complex III, and mutations in this protein result in decreased respiration, slow development, slow defecation, decreased brood size, and increased life span (FENG *et al.* 2001). Similarly, mutations in *sod-2*, which encodes the primary mitochondrial superoxide dismutase, result in decreased respiration, slow development, slow defecation, decreased brood size, and increased life span (VAN

RAAMSDONK and HEKIMI 2009). In fact, the association of disturbed mitochondrial function and increased life span has been clearly demonstrated in RNAi screens (LEE *et al.* 2003) and for proteins in all parts of the electron transport chain except complex II (DILLIN *et al.* 2002). The effect of inhibition of mitochondrial function by RNAi appears to be most critical during development as RNAi against mitochondrial proteins fails to extend life span in adult worms (DILLIN *et al.* 2002; REA *et al.* 2007). Thus, the maternal rescue observed in the *Clk* mutants can perhaps be explained by the presence of maternally deposited gene product at a critical period of development. In addition, it has recently been proposed that altered mitochondrial membrane potential may be a common mechanism underlying multiple longevity pathways (LEMIRE *et al.* 2009).

For those *clk* genes that have been identified, there exists at least some evidence linking their function to mitochondrial energy metabolism. While the connec-

tion of **CLK-1** to the mitochondria is straightforward, **CLK-2** has a somewhat more indirect link to mitochondrial function. Vertebrate **CLK-2** regulates a number of different phosphatidylinositol 3-kinase-related protein kinase targets (HAYASHI *et al.* 2006), one of which, the energy-sensing mTOR, is known to regulate and be regulated by mitochondrial function (SCHIEKE and FINKEL 2007). *gro-1* acts in both the cytoplasm and mitochondria; however, it is interesting to note that the mutant phenotype has been shown to be due only to the absence of **GRO-1** in mitochondria (LEMIEUX *et al.* 2001). Finally, **TPK-1** is involved in the synthesis of the cofactor thiamine pyrophosphate, which is necessary for several crucial biochemical reactions involved in mitochondrial energy metabolism (DE JONG *et al.* 2004).

Clk mutants are not more resistant to oxidative stress: When *C. elegans* mutants with decreased mitochondrial function were initially found to have increased life span, it was suggested that this may result from decreased production of ROS [*e.g.*, *isp-1* (FENG *et al.* 2001)]. However, since other mutations that decrease mitochondrial function result in increased ROS [*e.g.*, *mev-1* and *gas-1* (ISHII *et al.* 1990; KAYSER *et al.* 2001)], the effect of ROS production appears to depend on how mitochondrial function is decreased. As direct measurement of ROS levels *in vivo* can be unreliable (LAMBERT and BRAND 2009; MURPHY 2009), we instead chose to examine sensitivity to oxidative stress in the Clk mutants. While increased sensitivity to oxidative stress could result from either increased ROS production or decreased antioxidant defenses, in both cases the net result should be an increase in oxidative damage. We found that, with the exception of *clk-2* worms, which have previously been shown to be resistant to oxidative stress (JOHNSON *et al.* 2001), all of the Clk mutants exhibited increased sensitivity to oxidative stress during development, during adulthood, or both. As increased sensitivity to oxidative stress could result from increased ROS production or decreased antioxidant defense, we measured the levels of SOD protein and observed normal or increased levels in all of the Clk mutants except *clk-2* worms, which showed a decrease in **SOD-1** protein levels. In fact, many of the Clk mutants exhibited a significant increase in **SOD-2** levels, suggesting that increased mitochondrial ROS may be triggering the upregulation of **SOD-2**. The fact that SOD protein levels were not decreased suggests that the increased sensitivity to oxidative stress in the Clk mutants results from increased ROS production, although this was not directly tested. In addition, the levels of carbonylated proteins were not decreased in any of the Clk mutants, suggesting that their longevity is not dependent on lowering oxidative damage.

It is interesting to note that many of the Clk mutants exhibited increased expression of **SOD-2** in combination with increased sensitivity to oxidative stress. While

this appears to be counter-intuitive, there are two possible explanations. In cases such as *clk-5* and *clk-6*, where sensitivity to oxidative stress is observed in development but not in adulthood, it is possible that the upregulation of **SOD-2** (which was measured in adult worms) occurs in response to increased oxidative stress during development, thereby leading to normal stress resistance in adulthood. In cases where sensitivity to oxidative stress persists to adulthood, it is possible that the increased expression of **SOD-2** is effective at eliminating superoxide but the levels of hydrogen peroxide generated exceed the worms capacity to convert hydrogen peroxide to water. In support of this idea, it has been demonstrated that worms overexpressing *sod-1* exhibit increased sensitivity to paraquat, which is eliminated by increasing catalase activity (DOONAN *et al.* 2008).

Synergistic effect on life span reveals molecular interaction of *clk-1* and *clk-2* with the insulin/IGF-signaling pathway: To examine the interaction between life-span-extending mechanisms, we generated double mutants with *daf-2* worms, which have decreased insulin/IGF signaling (KENYON *et al.* 1993). Our laboratory has previously shown that *clk-1* acts in a distinct pathway from *daf-2* since the two mutations have synergistic effects on life span in *clk-1;daf-2* double mutants (LAKOWSKI and HEKIMI 1996). In contrast to *clk-1*, two other genes, *isp-1* and *sod-2*, which are thought to increase life span through decreasing mitochondrial function, did not show synergistic effects with *daf-2* life span (FENG *et al.* 2001; VAN RAAMSDONK and HEKIMI 2009), thereby suggesting that the marked interaction between *clk-1* and *daf-2* life-span extension does not result simply from decreased mitochondrial function but more likely results from a specific molecular interaction of *clk-1* and the insulin/IGF-signaling pathway. This conclusion is further supported by our present findings. We found that only *clk-1* and *clk-2* exhibit synergistic effects on the life span of *daf-2* worms. Similar to our previous study examining the *clk-1;daf-2* life span (LAKOWSKI and HEKIMI 1996), the effect of *clk-2* deletions on the *daf-2* life span is particularly dramatic as *clk-2* worms show the smallest increase in longevity of all the Clk mutants.

Rate of living theory and free radical theory of aging: The rate of living theory of aging proposes that organismal life span is inversely correlated with the rate of energy expenditure (PEARL 1922). Originally, this theory was supported by the observation that individuals belonging to species that have lower mass-specific metabolic rates tend to live longer than individuals belonging to species with higher mass-specific metabolic rates. While counter examples have cast doubt on the universality of this theory, there is also considerable support for this theory (SPEAKMAN 2005). In worms, there is a general trend that long-lived worms also have slow physiologic rates. For example, *clk-1*, *isp-1*, *eat-2*, and

sod-2 worms all develop slowly and show extended longevity. However, examples also exist of long-lived worms that have normal physiologic rates (*e.g.*, *age-1*) and of worms with a slow rate of living that have a shortened life span (*e.g.*, *mev-1*, *gas-1*). Note that most, if not all, worms in the latter category tend to be sick in appearance, as mutations with a toxic effect can shorten life span and tend to slow physiologic rates.

Our results provide further support for the rate of living theory in showing a link between energy metabolism and aging. In screening only for mutants with slow development and slow defecation, we found that 10 of 10 Clk mutants exhibited extended life span. *gro-1* mutant worms, which display the Clk phenotype but were not identified in our screens, also have extended life span. For comparison, KIM and SUN (2007) completed an RNAi screen for worms that are resistant to oxidative stress induced by paraquat and assessed which of the resulting worms had a 10% or greater increase in life span. They found that 13.8% (84/608) of paraquat-resistant worms had increased life span. While this success rate is much higher than random, our study suggests that slow development and slow defecation are a much better predictor of life span than resistance to oxidative stress.

The free radical theory of aging proposes that aging results from the accumulation of molecular damage caused by ROS, which eventually leads to cellular dysfunction and organismal death (HARMAN 1956). This theory has been extensively tested, and while numerous experiments support the theory, numerous others refute it (MULLER *et al.* 2007). In fact, a number of recent articles on *C. elegans* clearly demonstrate that increasing oxidative damage does not shorten life span (YANG *et al.* 2007; DOONAN *et al.* 2008; HONDA *et al.* 2008; VAN RAAMSDONK and HEKIMI 2009; YEN *et al.* 2009). Here, we show that the long-lived Clk mutants are not more resistant to oxidative stress but in most cases are in fact more sensitive to oxidative stress. This emphasizes the fact that increasing sensitivity to oxidative stress does not result in decreased life span and is, in fact, compatible with long life. Similarly, we have recently shown that deletion of *sod-2* results in increased sensitivity to oxidative stress and increased life span (VAN RAAMSDONK and HEKIMI 2009). Thus, our work here provides additional examples that contradict the predictions of the free radical theory of aging and suggests that decreased energy utilization can extend life span without decreasing oxidative damage.

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Supporting Information

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Decreased Energy Metabolism Extends Life Span in *Caenorhabditis elegans* Without Reducing Oxidative Damage

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FIGURE S1.—Embryonic development is delayed in Clk mutants. Examination of embryonic development in the Clk mutants reveals that all of the newly identified Clk mutants have slow embryonic development. This can be clearly seen by the fact that when 80% of wild-type embryos have developed only 0-20% of the embryos from any of the Clk mutants have reached the L1 stage.

FIGURE S2.—Characterization of *gro-1* mutant worms. To confirm our findings in the Clk mutants identified in our screens we characterized select phenotypes in *gro-1* worms which have been previously shown to exhibit the Clk phenotype. As with the other Clk mutants, *gro-1* worms were found to have increased lifespan (A) and decreased oxygen consumption (B). C. The levels of SOD1 and SOD2 protein were both increased in *gro-1* worms compared to wild-type. D. The *gro-1* mutation exhibited an additive effect on *daf-2* lifespan.

FIGURE S3.—Oxygen consumption is decreased in Clk mutants at day 1 but not at day 7. In order to determine how mitochondrial function changes over time we examined oxygen consumption at day 1 and day 7 in the Clk mutants and wild-type worms. A. At day1 we observed decreased oxygen consumption in all of the Clk mutants except for *clk-3* worms. B. In contrast, at day 7 oxygen consumption in the Clk mutant worms was similar to that in wild-type worms with the exception of *clk-8* worms which still showed decreased mitochondrial function. This indicates a more gradual decline in oxygen consumption in the Clk mutants.

* p<0.05, ** p<0.01, *** p<0.001.

TABLE S1
Phenotypic analysis of new *clk* genes

Genotype	N2	<i>clk-1</i> (<i>qm30</i>)	<i>clk-4</i> (<i>qm151</i>)	<i>clk-5</i> (<i>qm152</i>)	<i>clk-6</i> (<i>qm158</i>)	<i>clk-7</i> (<i>qm159</i>)	<i>clk-8/tpk-1</i> (<i>qm162</i>)	<i>clk-9</i> (<i>qm164</i>)	<i>clk-10</i> (<i>qm169</i>)
Embryonic Development (hours)	14.9±1.0 (n=297)	23.6±4.5 (n=70)	19.0±2.0 (n=73)	17.8±1.6 (n=104)	23.5±5.7 (n=46)	16.9±1.2 (n=80)	16.9±1.5 (n=116)	15.6±1.0 (n=177)	16.8±2.1 (n=166)
Post-embryonic Development (hours)	46.8±1.4 (n=200)	99.2±6.2 ^a (n=100)	94.6±7.2 (n=51)	73.5±3.3 (n=75)	72.0±3.8 (n=76)	61.0±6.9 (n=27)	59.1±5.2 (n=30)	68.4±3.8 (n=62)	70.7±10.9 (n=79)
Self Brood size	321.2±35.5 (n=10)	179.1±25.3 (n=10)	152.1±14.7 (n=10)	213.1±46.7 (n=10)	67.3±32.4 (n=10)	168.5±29.3 (n=10)	150.8±19.5 (n=10)	233.7±48.0 (n=10)	63.1±15.0 (n=10)
Lifespan ^b (days)	18.3±2.8 (n=74) max=23.3	27.5±7.1 (n=181) max=49.8	33.7±9.1 (n=144) max=48.7	27.2±4.2 (n=126) max=35.4	37.3±12.2 (n=131) max=69.6	29.7±6.0 (n=114) max=40.0	26.1±5.3 (n=137) max=38.9	27.7±5.2 (n=128) max=39.2	26.3±4.9 (n=140) max=36.6
Defecation cycle (seconds)	57.2±3.1 (n=25)	92.4±15.0 ^a (n=25)	132.1±15.7 (n=20)	98.6±11.8 (n=20)	90.0±10.4 (n=13)	94.0±13.5 (n=20)	115.9±19.7 (n=20)	101.9±24.1 (n=20)	106.4±26.8 (n=25)
Defecation cycle (seconds) [m ⁺ z ⁻] ^c	n.a.	53.0±3.6 (n=10)	58.2±4.0 (n=5)	67.9±3.8 (n=14)	62.5±5.9 (n=10)	65.5±5.3 (n=12)	59.6±1.4 (n=7)	52.92±2.3 (n=13)	51.5±4.2 (n=10)
Postemb. development (hours) [m ⁺ z ⁻] ^c	n.a.	n.d.	47.2±1.4 (n=38)	51.4±1.8 (n=35)	53.2±2.2 (n=37)	49.0±1.4 (n=45)	51.6±2.0 (n=31)	49.0±1.9 (n=44)	50.8±2.7 (n=24)

^aData from (WONG *et al.* 1995b). ^bThe life span of all mutants are significantly (P<0.05) longer than that of the wild type. ^cm⁺z⁻ denotes the first, maternally rescued, homozygous generation. n.d. not determined; n.a. not applicable.

TABLE S2**Summary of genetic mapping for 7 new *clk* genes**

Gene(allele)		Genetic mapping data ^a	
<i>clk-4(qm151)</i>	I	[<i>qm151/qm151</i>] ^b Linkage mapping ^c 14/55 Subregion mapping ^c 6/24	<i>stP124</i> <i>qm151</i> : 13.7cM <i>stP124</i> <i>qm151</i> : 13.4cM
<i>clk-5(qm152)</i>	III	[<i>unc-93 dpy-17/qm152</i>] [<i>unc-79 dpy-17/qm152</i>]	<i>unc-93</i> (6/7) <i>qm152</i> (1/7) <i>dpy-17</i> <i>unc-79</i> (6/6) <i>qm152</i> (0/6) <i>dpy-17</i>
<i>clk-6(qm158)</i>	III ^d	[<i>qm158 dpy-17/+ +</i>] ^e [<i>qm158 +/+ unc-32</i>]	65/102 <i>qm158 dpy-17</i> : 20.2cM 3/100 <i>qm158 unc-32</i> : 17.3cM
<i>clk-7(qm159)</i>	II	[<i>unc-4 bli-1/qm159</i>]	<i>unc-4</i> (17/27) <i>qm159</i> (10/27) <i>bli-1</i>
<i>clk-8(qm162)</i>	III	[<i>dpy-17unc-32/qm162</i>] [<i>dpy-17 qm162/+ +</i>] ^e	<i>dpy-17</i> (147/147) <i>unc-32</i> (0/147) <i>qm162</i> 268/280 <i>dpy-17 qm162</i> : 2.2cM
<i>clk-9(qm164)</i>	III	[<i>dpy-17 unc-32/qm164</i>]	<i>dpy-17</i> (8/21) <i>qm164</i> (13/21) <i>unc-32</i>
<i>clk-10(qm169)</i>	II	[<i>dpy-10 unc-4/qm169</i>] [<i>unc-85 dpy-10/qm169</i>]	<i>qm169</i> (17/17) <i>dpy-10</i> (0/17) <i>unc-4</i> <i>unc-85</i> (8/11) <i>qm169</i> (3/11) <i>dpy-10</i>

^a The genotypes given in square brackets are the relevant genotype of the animals whose descendants were scored to obtain multifactor or two-factor data.

^b *qm151* mutants were crossed with RW7000 hermaphrodites and resulting *qm151/qm151* progeny were assayed for the presence of STS markers.

^c *stP124* and *TCbn20* are polymorphisms on chromosome that were used for STS mapping.

^d Some mapping data for *clk-6* are contradictory. The data in the table suggest that it may be on chromosome III, other data (not shown) suggest it is not on any of the six chromosomes. The position of this mutation is still uncertain.

^e Two-factor data; the numerator of the fraction represents the number of self-progeny from animals of the given genotype which were homozygous for the doubly mutant chromosome. Only animals homozygous for the marker mutation were scored (the denominator).

TABLE S3**Correlation coefficients (R^2) between phenotypes measured in the Clk mutants**

	Lifespan	MOB	ED	PED	Brood Size	DEF	ATP	SOD1 Protein Level	SOD2 Protein Level	Juglone Sensitivity	Oxygen Consumption Day 1	Oxygen Consumption Day 7
Mobility-span	0.41											
ED	0.42	0.16										
PED	0.12	0.13	0.32									
Brood Size	0.14	0.02	0.17	0.21								
Defecation	0.24	0.30	0.01	0.28	0.28							
ATP	0.20	0.10	0.42	0.00	0.25	0.01						
SOD1 Protein Level	0.12	0.47	0.02	0.10	0.01	0.20	0.03					
SOD2 Protein Level	0.33	0.45	0.28	0.34	0.05	0.10	0.07	0.45				
Juglone Sensitivity	0.03	0.29	0.00	0.00	0.04	0.13	0.02	0.25	0.03			
Oxygen Consumption Day 1	0.02	0.24	0.12	0.43	0.41	0.79	0.05	0.07	0.11	0.01		
Oxygen Consumption Day 7	0.07	0.04	0.02	0.04	0.17	0.15	0.00	0.06	0.24	0.00	0.28	
Oxygen Consumption Ratio Day 7/Day 1	0.11	0.52	0.12	0.50	0.05	0.20	0.02	0.47	0.73	0.01	0.30	0.15

MOB = mobilityspan, ED = embryonic development time, PED = post-embryonic development time, DEF = defecation cycle length.