Knockdown of Mitochondrial Heat Shock Protein 70 Promotes Progeria-like Phenotypes in Caenorhabditis elegans

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Mitochondrial heat shock protein 70 (mthsp70) functions as a mitochondrial import motor and is essential in mitochondrial biogenesis and energy generation in eukaryotic cells. HSP-6 (hsp70F) is a nematode orthologue of mthsp70. Knockdown of HSP-6 by RNA interference in young adult nematodes caused a reduction in the levels of ATP-2, HSP-60 and CLK-1, leading to abnormal mitochondrial morphology and lower ATP levels. As a result, RNA interference-treated worms had lower motility, defects in oogenesis, earlier accumulation of autofluorescent material, and a shorter life span. These are the major phenotypes observed during the aging of worms, suggesting that the reduction of HSP-6 causes early aging or progeria-like phenotypes. The amount of HSP-6 became dramatically reduced at the expected mean life span in not only wild-type but also in long and short life span mutant worms (wild-type, daf-2, and daf-16). Mitochondrial HSP-60 and ATP-2 were also reduced following the reduction of HSP-6 during aging. These results suggest that the reduction of HSP-6 causes defects in mitochondrial function at the final stage of aging, leading to mortality.

Mitochondria are major organelles that carry out cellular oxidation and produce most of the cellular ATP by oxidative phosphorylation. Mitochondria also play essential roles in controlling cell viability and proliferation (1, 2). A large number of studies have shown the essential roles of mitochondria in development and differentiation (3, 4). Furthermore, mitochondria are thought to be deeply involved in the aging process, based on the free radical theory of aging, because mitochondria are a major source of reactive oxygen species (ROS) (5, 6). Accumulating evidence supports this idea. For example, abnormal mitochondria accumulate during aging (7), and enforced breakdown of mtDNA or their repair mechanism causes premature aging in mice (8, 9). In Caenorhabditis elegans, mutations in mev-1 (a subunit of the enzyme succinate dehydrogenase cytochrome b, a component of complex II of the mitochondrial electron transport chain) and gas-1 (a subunit of mitochondrial NADH-ubiquinone oxidoreductase, a component of complex I of the mitochondrial electron transport chain) increase ROS production and sensitivity to stress, resulting in a shorter life span (10–13).

A mammalian mitochondrial heat shock protein 70 (mthsp70, also known as mortalin or Grp75) has been shown to function as a mitochondrial protein import motor and is involved in mitochondrial biogenesis (14, 15). It has also been shown to be involved in the production of ROS (16), cell proliferation (17), and the regulation of life span (18) in mammalian cells. Increased expression of HSP-6 (hsp70F), the predicted C. elegans orthologue of mthsp70, by the introduction of an extra hsp-6 gene copies extended the life span of C. elegans (19). In contrast, deletion mutations of SSC1, the yeast orthologue of mthsp70, were lethal (20–22), and knockdown of mthsp70 caused growth arrest in human cancer cells (17, 23). Recently, it has been reported that the amount of mortalin/mthsp70 is reduced in Parkinson disease patients (24). In C. elegans, knockdown of hsp-6 by RNAi caused embryonic lethality, larval arrest, and sterile progeny (25, 26), suggesting that the gene is essential for development. However, the role of HSP-6 in adult worms, which consist of nondividing somatic cells, has not yet been analyzed. Therefore, we addressed this issue by knocking down HSP-6 in adult worms.

EXPERIMENTAL PROCEDURES

Strains and Culture Conditions—C. elegans strains (wild-type Bristol N2, daf-2(e1370), daf-16(mgDf50)) were obtained from the Caenorhabditis Genetics Center. Individuals were maintained at 20 °C using the standard technique (27). All experiments were carried out at 20 °C. For collecting synchronized eggs, gravid worms were placed on nutrient growth medium (NGM) agar plates seeded with Escherichia coli OP50,
allowed to lay eggs for 3–6 h, and then removed from the plate. Eggs were allowed to develop for 3 days until the young adult stage.

**Transgenic Worms**—For generation of the hsp-6:gfp reporter plasmid, a full-length hsp-6 cDNA and a 114-bp sequence at the 5′ end of hsp-6 containing a putative mitochondrial targeting signal (mts, N-terminal 38 residues) were amplified by PCR using *C. elegans* cDNA as a template. The following primers were used: 5′-GCTCTAGAGCATGCTTTCCGACGAT-3′ for hsp-6/mts forward primer, 5′-TCCCCGCGGGGATAAGTTTTGCTCC-3′ for hsp-6 reverse primer, and 5′-TCCCCGCGGGGAAAGATCAATTC-3′ for mts reverse primer. The constructs were confirmed by DNA sequencing. The amplified fragments were digested with XbaI/XhoI and cloned into an XbaI/XhoI-digested pPD129.36 (L4440) plasmid vector. Correct insertion of the fragments was confirmed by DNA sequencing. To generate a transgenic strain, hsp-6::gfp or mts::gfp plasmids (100 ng/μl) and pRF4 plasmid (20 ng/μl) were co-injected into the gonads of young adult N2 using standard methods (28).

**RNAi Treatment**—RNA interference (RNAi) was performed as described previously (29). For generation of an hsp-6 feeding RNAi construct, 502 bp at the 5′ end of hsp-6 were amplified by PCR from *C. elegans* cDNA using the following primers: forward 5′-GCTCTAGAGCATGCTTTCCGACGAT-3′ and reverse 5′-CCGCTCGAGCGGCGTTGTTGAC-3′. The amplified fragments were digested with XbaI/Xhol and cloned into an XbaI/Xhol-digested pPD129.36 (L4440) plasmid vector. The constructs were confirmed by DNA sequencing. *E. coli* strain HT115 (DE3) was transformed with a pPD129.36 plasmid containing the N-terminal 502 bp of hsp-6 cDNA or an empty pPD129.36 plasmid as a control (mock transformation) using standard CaCl2 transformation methods. Single colonies of transformed HT115 were inoculated onto 2× YT-containing ampicillin (100 μg/ml) and tetracycline (12.5 μg/ml) and incubated overnight at 37 °C. The pre-cultured *E. coli* was diluted 1:100 and grown to *A*600nm = 0.4. Isopropyl-β-D-thiogalactopyranoside was added (0.4 mM, final concentration), and the culture was incubated for 4 h at 37 °C. Additional ampicillin, tetracycline, and isopropyl-β-D-thiogalactopyranoside were added to the culture to double the concentration. Two to three drops of double-stranded RNA-induced *E. coli* culture were plated on NGM plates and stored at 4 °C until use. To begin the RNAi treatment, a young adult worm (3 days old) was transferred onto the RNAi plate. For longer culture, the worms were transferred onto a new plate every 2 days for the first 10–12 days and then onto a new plate every 4 days.

**Preparation of Antibody**—Anti-HSP-6 antibody was described previously (19). Anti-HSP-1 (hsp70A) polyclonal rabbit antibody was prepared as described previously (19). Briefly, a chemically synthesized peptide, NDQGNRTTPSYVC (Biologica Co., Nagoya, Japan), was conjugated with keyhole limpet hemocyanin. The antigen was injected subcutaneously. The resultant rabbit antiserum was used for Western blot analysis.

**Western Blotting**—Worms (50–100 individuals) were collected and washed with M9 buffer (22 mM KH2PO4, 42 mM Na2HPO4, 86 mM NaCl, 1 mM MgSO4), resuspended in lysis buffer (0.2 M Tris-HCl (pH 7.5), 0.1 M EDTA, 0.04 M EGTA, 20 mM phenylmethylsulfonyl fluoride, 1 mM NaN3, 1% SDS), sonicated six times for 25 s, and boiled for 5 min. This extract was centrifuged at 9,000 × g for 15 min, and the supernatant was stored at −80 °C until use. Protein concentration was measured by using the BCA protein assay kit (Pierce). Protein samples were separated by SDS-PAGE and then electrophoretically transferred to Immobilon-P (Millipore Corp., Bedford, MA). The membrane blots were incubated overnight at 4 °C with the following antibodies: anti-HSP-6 antibody at 1:3,000 dilution (19); anti-HSP-1 antibody at 1:1,000 dilution and anti-HSP-60 antibody (SPA-807; StressGen Bioreagents, MI) at 1:3,000 dilution; anti-actin monoclonal antibody (MAB1501R; Chemicon International, CA) at 1:30,000 dilution; anti-CLK-1 antibody (sc-925; Santa Cruz Biotechnology) at 1:200 dilution; and anti-C-V-β/ATP-2 antibody (MS503; MitoSciences LLC, OR) at 1:5,000 dilution. Then the membrane was washed with Tris-buffered saline containing Tween 20 (TBST; 137 mM NaCl, 2.7 mM KCl, 25 mM Tris-HCl (pH 7.4), 0.05% Tween 20), incubated with secondary antibody, and then washed with TBST. The blots were developed by using the enhanced chemiluminescence (ECL)-Plus detection system (Amersham Biosciences). Fluorograms were recorded by an LAS-1000 luminescence analyzer (Fujifilm, Tokyo, Japan), and the images were analyzed using ImageGauge version 3.1 software (Fujifilm).

**ATP Levels**—ATP levels were measured as described previously with slight modifications (30–32). Approximately 1200 young adults were transferred to hsp-6 RNAi plates or mock plates (9 cm diameter). All the plates contained 5′-fluorodeoxyuridine at a final concentration of 20 μg/ml to inhibit progeny production. Worms were incubated for 2 or 4 days at 20 °C, washed four times with M9 buffer, resuspended in cell lysis buffer, and immersed in liquid nitrogen immediately. The samples were stored at −80 °C. The frozen samples were boiled for 15 min to release ATP, and dilution buffer was added, and the samples were centrifuged at 15,000 × g for 5 min. The supernatant was diluted 100-fold with dilution buffer. ATP levels were measured by using the ATP Bioluminescent HSII kit (Roche Applied Science) with the Lumat LB9507 luminometer (Berthold Technologies, Bad Wildbad, Germany). ATP levels were normalized to protein concentration.

**Determination of Brood Size**—Young adult worms were transferred to a mock or hsp-6 RNAi plate (one worm per plate) and allowed to lay eggs. The worms were transferred to fresh plates every day, and the numbers of eggs remaining on the plates were counted for 3 days. Eggs that did not hatch after 2 days were counted as “unhatched.” For each experiment, 10 to 11 worms were analyzed.

**Morphological Observation and Motility Assay**—For observation by phase-contrast microscopy (Axioskop; Carl Zeiss, Jena, Germany), worms were placed on an agar pad in M9 buffer and covered with a coverslip. For transmission microscopy (MVX10; Olympus, Tokyo, Japan), worms were placed on an agar plate. For measuring the frequency of body bends, worms were placed in a dish containing a drop of M9 buffer and observed by transmission microscopy. Movement was recorded by a digital camera (Camedia C-4040ZOOM; Olympus), and the frequency of sinusoidal movements per min was counted (33). Experiments typically began with more than 50
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FIGURE 1. Localization of HSP-6 to mitochondria. A, transgenic worms expressing an HSP-6-GFP fusion protein (HSP-6::GFP, top panels) or a mitochondria targeting signal-GFP fusion protein (MTS::GFP, bottom panels) were produced as described under “Experimental Procedures.” Confocal images of the body walls are shown. Phase-contrast images are shown on the right. Both MTS::GFP and HSP-6::GFP (leftmost panels) co-localized with MitoTracker mitochondrial dye in muscle cells (2nd panels from the left, also see yellow color in merged images in the right panels). Scale bar = 10 μm. a.a., amino acids. B, wild-type worms (N2) were homogenized and cell-fractionated by differential centrifugation. 1 μg of mitochondrial fraction (MF) and 10 μg of post-mitochondrial supernatant (PMS) and the total fraction (TF) were analyzed by Western blotting as described under “Experimental Procedures.” The blot was probed for mitochondrial markers (HSP-6, HSP-60, ATP-2, and CLK-1) and cytoplasmic controls (HSP-1 and actin) using antibodies as indicated. An asterisk indicates a nonspecific reaction band.

Worms that crawled off the plate, exploded, or bagged were censored. The assays were repeated three times. Mini StatMate software (ATMS Co., Ltd., Tokyo, Japan) was used for producing Kaplan-Meier survival curves and statistical analysis.

Isolation of Mitochondria—Approximately 12,000 eggs were placed on large NGM agar plates (9 cm diameter) seeded with E. coli OP50 at 20 °C. When the worms reached the young adult stage (3 days old), 5′-fluorodeoxyuridine was added to the plates at a final concentration of 20 μg/ml to inhibit the development of progeny. The worms were harvested 2 days later and separated from bacteria and debris by 60% sucrose flotation. Mitochondria were isolated as described previously (34). Isolated mitochondrial pellets were resuspended in lysis buffer containing 1% SDS and boiled for 5 min. Then samples were centrifuged at 15,000 × g for 10 min, and the obtained supernatant was stored at −80 °C until use.

RESULTS

Mitochondrial Localization of HSP-6—HSP-6 is a predicted orthologue of mammalian mortalin/mthsp70/Grp75 and yeast Ssc1p. The amino acid sequence is 77% identical (87% similar) to that of mouse mortalin and 63% (78% similar) to that of yeast Ssc1p. The N-terminal sequence of HSP-6 is predicted to have an amphipathic helical structure with periodic appearance of basic residues, typical for mitochondrial targeting sequences. This region is similar in length to the mitochondrial targeting sequence of yeast Ssc1p and is significantly shorter than those of mammalian proteins. To confirm that HSP-6 is the orthologue of mortalin/mthsp70/Grp75, we first sought to determine its localization in mitochondria. We expressed tagged recombinant protein in a worm to analyze the subcellular localization. A fusion protein of HSP-6 and GFP was constructed and expressed in muscle cells using a myo-4 promoter (HSP-6-GFP). We chose muscle cells for observation because they have a simple and regular mitochondrial morphology (see below). When transgenic F2 progeny were observed for GFP fluorescence, most of the GFP fluorescence was observed with MitoTracker-positive structures, clearly indicating localization of the fusion protein to mitochondria (Fig. 1A, upper panels). Similar mitochondrial localization was observed using the 38-amino acid residue of HSP-6 that was predicted to contain a mitochondrial targeting signal (Fig. 1A, lower panels).

To confirm the localization of HSP-6 in mitochondria, we performed subcellular fractionation analysis. Worms were homogenized, and a mitochondria-enriched fraction was prepared by differential centrifugation. As shown in Fig. 1B, HSP-6 was clearly co-purified together with mitochondrial markers (HSP-60, a mitochondrial chaperone; ATP-2, the β-subunit of
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F1-ATPase; CLK-1, a subunit of ubiquinone synthetase), indicating the localization of HSP-6 in mitochondria. HSP-1 (hsp70A), which is most similar to HSP-6 (61%) and is localized in the cytoplasm, and actin were mostly recovered in the postmitochondrial supernatant and not in the mitochondrial fraction confirming the efficient removal of cytoplasm from the mitochondrial fraction. Taken together, these results show that HSP-6 localizes in mitochondria and that the N-terminal 38 amino acid residue functions as a mitochondrial targeting signal.

Effect of HSP-6 Knockdown on Mitochondrial Structure and Function—Because knockdown of HSP-6 caused embryonic lethality (25, 26), we started feeding RNAi to young adult worms (3 days old) to analyze the role of endogenous HSP-6 in adult worms. A significant reduction in HSP-6 level was observed after 2 days of feeding in wild-type N2 (Fig. 2A). The level of HSP-1 was not affected (Fig. 2A), suggesting that the RNAi effect was specific.

When young adults (3 days old) were stained with MitoTracker for 2 days, mitochondria were observed as distinct short rope-like structures regularly spaced and aligned in parallel along with myofibrils in the body wall muscle cells (Fig. 2B, top left panel) as reported previously (31, 35). In marked contrast, mitochondria were unevenly spaced, loosely aligned, and frequently interconnected across myofibrils in HSP-6-knockdown worms (hsp-6(RNAi)) (Fig. 2B, top right panel). In worms mock-treated for 4 days, the intestine was strongly stained with MitoTracker (Fig. 2C, left panels). In contrast, the fluorescence intensity of the intestine was lower in hsp-6(RNAi) (Fig. 2C, right panels). This result indicates that the uptake of MitoTracker was decreased in hsp-6(RNAi), suggesting that the mitochondrial membrane potential was decreased by the reduction of HSP-6. Correspondingly, the total ATP level of the worms was significantly decreased after 4 days of RNAi treatment, although not at 2 days of the treatment (Fig. 2D).

These results suggest that depletion of HSP-6 immediately causes defects in mitochondrial membrane potential and morphology, and prolonged or severe defects in mitochondria cause the reduction of the ATP level. ATP is synthesized by F0F1-ATPase in a mitochondrial inner membrane driven by a proton gradient produced by oxidative phosphorylation (36). We found that ATP-2, the β-subunit of the F1-ATPase (37, 38), was decreased in hsp-6(RNAi) (Fig. 3, upper panels). Similarly, CLK-1, a mitochondrial subunit of ubiquinone synthetase, and HSP-60, a mitochondrial chaperon, were decreased in hsp-6(RNAi) (Fig. 3, lower panels). The level of HSP-6 became 10–15% that in the control worms on the 2nd day of RNAi treatment (Fig. 3, D2). At this time point, the levels of ATP-2 and HSP-60 reduced to about 45 and 60% compared with the mock treatment, respectively (Fig. 3, D2). The reduction in ATP-2, HSP-60, and CLK-1 became pronounced after 4 days of RNAi treatment (D4–D10). At that time, although the level of HSP-6 was only slightly more reduced (to about 10%), the levels of ATP-2 and HSP-60 dropped to 40 and 50% of the mock treatment levels, respectively. A significant reduction in CLK-1 was also observed at this time point (about 60% compared with the mock treatment). This was coincident with the reduction of the total ATP level (Fig. 2D). These results strongly suggest that depletion of HSP-6 causes defects in the mitochondrial protein import system leading to the decline in various mitochondrial proteins, including ATP-2, HSP-60, and CLK-1, and resulting in defects in ATP synthesis.

Interestingly, mitochondria in the body wall muscle cells were noticeably thinner and less regularly shaped in untreated (not shown) and mock-treated worms by the 7th day of adulthood (Fig. 2B, left, D7). Mitochondria became thinner and fragmented in the older worms (Fig. 2B, right, D12). A similar aging effect was also observed in hsp-6(RNAi). The thinner and less regular mitochondrial structure was exaggerated in hsp-6(RNAi), and this may coincide with reduced function (membrane potential).

Defects in Gonadal Functions in hsp-6(RNAi)—Depletion of HSP-6 in adult worms neither produced an acute lethal effect nor extended the life span of worms, in contrast to the depletion of other mitochondrial proteins (31, 32, 39). Despite this, depletion of HSP-6 caused embryonic lethality, larval arrest, and sterile progeny (25, 26). Therefore, we closely inspected the oocytes and gonads in control (N2) and hsp-6(RNAi) for alterations. Control young adult worms (3 days old) laid normal eggs for the first 3 days and then laid unfertilized oocytes, which were less translucent and had brownish color, for several days on the culture plates. In contrast, hsp-6(RNAi) stopped laying
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![Image]

**FIGURE 3. Reduction of ATP-2, CLK-1, and HSP-60 in hsp-6(RNAi).** Whole-worm extracts (10 μg of protein for HSP-6, HSP-1, ATP-2, HSP-60, and actin; 30 μg for CLK-1) were prepared at the indicated time points (D1–D10, day 1 to day 10) and analyzed by Western blotting as described under "Experimental Procedures." m, mock treatment; i, RNAi treatment.

eggs by the 2nd day, and no oocytes were found on culture plates thereafter. 10 days after the RNAi treatment, oocytes accumulated markedly in the uterus (Fig. 4A, mock, asterisks). In contrast, the uteruses of hsp-6(RNAi) of the same age was empty (Fig. 4A). In addition, oocytes and gonads were less translucent and had brownish color, similar to the unfertilized oocytes (Fig. 4B, C). In mock-treated wild-type N2 worms, as reported previously (33, 43), a similar decline was observed in brood sizes of the worms were analyzed. As shown in Fig. 4C, the brood size was reduced about 36% in hsp-6(RNAi) compared with the mock treatment. About half of the laid eggs were infertile (unhatched; these eggs did not hatch for 3 weeks excluding the delay of hatching), indicating defects in early development. When unhatched eggs were carefully inspected under a microscope, cell division and morphogenesis appeared to be arrested at various developmental stages (Fig. 4D). Taken together, these results suggest that the depletion of HSP-6 disrupts gonadal function, possibly affecting oogenesis and causing embryonic lethality.

**Reduced Motility in hsp-6(RNAi)—**There have been reports suggesting that a decline in mitochondrial function diminishes motility in worms and humans (40–44). After we found that HSP-6 knockdown causes mitochondrial malfunction (Figs. 2 and 3), we next examined whether hsp-6(RNAi) had any effects in motility. First, we measured the frequency of body bends. As shown in Fig. 4A, the frequency of body bends gradually declined in mock-treated wild-type N2 worms, as reported previously (33, 45). A similar decline was observed in hsp-6(RNAi) during aging. However, the decline was more pronounced in hsp-6(RNAi) than in the mock-treated worms. The frequency of body bends per min was reduced to about 60 and 35% in RNAi-treated worms on the 6th and the 9th day, respectively, compared with mock-treated worms (Fig. 5A). A similar reduction in the frequency of body bends was also observed when HSP-6 was knocked down in long lived daf-2 mutant worms (Fig. 5B).

Next, we measured the pharyngeal pumping rate. Similar to the body-bending movements, the pharyngeal pumping rate gradually decreased in mock-treated and RNAi-treated worms during aging (Fig. 5C). Again, the reduction in the pumping rate was more pronounced in hsp-6(RNAi). The mean pumping rate was less than 80% in hsp-6(RNAi) compared with mock-treated worms on the 5th day and less than 60% on the 12th day (Fig. 5C). Taken together, these results strongly suggest that the depletion of HSP-6 reduces the rates of muscle contraction.

**Reduced Life Span in hsp-6(RNAi)—**We next analyzed whether HSP-6 knockdown affected the life span of worms. For each experiment, we started with 72–111 worms, and the numbers of live worms were counted every 2–4 days. Worms that crawled off the plate, exploded, or bagged were censored. The results of three independent experiments are shown in supplemental Table 5, and representative mean survival curves are shown in Fig. 5D. The log rank test revealed that the life span of hsp-6(RNAi) was significantly shorter than that of mock-treated worms for wild-type (N2) and also the long lived mutant...
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**FIGURE 5. Reduction of motility and life span in hsp-6(RNAi).** Worms were mock- or RNAi-treated as described above. A and B, the frequency of body bends was measured as described under “Experimental Procedures.” The mean ± S.E. is plotted for days under treatments. The result of each experiment and statistical analysis are shown in supplemental Table 3. A, N2; B, daf-2. C, pharyngeal pumping frequency was measured as described under “Experimental Procedures.” The mean ± S.E. (bar with error bars) are shown. The results of each experiment and statistical analysis are shown in supplemental Table 4. D, survival curve of mock- or RNAi-treated N2 and daf-2 worms. Curves were produced by combining the result of three independent experiments. Statistical analysis was performed as described under “Experimental Procedures.” The result of each experiment and statistical analysis are shown in supplemental Table 5.

daf-2 (supplemental Table 5). The relative reduction in life span was similar in N2 and daf-2 (~15%).

**Increased Autofluorescence in hsp-6(RNAi)**—Reduced motility is a prominent physiological alteration found in aged worms (33). In addition, it has recently been reported that worms with lower motility are expected to have a shorter life span (45). Therefore, the reduced motility and life span found in hsp-6(RNAi) in the present study were thought to be premature aging or progeria-like phenotypes. To examine this hypothesis, we assayed autofluorescence in hsp-6(RNAi) because lipofuscin-like fluorescent pigment is reported to accumulate with age and can be used as a general marker for aging in *C. elegans* (46–49). The autofluorescence in the intestine on the pharynx side of worms was photographed (Fig. 6A), and the average intensity was quantified as described under “Experimental Procedures” (Fig. 6B and supplemental Table 6). A low level of autofluorescence was detected in young mock-treated worms (Fig. 6, D2, mock). In contrast, the level was about 20% higher in hsp-6(RNAi) of the same age (Fig. 6, A and B, D2). The level of autofluorescence gradually increased in both mock- and RNAi-treated worms (D2 and D4) during aging, but the level of autofluorescence remained significantly higher in RNAi-treated worms up to 4th day of the RNAi treatment (D4, about 18%). This is consistent with progeria-like phenotypes in adult hsp-6(RNAi) similar to those found in *daf-16* and mev-1 mutants (10, 49). The average intensity of the autofluorescence was not significantly different at the 7th day after the RNAi treatment. It was thought that accumulation of autofluorescent material reached plateau by aging, and RNAi treatment did not exacerbate it.

**HSP-6 Is Reduced in Aged Worms**—Finally, we investigated the level of HSP-6 in progressively older worms. Wild-type (N2), long lived (daf-2), and short lived (daf-16) worms were collected at various time points, and the amounts of proteins were analyzed by Western blotting using actin as a control. Younger worms carried many fertilized eggs, which complicated the study of the proteins of adult body somatic cells. Therefore, we began the analysis using 10-day-old adult worms, which are post-reproductive. Live worms that responded to pricking were collected for the lysates. As shown in Fig. 7, the level of HSP-6 was fairly constant for most of the life span of worms. However, when worms reached their expected mean life span (15, 20, and 40 days for *daf-16*, N2, and daf-2, respectively), the level of HSP-6 dramatically reduced for all genotypes. A similar but slower decrease was observed for ATP-2 and HSP-60 in N2 and daf-16. These results indicate that there is a strong correlation between the decrease in HSP-6 and mortality.

**DISCUSSION**

The level of HSP-6 quickly declined at the early stage (2 days) of RNAi treatment (Figs. 2 and 3). At this time point, the levels of several mitochondrial proteins that are critical for mitochondrial function (ATP-2, HSP-60) were significantly reduced as well (Fig. 3). Interestingly, pronounced fusion or network formation of mitochondria was already observed at this time, along with a slight reduction in movement (Fig. 5A; about 90% compared with the mock treatment). A similar alteration of mitochondrial structure has been reported to occur in response to the reduction of several mitochondrial proteins that directly or indirectly affect mitochondrial electron transport or ATP synthesis (31) and also in response to the reduction of mitochondrial protein import receptors (35). A similar interconnected mitochondrial structure has also been observed in mutants of *SSC1*, a yeast orthologue of HSP-6 (50). Interestingly, defects in mitochondrial protein import have been reported to induce mitochondrial fusion in yeast and mammalian cells (51, 52). Therefore, it is possible that the morphological change found in *C. elegans* is caused by a similar mechanism. Intriguingly, we also observed a
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A

mock hsp-6(RNAi)

D2

D4

D7

B

FIGURE 6. Accumulation of autofluorescence in hsp-6(RNAi). Worms were mock- or RNAi-treated as described above for the indicated number of days (D2, D4, and D7, day 2, day 4, and day 7, respectively). Autofluorescence in the intestine on the pharynx side of worms was observed. A, six randomly selected worms were photographed (horizontal groups of panels). The intensity of the photograph was converted into pseudocolors (red, strong; green, medium; blue, low). B, average fluorescent intensity was quantified as described under “Experimental Procedures.” The means of three independent experiments are shown. Bars indicate S.E. The results of each experiment and statistical analysis are shown in supplemental Table 6.

FIGURE 7. Decrease of mitochondrial proteins in aged worms. N2, daf-2, and daf-16 were cultured in standard conditions, lysed, and analyzed by Western blotting as described under “Experimental Procedures.” Actin was probed as a control. The days after hatching are indicated at the tops of the panels. Five micrograms of protein was applied to each lane.

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significant alteration in mitochondrial morphology as a function of age. How or whether these morphological alterations affect mitochondrial function in nondividing somatic cells is a future area of investigation.

Gonadal function is also severely affected at the early time point (Fig. 4B, D2). This is consistent with the previous reports showing that knockdown of hsp-6 by RNAi causes embryonic lethality, larval arrest, and sterile progeny (25, 26). It is reasonable that HSP-6 knockdown more severely affects actively dividing cells because the amount of mitochondria must double in each round of cell division in those cells. In support of this possibility, depletion of Ssc1p in yeast and mortalin/mthsp70/Grp75 in mammalian cells has been reported to arrest cell division (17, 20). It is thought that the somatic cells of an adult worm do not divide, so a lesser amount of HSP-6 is necessary to sustain mitochondrial function sufficient to support cell survival.

The effect of HSP-6 knockdown became more pronounced in later time points. Although the level of HSP-6 was only slightly more reduced, the levels of ATP-2, HSP-60, and CLK-1 were reduced in much higher magnitude (Fig. 3, D4–D10). Concomitant reductions in mitochondrial membrane potential and total body ATP level were also observed (Fig. 2, C and D). Corresponding to the gradual development of mitochondrial defects, motility gradually and significantly declined in hsp-6(RNAi) compared with mock-treated worms (Fig. 5, A–C). A similar decline was also observed in hsp-6(RNAi) daf-2 individuals, which are long lived mutants (Fig. 5B). It has been reported that motility and life span are significantly linked, so a worm with lower motility is expected to have a shorter life span (45). In accordance with this relationship, life span was significantly shortened by HSP-6 knockdown in wild-type (N2) and daf-2 (long lived mutant) worms (Fig. 5D). Furthermore, greater and earlier accumulation of autofluorescence, which is a general marker for aging (46–49), was observed in hsp-6(RNAi) N2 (Fig. 6). A reciprocal finding that the overexpression of HSP-6 in muscle extends the life span (19) strongly suggests that the effect of HSP-6 knockdown is specific. Therefore, we interpret that the defects found in hsp-6(RNAi) to be progeria-like phenotypes in adults.

As discussed above, reduction of HSP-6 causes reduction of ATP-2 and CLK-1. The shortened life span of hsp-6(RNAi) seems to contradict previous reports showing that loss of function mutants of those proteins, ATP-2 and CLK-1, have extended life span (37, 40). In accordance with these reports, isp-1, a mutant of iron-sulfur protein of mitochondrial complex III, has also been reported to have an extended life span (53). Furthermore, comprehensive RNAi-based studies have revealed that depletion of mitochondrial proteins, including several electron transport chain components and a subunit of ATP synthase, generally extend life span (31, 32). In addition, reduced expression of frh-1, a C. elegans orthologue of frataxin and yeast Yfh1p (responsible for Friedreich ataxia and shown to be involved in the function of respiratory chain I–III function and mitochondrial iron metabolism), was reported to extend life span, although there is a conflicting report showing reduction of life span (54, 55). It is possible that mitochondrial function is more severely affected by the reduction of HSP-6 compared with the reduced function associated with reduction in ATP-2 or CLK-1 alone because the reduction of HSP-6 would be expected to reduce general protein import into mitochondria. In addition, the concomitant reduction of mitochondrial HSP-60 (Fig. 3) is expected to cause some defects in the quality control of proteins in mitochondria. Therefore, the life span extension induced by the reduction of ATP-2 or CLK-1 may be masked by severe mitochondrial dysfunction. In supporting this notion, the reduction of components of mitochondrial protein import receptors by RNAi was reported to cause life span shortening (35). Interestingly, it has been reported that depletion of CYC-1, a component of mitochondrial electron transport com-
plex III, and ATP-3, a component of complex V, in adult worms did not extend the life span of worms, except when RNAi treatment was started from the time of hatching (32). Therefore, it is possible that depletion of ATP-2 or CKL-1 alone in an adult worm does not affect the life span of worm.

Similar to hsp-6(RNAi), accumulation of autofluorescence and short life span have been observed in mutants of mev-1 (kn1) and gas-1 (fc2) (12, 47, 56, 57). Therefore, it is possible that the main effect of a reduction of HSP-6 is on these proteins.

A reduction in HSF-1, a heat shock factor, also reduced life span (48, 58). HSF-1 is a master regulator of heat-shock response and is known to induce mthsp70 (59, 60). Consistently, we found several potential heat shock elements in the promoter region of hsp-6 (61), and we actually observed an increase in HSP-6 in response to heat shock (19). Therefore, it is possible that the reduction in HSF-1 induced the reduction of HSP-6, resulting in a shorter life span.

In this study, the amount of HSP-6 was found to be constant over most of the life span of worms, irrespective of the life span phenotype (Fig. 7). This suggests that some physiological aging processes are independent of the reduction in HSP-6, i.e. the reduction in motility and accumulation of autofluorescent material that is found during the physiological aging process (45, 46) are not the result of the reduction in HSP-6. For example, the reduction in motility can be much affected by the degeneration of muscle cells (62). Autofluorescent material accumulates as a function of age, but the involvement of mitochondrial respiratory activity in this process remains obscure (49). Because the function of mthsp70 is thought to be strongly conserved in yeast, nematode, and mammalian cells, it is possible that defects in mthsp70 cause progeria-like phenotypes in other organisms, including humans. If so, knockdown of HSP-6 in the adult worm may serve as a model system for developing anti-progeria drugs.

Interestingly, the amount of HSP-6 dramatically reduced when the expected mean life span had been reached not only in the wild type but also in longer and shorter life span mutants (Fig. 7). Mitochondrial HSP-60 and ATP-2 were also reduced following the knockdown of mtHSP70, leading to locomotor defects (62). Therefore, it is possible that the reduction in HSF-1 induced the reduction of HSP-6, resulting in shorter life span.

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