

Original Article

# Life-Span Extension by Axenic Dietary Restriction Is Independent of the Mitochondrial Unfolded Protein Response and Mitohormesis in *Caenorhabditis elegans*

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## Abstract

In *Caenorhabditis elegans*, a broad range of dietary restriction regimens extend life span to different degrees by separate or partially overlapping molecular pathways. One of these regimens, axenic dietary restriction, doubles the worm's life span but currently, almost nothing is known about the underlying molecular mechanism. Previous studies suggest that mitochondrial stress responses such as the mitochondrial unfolded protein response (UPR<sup>mt</sup>) or mitohormesis may play a vital role in axenic dietary restriction-induced longevity. Here, we provide solid evidence that axenic dietary restriction treatment specifically induces an UPR<sup>mt</sup> response in *C. elegans* but this induction is not required for axenic dietary restriction-mediated longevity. We also show that reactive oxygen species-mediated mitohormesis is not involved in this phenotype. Hence, changes in mitochondrial physiology and induction of a mitochondrial stress response are not necessarily causal to large increases in life span.

**Keywords:** Axenic culture—UPR<sup>mt</sup>—Longevity

Dietary restriction (DR), the restriction of nutrients without malnutrition, has been shown to extend life span in a wide variety of species (1). In *Caenorhabditis elegans*, a broad range of DR regimens has been proposed and they all extend life span to various degrees by separate or partially overlapping molecular pathways (2). DR-like phenotypes are also induced using sterile, chemically defined or undefined liquid media, called axenic media (3–5). Axenically cultured worms share many characteristic traits with worms subjected to other DR methods: slowed development, reduced fecundity, a slender appearance, and prolonged life span and hence the term axenic dietary restriction (ADR) is often applied (3).

Although the *C. elegans* life span is doubled under ADR, the underlying molecular mechanisms supporting this robust longevity effect still remain enigmatic. Probably, this mechanism is distinct from that of most other DR regimens, as was recently shown in a genetic screen (6). Our previous findings suggest that increased stress resistance and enhanced mitochondrial function may play a vital role in ADR-induced longevity (3,7,8). These observations lead us to investigate the role of the mitochondrial unfolded protein

response (UPR<sup>mt</sup>) and mitohormesis in ADR-mediated life-span extension.

The UPR<sup>mt</sup> is a highly conserved cellular stress response that activates nuclear-encoded mitochondrial chaperone genes to maintain protein homeostasis in the mitochondria (9). The UPR<sup>mt</sup> is activated upon different forms of mitochondrial stress including the misfolding of mitochondrial proteins or stoichiometric abnormalities of large multimeric complexes, such as electron transport chain (ETC) complexes (10,11). In *C. elegans*, the UPR<sup>mt</sup> is essential for the life span-extending effect caused by inhibition of mitochondrial respiration (12). Mitonuclear protein imbalance by disturbance of mitochondrial protein synthesis also activates the UPR<sup>mt</sup> and extends *C. elegans* life span (13). However, a more recent genome-wide RNAi screen showed that there is no straightforward correlation between UPR<sup>mt</sup> activation and life-span extension in *C. elegans*. Constitutive activation of the UPR<sup>mt</sup> in the absence of mitochondrial stress also fails to extend life span (14,15). These findings cast doubt on the unique importance of the UPR<sup>mt</sup> in longevity. One possible explanation is that the nature of the mitochondrial proteotoxic stress may

differ in various conditions. Nevertheless, the UPR<sup>mt</sup> has been implicated in life-span extension of worms, flies, and mice, suggesting a conserved role in cellular homeostasis (16).

The free radical theory of aging proposes that mitochondria are the main source and prime target of reactive oxygen species (ROS) and it is a widely held view that this might cause or at least contribute to the aging process (17). In recent years, a different view has gained popularity: ROS produced by mitochondria result in an adaptive response, called mitohormesis, which promotes cellular health and organismal longevity (18,19). The positive effect of mitohormesis on life span only occurs within a limited range of relatively low intracellular ROS levels. High ROS levels may not be compensated for by the mitohormetic response, which may explain the wide range of life spans observed for different respiratory chain mutants (20). Although the detailed mechanism of ROS-mediated mitohormesis has not been fully elucidated, several key factors, such as PMK-1 (p38 MAP kinase), HSF-1 (heat shock factor), SKN-1 (NRF-2), DAF-16 (FOXO), and PRDX-2 (peroxiredoxin) are involved for the mitohormetic response (18,19,21,22).

Here, we provide evidence that ADR treatment specifically induces a UPR<sup>mt</sup> response in *C. elegans* but this induction is not required for the ADR-mediated longevity. We also show that ROS-mediated mitohormesis is probably not involved in this longevity phenotype.

## Methods

### *C. elegans* and RNAi Strains

The wild-type *C. elegans* used was Bristol N2 male stock (Caenorhabditis Genetics Center [CGC]). The mutant and transgenic strains VC289 *prdx-2(gk169)*, VC1024 *pdr-1(gk448)*, RB2547 *pink-1(ok3538)*, SJ4100 *zcls13(hsp-6p::gfp)*, SJ4058 *zcls9(hsp-60p::gfp)*, CL2070 *dvls(hsp-16.2p::gfp)*, and SJ4005 *zcls4(hsp-4p::gfp)* were also obtained from CGC. QC117 (*atfs-1[et17]*), QC118 (*atfs-1[et18]*), and *atfs-1(tm4525)* were provided by Dr. Matt Kaeberlein. dsRNA expressing bacterial strains targeting *ubl-5* and *hsp-6* were from the genomic RNAi library (produced by J. Ahringer). As a control, the bacterial strain containing the empty vector L4440 was used.

### Worm Maintenance

Axenic and monoxenic culture conditions were adapted as previously described (6). To avoid contamination, 100 µg/mL ampicillin was added to both axenic and monoxenic cultures. The day of L4-to-adult transition was determined as Day 0 of adulthood. For monoxenic and axenic cultures, it took 3 and 6 days on average to reach this stage at 20°C, respectively. Worm samples were collected and cleaned by gravitational settling followed by three to five washes in S-basal.

Briefly, for monoxenic culture, eggs prepared by hypochlorite treatment of gravid adults were allowed to hatch overnight in S basal (23) and the resulting first stage larvae (L1s) were inoculated into Fernbach flasks containing 250 ml of growth medium (S-basal containing approximately  $3 \times 10^9$  bacterial cells/mL and 5 µg/mL cholesterol). Bacteria were added as frozen beads consisting of equal volumes of pelleted bacteria and S basal. The flasks were shaken in a temperature controlled (20°C) gyrotory shaker at 120 oscillations/min. For axenic culture, the basal medium consists of 3% soy peptone (Sigma-Aldrich, St. Louis, MO) and 3% yeast extract (Becton-Dickinson, Franklin Lake, NJ), final concentrations (f.c.). Since *C. elegans* is not capable of heme synthesis, after autoclaving

the basal medium was supplemented with 0.05% hemoglobin f.c. (bovine; Serve, Heidelberg, Germany) diluted from a 100× stock in 0.1M KOH (autoclaved for 10 minutes). Axenic medium is very rich in nutrients and easily gets contaminated by microbes. Hence, all equipment and preparations should be handled in a laminar flow cabinet to ensure sterility.

### Life-Span Assay

Life-span assays were performed as described previously (6). ADR was performed in liquid axenic culture while fully-fed (FF) assays were carried out on standard solid nematode growth medium (NGM) plates seeded with bacteria (23). Briefly, gravid adults were subjected to a microbleaching procedure in which about 10 worms were brought in a 10 µL drop of sterile distilled water. Then 10 µL alkaline bleach (12° hypochlorite and 1M NaOH f.c.) was added and left to incubate until all adults were dissolved or for maximally 10 minutes. Then 5 mL of axenic medium containing 20% sterile skimmed milk was added. Milk was added to stimulate and synchronize larval growth and we confirmed that this component did not cause UPR<sup>mt</sup> (data not shown). The eggs were allowed to hatch and were incubated at 20°C until adulthood. At L4 stage (around 5 days after bleaching), 50 µM of 5-Fluoro-2'-deoxyuridine (FUDR) was added to prevent progeny production. At adulthood, worms were transferred and exposed to the experimental conditions. Worms were transferred to fresh NGM plates every 2 days and checked for survival. NGM plates with antioxidants were freshly prepared for every transfer, whereas for axenic cultures, antioxidants were added to final concentration only once. For butylated hydroxyanisole (BHA), the compound was added from a 1,000× concentrated stock in dimethyl sulfoxide (DMSO) and equal volume of DMSO was added to the respective controls.

For RNAi-based life-span assay, worms were cultured on standard NGM agar plates seeded with the *Escherichia coli* OP50 bacteria until adulthood. RNAi was carried out as previously described (6) and performed on adult worms for 5 days before they were transferred to the experimental conditions. For each strain, around 100 worms were placed on small NGM plates (10 per plate) seeded with *E. coli* OP50 as FF control. For ADR, about 100 worms were transferred to small screw-cap tubes (three to five worms per tube) containing 0.3 mL of liquid axenic medium (ADR). Progeny production was avoided by the addition of FUDR at 100 and 50 µM f.c. for FF and ADR, respectively. Survival was scored at regular time intervals: daily for the FF condition, every other day for the ADR conditions. In solid conditions, worms were considered dead if they did not respond to gentle prodding with a platinum wire. In liquid conditions, worms were scored dead if no movement could be detected, even after gently tapping the tubes. Worms that died of protruding vulva or crawling off the plates were censored. All life-span assays were conducted at 20°C and were repeated at least twice independently (pooled data is shown).

Data were analyzed with the online application for survival analysis (OASIS) as described in Yang and colleagues (24). In all cases, life-span data is indicated as mean ± standard error of the mean and *p* values were calculated using the log-rank (Mantel-Cox) method. To evaluate the impact of genes or treatments on life-span extension, the relative importance for each experimental setup was calculated based on the formula previously described (6).

### GFP Expression and Quantification

Eggs from gravid worms of the appropriate green fluorescent protein (GFP) reporter strains were collected and hatched overnight. L1 worms were grown monoxenically or axenically. For single-time point experiments, animals were harvested and tested at the first day

of adulthood. For the longitudinal experiments, worms were collected and analyzed at regular time intervals.

Fluorimetry assays were performed using black 96-well plates in a Victor2 plate reader (Perkin Elmer, Waltham, MA). Fluorescence was normalized to protein content (standard bicinchoninic acid method) of the worms recovered from the fluorimetry plate. Each experiment was repeated independently at least three times. Images were taken from at least 10 randomly picked worms from each culture condition, using a Olympus SZX12 fluorescence stereomicroscope. Only for longitudinal experiments, FUdR was added (100 and 50  $\mu$ M f.c. for FF and ADR, respectively) to prevent progeny production. Worms were paralyzed with 0.1 mg/ml levamisole (Sigma) for image capture.

## Results

### ADR Specifically Activates the Mitochondrial UPR

DR regimens, such as bacterial dilution and *eat-2* mutation, were shown to increase life span independently from the UPR<sup>mt</sup> response (12). Also, the DR mimetic metformin operates independently of this response (21). Moreover, dietary interventions that suppress the reduced life span of *phb-2* (RNAi) block UPR<sup>mt</sup> induction (25). These observations suggest that DR may not cause mitochondrial proteotoxic stress and its beneficial effect on life span is independent of the UPR<sup>mt</sup>. On the other hand, we found enhanced mitochondrial performance (8) and increased stress resistance (3) in ADR worms, hinting at enhanced proteostasis. Therefore, we reasoned that unfolded protein responses and in particular the UPR<sup>mt</sup> may underlie the life

span-doubling effect of ADR. The bloated mitochondrial morphology of ADR worms may also reflect UPR<sup>mt</sup> activation (Supplementary Figure 1) (26).

In order to test this hypothesis, worm strains, expressing specific reporters for UPR<sup>mt</sup> (*hsp-6p::gfp* and *hsp-60p::gfp*), UPR<sup>er</sup> (*hsp-4p::gfp*) and cytosolic UPR (*hsp-16.2p::gfp*) were subjected to ADR. The UPR<sup>mt</sup> response is clearly activated in ADR-treated worms compared to controls (Figure 1A and B), whereas the UPR<sup>er</sup> and cytosolic UPR reporter expression does not change upon exposure to axenic medium (Figure 1C and D). These observations show that ADR specifically activates the UPR<sup>mt</sup>.

### ADR-Dependent Activation of UPR<sup>mt</sup> Peaks Near Juvenile-to-Adult Transition

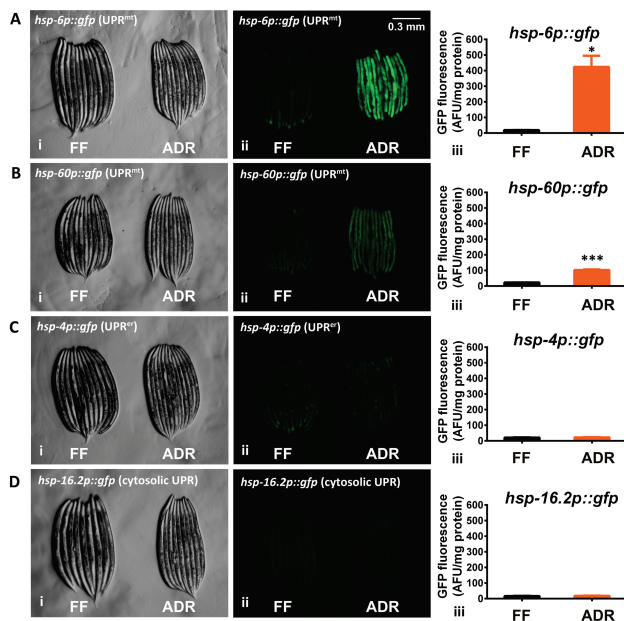
Life-span extension by RNAi knockdown of electron transport chain components has a specific temporal requirement during the L3/L4 stages of larval development (27,28), a period that coincides with strong mitochondrial proliferation (29). Furthermore, the UPR<sup>mt</sup> marker *hsp-6p::gfp* shows its greatest activation at the L4 stage when worms are challenged with mitochondrial stress (11), we speculated that the ADR-dependent activation of the UPR<sup>mt</sup> also has a distinct temporal pattern that may underlie the longevity phenotype.

To study this, *hsp-6p::gfp* worms were cultured in both axenic and monoxenic medium from embryo to young adulthood. Worms were imaged and GFP fluorescence was measured for each culture condition at regular time intervals. Animals in monoxenic culture only showed background levels of GFP fluorescence, whereas worms collected from axenic medium increasingly expressed GFP up to L4 (Figure 2A and B), the stage which corresponds to the increased mitochondrial density. After the L4 peak, the UPR<sup>mt</sup> response decreased in young adult worms and stabilized from the second day of adulthood at a level which was still significantly higher than that of the monoxenic controls. This level was maintained throughout life (Supplementary Figure 2), suggesting that ADR causes mitochondrial stress. Hence, activation of UPR<sup>mt</sup> along life might promote longevity and knockdown of its components should abolish ADR-mediated life-span extension.

### Activation of the UPR<sup>mt</sup> Is Not Required for Life-Span Extension by ADR

To assess whether induction of the UPR<sup>mt</sup> contributes to the strong longevity phenotype caused by ADR, we analyzed worm survival under FF and ADR conditions following RNAi knockdown of UPR<sup>mt</sup> pathway components.

We found that RNAi knockdown of *ubl-5* failed to prevent life-span extension by ADR, either treated from the L1 stage to early adulthood (Figure 3A; Supplementary Table 1) or during the first 5 days of adulthood (Figure 3B; Supplementary Table 1). Even a three-generation RNAi treatment failed to abolish life-span extension under ADR (Figure 3C; Supplementary Table 1). Although a minor reduction of ADR-induced longevity seems to occur in *ubl-5* RNAi-treated worms, the relative importance of this gene for ADR longevity is not significant when RNAi was performed during juvenile stages or over three generations. Only for young adult RNAi treatment, the 10% rescue effect on ADR-induced life-span extension reaches borderline significance (Supplementary Table 2). Hence, *ubl-5* seems not essential for life-span extension by ADR (Supplementary Tables 1 and 2). Inhibition of other UPR<sup>mt</sup>-related key factors by RNAi, such as *haf-1* and *hsp-6*, also has no or limited effect on worm life span (Figure 3D and E; Supplementary Table 1).



**Figure 1.** Axenic dietary restriction specifically induces UPR<sup>mt</sup>. The UPR<sup>mt</sup> reporter strains *hsp-6p::gfp* (A) and *hsp-60p::gfp* (B) show enhanced green fluorescent protein (GFP) expression under axenic dietary restriction (ADR) compared to fully-fed (FF) control. Strains carrying reporters for the UPR<sup>er</sup> *hsp-4p::gfp* (C) and cytosolic UPR *hsp-16.2p::gfp* (D) show no GFP induction when cultured in axenic medium. Panels (i) and (ii) show light and fluorescence images of the same worm clusters, respectively. Panels (iii) display microplate-based fluorimetric GFP quantification of worm populations, normalized to protein content. Error bars indicate standard error (standard error of the mean) of at least three independently grown replicates. \**p* < .05. \*\**p* < .01. \*\*\**p* < .001. Student *t* test was used for statistical analysis.



Again, the relative importance for each of these genes to the ADR-mediated longevity was not significant (Supplementary Table 2). Taken together, these observations provide primary evidence that induction of the UPR<sup>mt</sup> seems not to underlie the life-span extension caused by ADR.

Since UPR<sup>mt</sup> activation cannot be blocked by RNAi knockdown of *baf-1* (15) and inhibition of *hsp-6* can induce UPR<sup>mt</sup> (11), we next set out to analyze the relation between UPR<sup>mt</sup> and ADR-mediated longevity using the *atfs-1(tm4525)* mutation, which has been shown to completely prevent induction of the UPR<sup>mt</sup> (30). No effect on life span was observed in the *atfs-1(tm4525)* background, as deletion of *atfs-1* failed to significantly block the ADR-induced life-span extension (Figure 3F). Recently, constitutively active alleles of *atfs-1* have been described, which result in induction of the UPR<sup>mt</sup> in the absence of exogenous mitochondrial stress (31). To determine whether constitutive activation of the UPR<sup>mt</sup> would further extend life span in ADR, we performed survival assays of strains carrying either the *atfs-1(et17)* or *atfs-1(et18)* gain-of-function alleles. Counter to expectation, the life span of *atfs-1(et17)* was slightly reduced under ADR, indicating that overactivity of UPR<sup>mt</sup> tends to decrease the life span-extending properties of ADR (Figure 3G, Supplementary Table 1). Similar results were obtained for *atfs-1(et18)* (Figure 3H).

In order to further confirm our results, as an alternative approach to investigate the importance of the UPR<sup>mt</sup> for ADR longevity, we avoided early UPR<sup>mt</sup> induction by culturing juvenile worms on monoxenic plates. Once they reached adulthood, half of the population was switched to axenic medium, whereas the control worms were transferred to fresh monoxenic plates and life span was assessed. In a reverse setup, juveniles were grown under ADR, allowing UPR<sup>mt</sup> induction, and switched upon adulthood to monoxenic plates or fresh axenic cultures for the controls (Figure 4A).

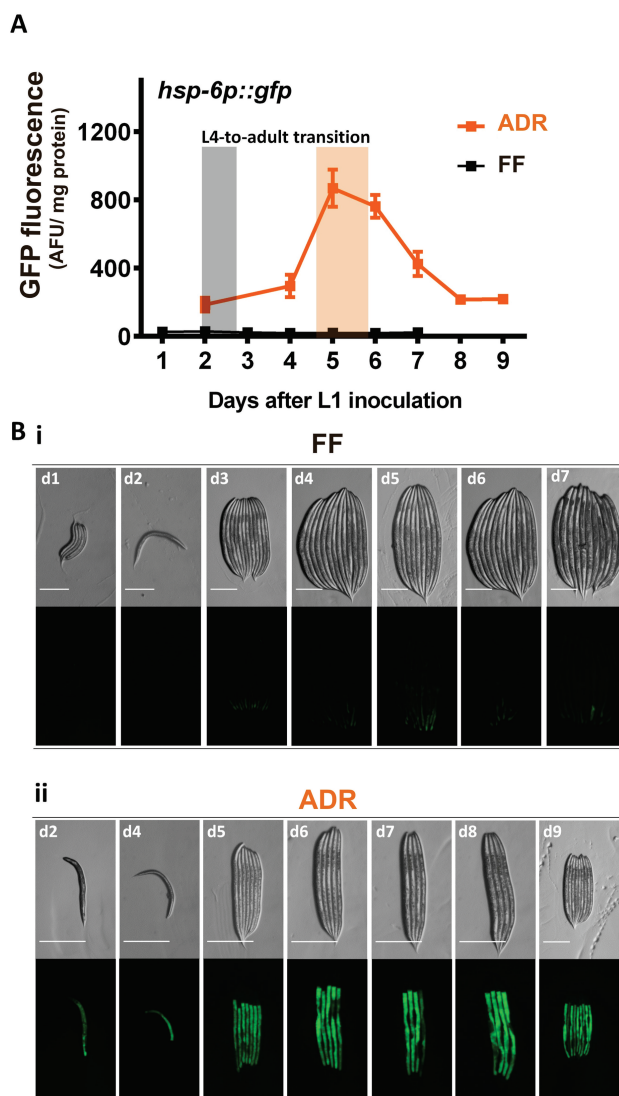
Interestingly, no obvious GFP expression was observed in animals grown monoxenically and subsequently transferred to axenic medium when reaching adulthood (Figure 4B), clearly demonstrating that the UPR<sup>mt</sup> is not activated in adults experiencing ADR. However, these worms still exhibit full life-span extension, comparable to those cultured axenically from L1 stage (Figure 4C). Besides, GFP expression gradually disappears once worms are transferred from axenic to monoxenic culture, indicating inactivation of UPR<sup>mt</sup> and alleviation of mitochondrial stress (Figure 4B). Despite their history in axenic medium and UPR<sup>mt</sup> induction during larval development by ADR is not sufficient to confer increased life span.

Overall, we conclude that UPR<sup>mt</sup> activation by ADR is specific and life stage-dependent but ADR-mediated longevity can be uncoupled from this stress response.

### Mitohormesis Is Not Involved in the Life-Span Extension by ADR

There is a growing body of research emphasizing the importance of ROS as crucial cell signaling molecules rather than damaging agents (18,32,33). Our previous study indicated that young worms, exposed to ADR, show no increase in in vitro ROS production capacity as well as in vivo H<sub>2</sub>O<sub>2</sub> levels compared to monoxenically grown controls (8), arguing against a role of mitohormesis in ADR longevity. However, it is possible that low or local levels of ROS may act as signaling molecules and potentially serve as the mitokine or intermediary signal to elicit a nuclear response (12).

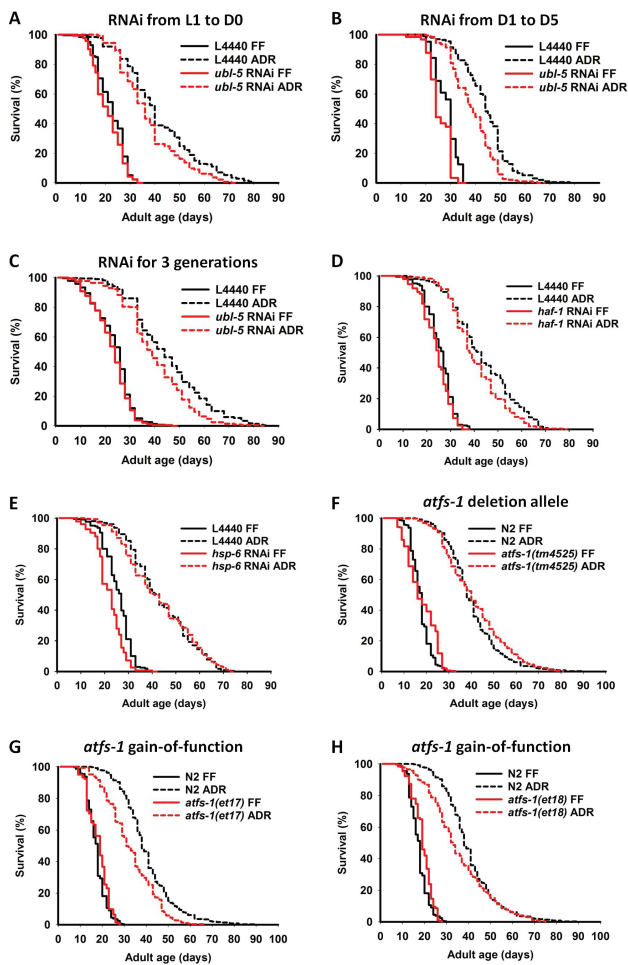
In order to further study whether a mitohormetic response contributes to the twofold life-span extension under ADR, worms



**Figure 2.** ADR-dependent activation of UPR<sup>mt</sup> peaks near juvenile-to-adult transition. Expression of the UPR<sup>mt</sup> reporter *hsp-6p::gfp* was followed over time by collecting and analyzing worms regularly as indicated. (A) Microplate-based fluorimetric GFP quantification of worm populations, normalized to protein content. (B) Panels (i) and (ii) show light and fluorescence images of the same worm clusters from monoxenic culture and axenic culture, respectively. The days after L1 inoculation in each condition is indicated up-left. Error bars indicate standard error (standard error of the mean) of at least three independently grown replicates. \**p* < .05. \*\**p* < .01. \*\*\**p* < .001. Scale bar: 0.3 mm. ADR = axenic dietary restriction; FF = fully fed; GFP = green fluorescent protein.

were exposed to ROS scavengers in axenic medium. Life-long treatment with the antioxidants N-acetyl-l-cysteine (NAC, 5 mM) or BHA (25 μM) did not block ADR-induced life-span extension. However, a slight life-span reduction, of which the relative importance was not significant, was found in ADR animals treated with antioxidants (Figure 5A and B; Supplementary Tables 1 and 2). When antioxidant levels were doubled, still no clear effect was found for BHA (Figure 5D; Supplementary Tables 1 and 2). However, 10 mM NAC had opposite effects on life span in monoxenic and axenic conditions. Although this high dose of NAC extends life span in monoxenically cultured worms with 17%, it slightly shortens life span with 11% under ADR. In such case,





**Figure 3.** Inactivation of the UPR<sup>mt</sup> pathway by RNAi knockdown does not prevent ADR-induced life-span extension. (A) Effect of *ubl-5* RNAi knockdown during worm development (L1 stage to first day of adulthood) on worm longevity under FF and ADR conditions. (B) Same as in (A) but *ubl-5* knockdown was performed during the first 5 days of adulthood. (C) Same as in (A) with RNAi knockdown of *ubl-5* for three generations. (D, E) Effect of *haf-1* (D) and *hsp-6* (E) knockdown during the first 5 days of adulthood on worm longevity under FF and ADR conditions. (F) Effect of the *atfs-1* deletion allele *tm4525* on life span in FF and axenic (ADR) condition. (G, H) Effect of constitutively active alleles of *atfs-1(et17)* and *atfs-1(et18)* on life span in FF and axenic (ADR) condition. All data are summarized in Supplementary Tables 1 and 2. ADR = axenic dietary restriction; FF = fully fed.

it significantly decreased the life-span extension ( $p = .016$ ) and the relative importance of 10 mM NAC treatment was 49.5% (Figure 5C; Supplementary Tables 1 and 2). Hence, where NAC treatment is beneficial to worms in bacterial culture, it seems harmful for worms under ADR.

NAC and BHA treatments did not interact with the UPR<sup>mt</sup> response as treatment of the axenically grown UPR<sup>mt</sup> reporter strain *hsp-6b::gfp* with NAC (5 mM) and BHA (25  $\mu$ M) did not decrease reporter fluorescence (Supplementary Figure 3).

In *C. elegans*, the peroxiredoxin PRDX-2 is involved in the pro-longevity signal of the mitohormetic response (21). We did not observe life-span reduction in *prdx-2* mutants compared to controls in axenic culture (Figure 5E; Supplementary Tables 1 and 2). Previously, we had shown that ADR-mediated longevity is independent of other players in the mitohormetic response such as DAF-16,

HSF-1, and SKN-1 and it is only partially dependent of AAK-2 (6). Therefore, it is unlikely that ROS-mediated mitohormesis plays an important role in the life span–doubling effect by ADR in *C. elegans*.

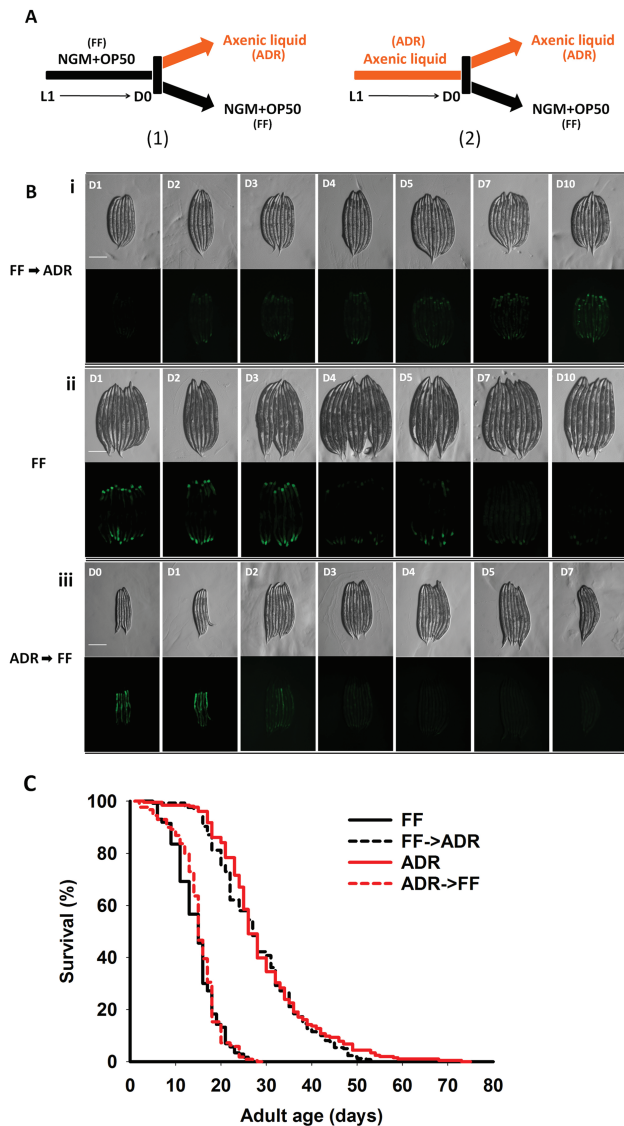
## Discussion

Chemically defined axenic medium was established several decades ago, in a search for nematode nutritional requirements (5,34). An easy-to-prepare alternative, used in this study, is the undefined axenic medium based on a mixture of soy peptone and yeast extract, supplemented with heam (3). Axenically cultured worms show typical morphological features of DR and their life span is often doubled, making it the largest life-span increase of any DR method reported (2–4). A recent genetic screen for key regulators of ADR-mediated longevity showed that the Insulin/IGF pathway and oxidative stress response pathways are not involved in this remarkable phenotype (3,6). Mitochondria are often considered as the major culprits behind the aging process (17), yet their density and coupling were shown to be increased under ADR (8). We wondered whether mitochondrial stress responses such as the UPR<sup>mt</sup> and mitohormesis are required for ADR longevity.

In this study, we provided evidence that ADR, unlike other DR regimens, specifically activates the UPR<sup>mt</sup> while cytosolic and ER unfolded protein responses are unaffected. This suggests that axenic medium causes some sort of specific mitochondrial perturbation. In *C. elegans*, the UPR<sup>mt</sup> can be triggered by ETC disturbance, by disrupted mitochondrial protein homeostasis or by mitonuclear protein imbalance (9,12–15). More recent studies indicate that both chromatin remodeling and histone modification are critical for the activation of UPR<sup>mt</sup> and its role for mitochondrial perturbation-mediated longevity (35,36).

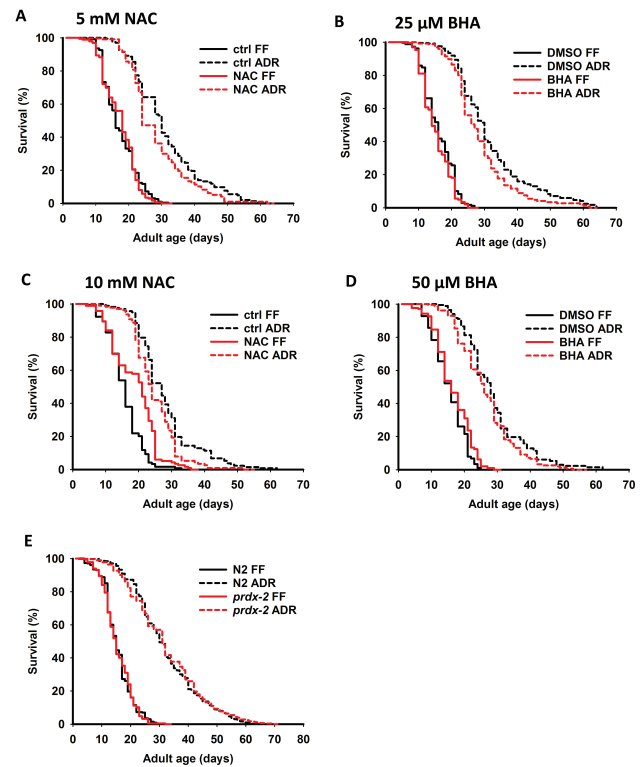
Although UPR<sup>mt</sup> induction in axenic medium can be observed over the entire life span of the worms, it specifically peaked near L4-to-adult transition. This coincides with the period of rapid expansion of the mitochondrial compartment during development (29). It may seem plausible that the limited nutritional uptake under ADR cannot support the late juvenile mitochondrial expansion which in turn may trigger the UPR<sup>mt</sup> response. However, this scenario is unlikely as ADR worms have increased mitochondrial density, coupling and respiration (3,8). The link between juvenile UPR<sup>mt</sup> and longevity has also been found in worms suffering mitochondrial lesions such as knockdown of ETC subunits (12,13), although general causality has been questioned (15). Hence, an important question is whether juvenile UPR<sup>mt</sup> provokes the ADR longevity phenotype. A simple medium switch experiment showed that this is not the case: juveniles raised in axenic medium, showing induction of the UPR<sup>mt</sup>, were not long lived when switched to monoxenic medium at adulthood. On the other hand, worms that spent their juvenile stages on monoxenic plates and were transferred to axenic medium at adulthood displayed the full ADR longevity phenotype. Therefore, juvenile UPR<sup>mt</sup> induction and life-span extension can be uncoupled in ADR-treated worms.

Although UPR<sup>mt</sup> peaks at L4-to-adult transition in ADR worms, it is still activated during adulthood as well and may support ADR longevity at that stage. This assumption was tested by assessing life span of ADR worms in which key players of the UPR<sup>mt</sup>, such as *ubl-5*, *haf-1*, *hsp-6*, and *atfs-1* (30,37) were knocked down or mutated. Worms unable to induce the UPR<sup>mt</sup> were still long lived under ADR conditions, showing that the ADR longevity phenotype does not rely on UPR<sup>mt</sup> at any life stage. As homozygous mutants of *ubl-5*, *haf-1*, and *hsp-6* are not viable, the role of these UPR<sup>mt</sup> genes had to be assessed by RNAi knockdown.



**Figure 4.** Activation of the UPR<sup>mt</sup> can be uncoupled from life-span extension by ADR. (A) Experimental setup: (1) hatched L1s were grown on solid nematode growth medium (NGM) plates seeded with *Escherichia coli* OP50 as fully-fed (FF) condition till young adulthood after which they were switched to either liquid axenic medium (ADR) or fresh *E. coli* plates (2); after microbleaching, hatched L1s were cultured in axenic medium until young adulthood. Afterwards, animals were transferred to fresh axenic medium or NGM plates seeded with *E. coli* OP50. (B) Images of worm clusters, grown according to the experimental setup in A. i: juveniles grown monoxenically and transferred to axenic medium at the first day of adulthood, ii: monoxenically grown worms, and iii: juveniles grown in axenic medium and transferred to monoxenic culture at the first day of adulthood. Top row: worms light image, bottom row: the same clusters imaged with fluorescence microscopy. The days of adult age are indicated in the upper left corners of the light images. (C) Survival of worms cultured according to experimental setup (1) and (2). Data are summarized in Supplementary Tables 1 and 2. Scale bar: 0.3 mm.

As there is currently no efficient sterile RNAi soaking procedure available, genes were knocked down by bacterial feeding during the first 5 days of adulthood, with the intention to knock down the UPR<sup>mt</sup>, after which worms were switched back to axenic medium with antibiotics. However, it must be noted that bacterial feeding

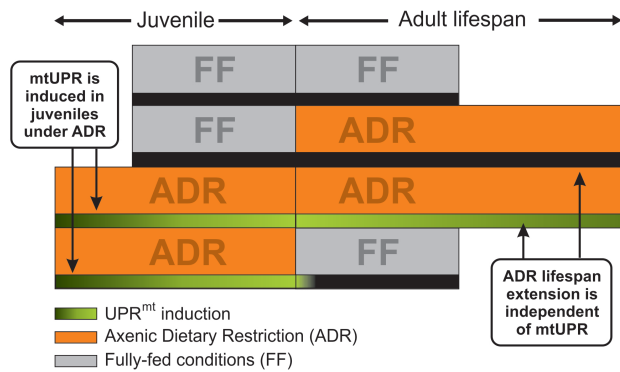


**Figure 5.** Mitohormesis is not involved in life-span extension by ADR. Survival analysis of worms treated with and without 5 mM NAC (A), 25 μM BHA (B), 10 mM NAC (C), and 50 μM BHA (D) under fully-fed (FF, full line) and axenic dietary restriction (ADR, dashed line) conditions. (E) Effect of *prdx-2* mutation on life span under both FF and ADR conditions. Survival data are summarized in Supplementary Tables 1 and 2. ADR = axenic dietary restriction; BHA = butylated hydroxyanisole; DMSO = dimethyl sulfoxide; FF = fully fed; NAC = N-acetyl-L-cysteine.

by itself is sufficient to inhibit UPR<sup>mt</sup> (Figure 4B) and thus may act redundantly to genetic UPR<sup>mt</sup> knockdown. Nevertheless, this leaves the conclusion unaltered: UPR<sup>mt</sup> shutdown does not inhibit ADR longevity.

In testing the homozygous *atfs-1* mutants, there were no bacterial feeding issues. The knockout allele *tm4525*, deleting almost the entire exons 2 to 4 and causing lack of UPR<sup>mt</sup> induction under *spg-7* knockdown (38), did not influence ADR longevity. Also, two *atfs-1* gain-of-function alleles failed to extend ADR life span. These data corroborate the finding that the UPR<sup>mt</sup>-related transcription factor ATF5-1 is not required for life-span extension in *C. elegans* (15,30).

Our observations also raise the question whether axenic culture is stressing for larvae yet harmless for adult worms. It is commonly observed that worm development under ADR is slow (about 6 or more days compared to 3 days in monoxenic cultures), which may be result of dietary challenges experienced by the larvae. However, development in axenic medium can be restored to about 3–5 days by adding skimmed milk to the cultures, a treatment that does not quench the UPR<sup>mt</sup> response nor does it abolish life-span extension (data not shown). Hence, UPR<sup>mt</sup> activation is not related to slow development but rather to other physiological adaptations under ADR. It remains to be discovered whether the absence or relative overabundance of specific compounds in the axenic medium leads to UPR<sup>mt</sup> induction or increased life span. Likely, the absence of a putative nondiffusable heat-labile *E. coli* factor is responsible for ADR longevity (39).



**Figure 6.** The relation between UPR<sup>mt</sup> activation and dietary regimen in juvenile and adult stages of *Caenorhabditis elegans*. ADR = axenic dietary restriction; FF = fully fed.

Next, we investigated whether mitohormesis is involved in ADR longevity, referring to the concept that an adaptive and protective antioxidant response is activated in mitochondria by moderate levels of ROS (33). This protective response may increase life span and can operate independently of the UPR<sup>mt</sup> (21). By treating worms with the ROS scavengers NAC or BHA, the mitohormesis effect can be blocked efficiently (18,22). We found that antioxidant treatment did not consistently abrogate ADR longevity, suggesting the absence of a clear mitohormetic effect. This is further supported by the fact that DAF-16 and SKN-1, two transcription factors that act in the mitohormesis pathway are not involved in ADR longevity, as was shown in our earlier studies (3,6). Furthermore, PRDX-2, known to mediate the beneficial effect of metformin treatment by activating mitohormesis (21), does not play a role in ADR longevity. Other genes involved in mitohormesis, such as *pmk-1*, contribute to the longevity of *C. elegans* through their essential role in innate immunity against bacterial infection in the intestine (19). However, in axenic culture conditions, bacterial infections at late life stage are excluded because of sterility of the medium.

Interestingly, we found that 10 mM of NAC is beneficial to worms under monoxenic conditions (17% life-span increase), an effect that was not observed before (40), whereas this treatment is slightly toxic under axenic conditions. Dietary supplements of antioxidants were previously reported to have positive effects on longevity, whereas other studies reported controversial results (32). Catalase and superoxide dismutase activities are strongly upregulated in axenically cultured worms (3). Adding high levels of antioxidants to such conditions may cause reductive stress and mild life-span shortening.

## Conclusion

We provide solid evidence that, under ADR, the UPR<sup>mt</sup> is activated in *C. elegans* larvae but this induction is not responsible for the extreme life-span extension (Figure 6). We further show that ROS-mediated mitohormesis is not required for this longevity phenotype, either. Despite clear improvements in mitochondrial function and changes in mitochondrial morphology in axenically cultured worms (7,8), UPR<sup>mt</sup> and mitohormesis do not seem to be involved in its life span-doubling effect. This leaves the dietary treatment with the largest life-span effect still the least characterized.

## Supplementary Material

Supplementary data are available at *The Journals of Gerontology, Series A: Biological Sciences and Medical Sciences* online.

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## Conflict of Interest

The authors declare no conflict of interest.

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