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Effects of 5'-Fluoro-2-deoxyuridine on Mitochondrial Biology in *Caenorhabditis elegans*

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Abstract

5-Fluoro-2'-deoxyuridine (FUdR) is a DNA synthesis inhibitor commonly used to sterilize *Caenorhabditis elegans* in order to maintain a synchronized aging population of nematodes, without contamination by their progeny, in lifespan experiments. All somatic cells in the adult nematode are post-mitotic and therefore do not require nuclear DNA synthesis. However, mitochondrial DNA (mtDNA) replicates independently of the cell cycle and thus represents a potential target for FUdR toxicity. Inhibition of mtDNA synthesis can lead to mtDNA depletion, which is linked to a number of diseases in humans. Furthermore, alterations in mitochondrial biology can affect lifespan in *C. elegans*. We characterized the effects of FUdR exposure on mtDNA and nuclear DNA (nucDNA) copy numbers, DNA damage, steady state ATP levels, nematode size, mitochondrial morphology, and lifespan in the germ line deficient JK1 107 *glp-1(q244)* and PE255 *glp-4(bn2)* strains. Lifespan was increased very slightly by 25 μ M FUdR, but was reduced by 400 μ M. Both concentrations reduced mtDNA and nucDNA copy numbers, but did not change their ratio. There was no effect of FUdR on mitochondrial morphology. Although both concentrations of FUdR resulted in smaller sized animals, changes to steady-state ATP levels were either not detected or restricted to the higher dose and/or later timepoints, depending on the method employed and strain tested. Finally, we determined the half-life of mtDNA in somatic cells of adult *C. elegans* to be between 8 and 13 days; this long half-life very likely explains the small or undetectable impact of FUdR on mitochondrial endpoints in our experiments. We discuss the relative pitfalls associated with using FUdR and germline deficient mutant strains as tools for the experimental elimination of progeny.

Keywords

mitochondrial DNA; mitochondrial DNA half-life; mitochondrial toxicity; FUdR; copy number; lifespan; aging; *Caenorhabditis elegans*

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Introduction

Caenorhabditis elegans is a free-living nematode found largely in decaying organic matter such as leaf litter [1]. It is widely used as a model organism in studies of aging, due mainly to this organism's short lifespan of approximately 2–3 weeks, as well as its completely sequenced and annotated genome [2], the availability of mutant strains, and the ease of gene knockdown via RNA interference [3]. *C. elegans* has played a role in the discovery of a number of cellular pathways that influence aging, including IGF/insulin signaling [4], dietary restriction [5], and inhibition of mitochondrial respiration [6, 7].

Hermaphroditic nematodes normally lay 200–300 eggs, which upon hatching are difficult to separate from an age-synchronized adult population. To overcome this, it is common practice to add 5-fluoro-2'-deoxyuridine (FUDR) to the nematode growth media [8]. FUDR is a DNA synthesis inhibitor that acts by inhibiting the enzyme thymidylate synthase. FUDR and its metabolites can also be incorporated into RNA, replacing uracil and affecting RNA synthesis [9]. There is evidence that FUDR causes chromosomal breaks specifically during S-phase of the cell cycle [10]. *C. elegans* undergo a finite number of cell divisions during larval development; therefore, after reaching maturity, DNA synthesis is not required for survival, though its inhibition effectively sterilizes nematodes [8]. Early studies demonstrated that exposure to FUDR from the L4 larval stage (the final larval stage) or later results in a small decrease in nematode length, and an increase in lifespan of 39% in axenic medium and 7% in monoxenic medium [8]. Since that early report, there have been conflicting results reporting that FUDR exposure either has no effect on lifespan [11, 12] or that it can extend lifespan, particularly in specific mutant backgrounds [13, 14]. FUDR also significantly alters the metabolomic profile of *C. elegans*, and may do so differently in different mutant backgrounds [15].

While the effects of FUDR on nuclear DNA (nucDNA) have been relatively well studied, its effects on mitochondrial DNA (mtDNA) have not. Mitochondria are responsible for producing the majority of cellular ATP and their proper function is critical to cellular and organismal health [16]. Mitochondria contain multiple copies of their own genomes which encode approximately 13 (depending on the species) proteins that are all essential for the process of oxidative phosphorylation [17]. The importance of mtDNA integrity has recently been more fully appreciated, as mutations, deletions, and depletion of mtDNA have all been linked to human disease [18–20]. Importantly, mtDNA replication occurs independently of the cell cycle [21], raising the concern that FUDR inhibition of DNA synthesis may interfere with normal mtDNA replication even after nematodes have completed their final somatic cell divisions. Interestingly, the original report on the use of FUDR in *C. elegans* [8] included observations of decreased pharyngeal pumping and slow movement, which could both result from decreased energy availability. Other studies in *C. elegans* have identified abnormalities in FUDR-treated nematodes [12, 22], and research in other organisms has also suggested that FUDR has mitochondrial toxicity [23–25]. While it has been argued that the effects of FUDR on nucDNA replication are greater than the effects on mtDNA replication [26], it is unclear whether this would be true in postmitotic tissues. Potential FUDR-mediated mitochondrial toxicity is of concern for lifespan studies because inhibition of mitochondrial respiration during larval development extends nematode lifespan [6, 7].

These experiments were designed to test the hypothesis that FUDR exposure inhibits mtDNA replication and mitochondrial function. We investigated impacts of FUDR on mitochondrial function, including mtDNA and nucDNA copy numbers, DNA damage levels, steady state ATP levels, and mitochondrial morphology in response to FUDR exposure. To place our experiments in the context of other reports on the effects of FUDR on lifespan, we also measured lifespan. FUDR is typically applied to worm cultures at the L4 larvae or young adult stages of development. In order to err on the side of detecting any potential phenotypes, we exposed nematodes to the highest and lowest commonly used FUDR concentrations (25 μ M and 400 μ M) slightly earlier in development, starting at the L3/L4 transition.

Methods

Strains and culture conditions

Populations of *C. elegans* were maintained on K agar plates seeded with OP50 *E. coli*. The wild-type (N2), JK1107 *glp-1(q224)*, and SJ4103 strains were obtained from the Caenorhabditis Genetics Center (CGC, University of Minnesota). The PE255 *glp-4(bn2)* strain was generously provided by Cristina Lagido, University of Aberdeen (Aberdeen, UK). All strains were maintained at 15°C prior to experiments. Synchronized L1 populations were obtained by bleach-sodium hydroxide isolation of eggs followed by overnight hatch in liquid K-medium at 20°C with shaking [27]. Synchronized L1 larvae were transferred to K agar plates seeded with OP50 and incubated at 25°C for approximately 24 hours prior to being transferred to K agar plates containing 0 μ M, 25 μ M or 400 μ M FUDR. Worms were washed and transferred to fresh plates daily to reduce the potential for bacterial contamination. Nematode samples were removed at 4, 8 and 12 days post FUDR exposure for experimental analysis.

Genome Copy Number Analysis

mtDNA and nucDNA copy numbers were measured before (day 0) and 4, 8 and 12 days post FUDR exposure, in both JK1107 *glp-1(q244)* and PE255 *glp-4(bn2)* strains as previously described [28]. Briefly, 6 worms were transferred to 90 μ L proteinase K-containing lysis buffer using a platinum worm pick, and lysed and digested by freezing at -80°C followed by thawing, and incubation at 65°C for one hour. Crude worm lysate was used as template DNA for real-time PCR based determination of mtDNA and nucDNA copy numbers. A plasmid-based standard curve for mtDNA is employed, allowing for the determination of absolute mtDNA copy number [28]. 3 samples per treatment per time point were measured in triplicate PCR reactions and averaged. 3 individual experiments were performed.

DNA Damage Analysis

mtDNA and nucDNA damage levels were measured in wild type N2 nematodes at both 15°C and 20°C by the quantitative PCR (QPCR) method, essentially as previously described [29, 30]. Nematodes were synchronized and grown to L4, and were then transferred to K-agar plates containing 0 μ M, 25 μ M or 400 μ M FUDR. 3 samples of 6 worms each were picked at days 4 and 8 of treatment for analysis. 2 individual experiments were performed.

Steady State ATP Level Analysis

Steady state ATP levels were determined in two strains, by two different methods.

First, using both the JK1107 *glp-1(q244)* and PE255 *glp-4(bn2)* strains of nematodes, ATP levels were determined as described in [31]. Briefly, approximately 500 worms were washed and frozen at -80°C in 100 μL of K-medium. Samples were removed from the freezer and 200 μL 10% trichloroacetic acid (TCA) was added while samples were still frozen, and allowed to thaw on ice. 0.5mm diameter zirconia beads were added to each sample (approx. 250 μL), and two 30 second pulses at maximum speed in a Bullet Blender (Next Advance, Averill Park, NY) were performed to lyse the worms. Lysates were neutralized by the addition of 100 μL of 1.33 mM KHCO_3 and 100 μL Sigma water (St. Louis, MO, USA). 50 μL -150 μL aliquots were removed at this time for total protein determination. Samples were vacuum centrifuged for 10 minutes to remove bubbles, and then centrifuged at 14,000 rpm for 8 minutes at 4°C to pellet protein. The ATP containing supernatants were removed and transferred to sterile tubes. ATP was measured from 1:50 and 1:100 dilutions of these samples using the Molecular Probes ATP determination Kit (Invitrogen/Life Technologies, Carlsbad, CA, USA). Luminescence was measured every 2 minutes for 30 minutes after the addition of the luciferin/luciferase reagent using a FLUOstar Optima plate reader (BMG Labtech, Offenburg, Germany) equipped with a luminescence optic. ATP concentrations were determined by comparing luminescence values to an ATP standard curve measured at the same time points. The calculated ATP concentrations were then averaged over the 15 time points, and normalized to total protein concentrations as measured with the BCA method (Thermo Fisher Scientific, Rockford, IL.). One or two technical replicates (averaged) were performed in 3 separate experiments, resulting in a statistical n of 3.

Second, the firefly luciferase expressing PE255 *glp-4(bn2)* strain (only) was used to investigate relative, steady state ATP levels *in vivo*, in live nematodes, as previously described [32–34]. Worms were washed with K-medium, and approximately 100 worms in 100 μL K-medium were aliquoted into wells of a white 96 well plate. 4 to 5 technical replicates (wells) were averaged per treatment per time point. All measurements were made using a FLUOstar Optima microplate reader. First, GFP fluorescence was measured with an excitation wavelength of 485 nm and an emission wavelength of 520 nm. Then, luminescence was measured 3 minutes after the automated addition of luminescence buffer consisting of citrate-phosphate buffer (pH 6.5), 0.1 mM D-luciferin, 1% DMSO, and 0.05% Triton-X. Luminescence values were normalized to GFP fluorescence at each time point, as the transgene expressed in the PE255 *glp-1(bn2)* strain is a luciferase-GFP fusion, and consequently GFP fluorescence can be used to control for the amount of luciferase enzyme in each well. Three individual experiments were conducted.

Size Analysis

Nematode size was determined by light microscopy in both JK1107 *glp-1(q244)* and PE255 *glp-4(bn2)* strains. Small samples of worms were frozen in K-medium, thawed, and imaged at 10x magnification on a Zeiss Axioskop. Images were analyzed using NIS elements BR software (Nikon Inc. Melville, NY, USA). Length and total area of approximately 10

individual worms per treatment was determined. This was conducted twice for a total of approximately 20 individual worms per treatment.

Mitochondrial Morphology

Mitochondrial morphology was visualized at 6 days post FUDR exposure in the SJ4103 strain, which expresses a GFP transgene in body wall muscle cells that contains a mitochondrial matrix localization sequence. Worms were paralyzed in 10mM levamisole and mounted on 10% agar pads. GFP tagged mitochondria were imaged using a Zeiss LSM 510 upright confocal microscope.

Lifespan Assay

All assays were performed at 25 °C. Strain JK1107 *glp-1(q224)* nematodes were cultured as described above. Synchronized L1 larvae were placed on K agar plates seeded with OP50 bacteria and incubated at 25°C for 24 hours. 25 individuals were then transferred to K agar plates seeded with OP50 bacteria that contained 0µM, 25 µM or 400 µM FUDR. Nematodes were monitored daily and scored as dead when they failed to move in response to repeated probing. The lifespan of each individual was calculated from L1 to death. The data presented are for 50 individuals assayed in 2 individual experiments separated in time.

mtDNA Half-life Analysis

Strain JK1107 *glp-1(q224)* nematodes were synchronized as described above, grown for 48 hours at 25°C (approximately young adult stage), and were then transferred to K agar plates containing either 0 µg/mL or 5 µg/mL ethidium bromide (EtBr). mtDNA and nucDNA copy numbers were determined in 3 to 4 samples of 6 worms each at days 3, 6, 9 and 12 of treatment, as described above. 4 individual experiments were performed.

Results

Genome Copy Number and Damage Analysis

We hypothesized that inhibition of DNA synthesis by FUDR exposure would reduce mtDNA copy number in post mitotic *C. elegans* over time, as a result of FUDR-mediated inhibition of adult mtDNA replication. Of note, we carried out the majority of these experiments in germ cell-deficient nematodes, because the very number of mtDNAs produced associated with germ cell production [35] would likely obscure any ability to detect an effect of FUDR on mtDNA metabolism.

The nematode mitochondrial respiratory chain (MRC) consists of over 75 protein subunits, most of which are encoded in nuclear DNA and are then translocated to the mitochondria [36]. However, 12 [36] or 13 [37] of these subunits are encoded in the mtDNA, and proper stoichiometric balance between nuclear- and mitochondrial-encoded proteins is important for normal MRC function [38]. Furthermore, mtDNA depletion leads to disease [20, 39]. Thus, proper maintenance of mtDNA copy number is critical to organismal health. FUDR and other DNA synthesis inhibitors have been shown to block replication of mtDNA and lead to reductions in mtDNA copy number. Ethidium bromide, a DNA intercalating agent that blocks mtDNA replication, leads to a reduction in mtDNA copy number over time in

exposed nematodes [35], and FUDR reduces mtDNA content and cytochrome C oxidase expression in cultured lymphoblasts [23].

mtDNA copy number per worm (fig. 1a) was significantly reduced in the JK1107 *glp-1(q244)* worms by FUDR treatment as analyzed by two way ANOVA (significant main effects of time ($p < 0.0001$) and treatment ($p < 0.0005$) but not their interaction ($p = 0.057$)). However, nucDNA copy number per worm (fig. 1b) was also altered by FUDR exposure (main effects of time ($p < 0.0001$), treatment ($p < 0.0001$) and their interaction ($p < 0.005$) are all significant). The resulting mtDNA / nucDNA copy number ratio (fig. 1c) was not affected by FUDR treatment, although there was a significant decrease in mtDNA / nucDNA ratio over time ($p < 0.0001$). Thus, the observed reduction in mtDNA copy number per worm can be attributed to the reduction in nucDNA copy number, as this ratio is unchanged. An age-related decline in nucDNA copy number in a germline deficient strain (*glp-4*) of *C. elegans* has previously been reported [40].

The approximate expected number of nucDNA copies in germ cell-deficient adult *C. elegans* is 3134 (959 2n somatic cells, 34 32n intestinal cells, and 98 4n hypodermis cells) [40, 41]. Our FUDR treated worms, however, only achieve slightly over 2000 nucDNA copies. As observed previously by other researcher using a germline deficient (*glp-4*) strain [40], our control (0 μ M FUDR) worms never reach the expected number of nucDNA copies (or potentially do so before day 4 which would not be detected given our experimental design). It is not possible from our data to determine the mechanism of the FUDR-induced decrease nucDNA copy number; we speculate that it may be a result of fewer somatic cells in the FUDR treated groups, a reduction in the endoreduplication that occurs in the intestinal and hypodermal cells, or a combination of the two.

The lack of a difference in mtDNA:nucDNA ratio after FUDR could be explained in two ways. It is possible that the same number of mtDNAs are present per cell, and there are simply less cells in the FUDR treated groups. Or, if some of the difference in nucDNA copy number is attributable to a lack of endoreduplication, mtDNA copy number per cell may in fact be reduced by FUDR treatment.

Copy number determination experiments were also performed using the PE255 *glp-4(bn2)* strain and again FUDR decreased both mtDNA and nucDNA copy numbers, but did not alter their ratio. However, in this strain, the dynamics of mtDNA copy number with age were different (irrespective of FUDR treatment). mtDNA copy number per worm (fig S1 a) increased throughout the length of the experiment in control and 25 μ M FUDR treated worms, and only decreased slightly at day 12 in the 400 μ M treatment group. The main effects of time ($p < 0.0001$) and treatment ($p < 0.0001$) as well as their interaction ($p < 0.001$) were all significant. nucDNA copy number (fig S1b) showed a similar trend with controls increasing throughout, 25 μ M treated decreased slightly from days 4 to 8 but increased again at day 12, and the 400 μ M group plateaued at roughly 1500 copies at day 4 and remained unchanged thereafter. Main effects of time ($p < 0.0001$) and treatment ($p < 0.0001$), and their interaction ($p < 0.0001$) were all significant. This resulted in an mtDNA / nucDNA ratio that increased slightly over the 12 day exposure for all treatments ($p < 0.0001$), but was unaltered by FUDR treatment. Thus, the trends in copy numbers were different than what was seen in

the JK1107 *glp-1(q244)* strain, and while we do not have an explanation for the differences seen between the strains, we have seen similar trends in copy number in the PE255 *glp-4(bn2)* strain in other experiments (unpublished). We have also seen differences in gene expression between wild-type N2 worms and the PE255 N2 (wild type background) strain in response to UVC treatment that inhibits mtDNA replication during development [42].

Finally, we tested for DNA damage in both genomes of wild type (N2 Bristol strain) nematodes, grown at both 15°C and 20°C, at 4 and 8 days of exposure to 0, 25, or 400 µM FUDR. We did not detect any damage (Table S1). The limit of detection of this assay is approximately 1 lesion/10⁵ nucleotides [29].

Steady State ATP Levels

Next, we investigated whether FUDR exposure would alter ATP levels in post-mitotic, adult *C. elegans*. If mtDNA copy number were in fact reduced by FUDR treatment, or if FUDR affected RNA or protein synthesis in the mitochondria [23], it is possible that MRC function could be altered, leading to reductions in steady state ATP levels.

ATP levels were measured in JK1 107 *glp-1(q244)* mutants at 4 and 8 days post FUDR treatment. Unfortunately, too few worms survived until day 12 to obtain reliable measurements of ATP levels. ATP levels per unit protein (fig 2) were unaffected by all FUDR concentrations at both days 4 and 8 post treatment. Interestingly, we observed a very slight decrease in ATP levels per unit protein from day 4 to 8, whereas previous reports in N2 nematodes have shown decreases closer to 50% over that time [6].

ATP levels were also measured in the PE255 *glp-4(bn2)* *C. elegans* strain, and using this method, the results were different. The PE255 transgenic strain expresses a luciferase-GFP fusion protein, and relative ATP levels are measured as light output from live worm. Importantly, ATP levels are normalized to GFP levels as opposed to protein concentrations. ATP levels were slightly higher in 25 µM FUDR treated worms, and slightly lower in 400 µM treated worms at day 4. However, at days 8 and 12 ATP levels were significantly higher in the control worms than in either of the treatment groups (Fig. S2).

Lastly, we measured ATP levels in the PE255 *glp-4(bn2)* strain using the same method that was used with the JK1 107 *glp-1(q244)* strain, to determine if the differences in ATP level responses to FUDR were attributable to different biological responses between the strains or differences in the measurement techniques used. ATP levels (fig. S3) appear to decrease with FUDR treatment, and in fact, there is a significant main effect of treatment ($p < 0.005$). However, this effect is driven solely by the 400 µM FUDR exposed nematodes. These nematodes were much smaller compared to other treatments, and it was difficult to extract significant quantities of ATP and of total protein, making this data questionable. Without the 400 µM FUDR exposed nematodes, the significant main effect is lost ($p = 0.126$), though the trend towards lower ATP still appears.

Nematode Size

FUDR can cause a reduction in overall nematode length depending on when it is administered. For example, a dose of 400 µM reduced length by approximately 20% when

nearly mature nematodes were exposed [8]. We measured the length and area of both JK1 107 *glp-1(q244)* and PE255 *glp-4(bn2)* worms exposed to FUdR beginning at the L3/L4 transition (as described above) on days 4, 8 and 12 of treatment.

In the JK1 107 *glp-1(q244)* average nematode area was significantly reduced by FUdR (main effects of treatment ($p<0.0001$) and time ($p<0.0001$)), as was length (treatment ($p<0.0001$), time ($p<0.005$) and their interaction ($p<0.005$) were all significant) as shown in figure 3. The maximum reduction in area for the 25 μM dose was 33% and occurred on day 4, and for 400 μM was 68% occurring on day 12. Maximum reduction in length at 25 μM FUdR was 15% on days 4 and 8, and at 400 μM was 45% at day 12.

The PE255 *glp-4(bn2)* nematodes were also significantly smaller when exposed to FUdR (Figure S4). Area was significantly reduced (main effects of treatment ($p<0.0001$), time ($p<0.05$) and their interaction ($p<0.0001$), as was length (main effect of treatment ($p<0.0001$) and treatment by time interaction ($p<0.005$)). The maximum reduction in area at the 25 μM concentration was 65% on days 4 and 8, and at 400 μM was 77% on day 12. Length was maximally reduced at 25 μM FUdR by 33% on day 8, and at 400 μM by 49% on day 12.

Mitochondrial Morphology

Mitochondria are dynamic structures; their morphology responds to many types of damage through the processes of fusion and fission, and they can be selectively degraded through mitophagy [43–45]. We examined the mitochondrial morphology of the SJ4103 strain of *C. elegans*, which expresses a GFP protein with mitochondrial matrix localization sequence under the control of a body wall muscle cell promoter, after 8 days of exposure to 25 μM and 400 μM FUdR. There were no obvious differences in mitochondrial morphology in any worms examined (representative images can be found in figure S5).

Lifespan

Since the somewhat conflicting reports of the effects of FUdR on lifespan found in the literature suggest that experimental conditions may alter those effects, we tested the effects of FUdR on lifespan in our experimental conditions. We measured the lifespan of the germline proliferation defective JK1107 *glp-1(q244)* mutant strain of *C. elegans* in response to lifelong treatment with 0 μM (control), 25 μM and 400 μM FUdR beginning from the L3/L4 transition.

Compared to controls (0 μM FUdR), 400 μM FUdR resulted in a significant ($p<0.0001$) reduction in mean lifespan by 6 days. Conversely, 25 μM FUdR significantly ($p<0.0427$) increased mean lifespan by approximately 1 day. Lifespan data were statistically analyzed using the Mantel-Cox test (fig S6).

mtDNA Half-life Analysis

The lack of mtDNA depletion seen in response to FUdR treatment prompted us to measure the half-life of mtDNA, as a long half-life is one potential explanation for these results. To measure half-life, we transferred young adult JK1107 *glp-1(q244)* nematodes to K-agar

plates containing ethidium bromide (EtBr). EtBr is a DNA intercalating agent that blocks mtDNA replication by impeding the mtDNA polymerase, including in nematodes [35]. By measuring the decrease in mtDNA copy numbers in the presence of EtBr we can approximate the half-life of mitochondrial genomes in adult worms.

Based on the decrease in mtDNA copy number from days 3 to 12 in the presence of EtBr, we determined the mtDNA half-life to be 8.2 – 13.2 days (fig 4). Interestingly, we saw nearly the same rate of decline (half-life of 6.5 to 11.7 days) in untreated nematodes, however mtDNA copy number did not begin to decrease until day 6. Half-life was determined by linearizing the portion of the graph that showed a consistent decrease (day 3–12 for EtBr treated, and day 6–12 for control) by plotting the natural log (LN) of the mtDNA / nucDNA ratio vs. time (fig. S7). The slopes of these lines were used to calculate half-life (half-life = $\text{LN}(2)/\text{slope}$). The range of the estimates of half-life were made by plotting the steepest and least steep possible lines based on the SEM at the first and last points used in the plot.

Statistical analysis by ANCOVA did not reveal an interaction between treatment and day ($p=0.62$) indicating that there was not a statistically significant difference in the slopes of the control and EtBr treated lines, and therefore no significant difference in the half-life of mtDNA between treatments.

Discussion

We have investigated the impact of FUdR exposure, a DNA synthesis inhibitor commonly used to sterilize nematodes in aging and lifespan studies, on mitochondrial biology in *C. elegans*. We have also determined the half-life of mtDNA in adult germ cell-deficient nematodes.

Use of Germ Line Deficient Strains

Mutations in the *glp-1* gene cause germ cells to enter meiosis as opposed to mitosis, and terminally differentiate, resulting in sterility [46]. The exact function of the *glp-4* gene is less well characterized, however mutations also result in a temperature sensitive sterility phenotype. Nematodes with *glp-1(q244)* or *glp-4(bn2)* mutations are reproductively competent when maintained at 15°C, but are sterile at the restrictive temperature of 25°C [46–48]. We chose to perform the majority of the experiments in these strains to specifically remove the influence of germ cells on the results, as we are interested in the effects of FUdR on mitochondrial function in somatic cells. Nematodes that lack germ line stem cells live longer than those that contain them [49, 50], and while this would not be altered by FUdR exposure, there is evidence that lack of fertility can influence stress resistance [51, 52] and that FUdR alters metabolism [15]. Furthermore, mtDNA copy number in wild type nematodes increases dramatically during egg laying [35], and would therefore obscure any comparisons between untreated and FUdR treated wild type animals.

Copy number

There are two potential explanations for the observed lack of FUdR-mediated reduction in mtDNA/nucDNA copy numbers. The first is that mitochondrial dTTP pools are not depleted

by FUDR treatment, and mtDNA replication continues as normal. mtDNA replication rates in cultured mouse L-cells are less sensitive to inhibition by FUDR than are nucDNA replication rates [53]. Also, mtDNA precursor pools in cultured HeLa cells expand when treated with FUDR, while nuclear dNTP pools are depleted [54]. The second possibility is that the half-life of mitochondrial DNA in adult *C. elegans* is long enough that inhibition of mtDNA replication has little effect on copy number. This is supported by the relative lack of a decline in mtDNA copy number in adult nematodes in the presence of ethidium bromide [35]. A sharp decline was seen after nematodes exposed from the L3 stage laid their broods, but copy number then remained relatively constant during adulthood [35]. If mtDNA were turning over at an appreciable rate it would be expected that copy number would continue to decline. Supporting that observation, our own unpublished results also indicate that exposure to ethidium bromide in adult *glp-1* nematodes resulted in a maximal decrease of only 25% in mtDNA copy number throughout adulthood.

Nonetheless, an effect on mtDNA copy number in specific tissues or after exposure to mtDNA genotoxins remains an important concern. While the half-life of mtDNA is not well-studied, it has been investigated in rats and reported to be 6.7 days in heart, 9.4 days in liver, 10.4 days in kidney, and 31 days in brain [55]. Organellar half-life has been measured in rat livers as 3.8 days using radioactive labeling of proteins [56]. More recently, a proteomics approach has demonstrated that median half lives of mouse liver and cardiac mitochondrial proteins are 4.26 and 17.2 days, respectively, and that individual proteins turn over at different rates [57]. While these are not all direct measures of mtDNA turnover, they do suggest that turnover rates may differ by tissue type. It is also possible that mtDNA half-life is much shorter during development, or that turnover may be influenced by environmental factors that, for example, increase autophagy rates. mtDNA damaged by ultraviolet C radiation both induces autophagy and is removed slowly by mechanism that is dependent on it [34] and the mitochondrial DNA polymerase, pol γ , is upregulated 3.4 fold by UVC exposure in young adult *glp-1* nematodes [58]. Thus, FUDR exposure may have significant effects on mitochondrial biology in the context of mitochondrial or mtDNA damage.

ATP Levels

In the context of a lack of depletion of mtDNA copy number per cell, it is not surprising that ATP levels per unit protein in adult nematodes were not significantly affected by FUDR treatment. While mtDNA depletion is certainly not the only mechanism by which mitochondrial dysfunction can be induced, it appears to be the most likely way that FUDR would do so. There is some evidence that suggests FUDR can inhibit RNA synthesis [9]. If this were to occur in the mitochondria it would be possible that ATP production could be altered without mtDNA depletion. However, this seems less likely as potential mechanism, especially when considering the multiplicity of mitochondrial genomes per cell and the ability of individual mitochondria to transfer contents and functionally complement others [59]. Furthermore, our results do not support this possibility, since we do not observe altered steady state ATP levels in response to FUDR.

mtDNA Half-life

To investigate the possibility that a long mtDNA half-life is the explanation for a lack of FUdR induced mtDNA depletion, we measured the rate at which mtDNA decreased in the presence of EtBr. To our knowledge, this is the first reported measurement of mtDNA half-life in *C. elegans*, and at 8.2 – 13.2 days it is surprisingly similar to that measured in rats. This suggests that in adult nematodes, there is very little turnover of mtDNA. Furthermore, the rate of decline of mtDNA in untreated animals was the same as that in EtBr treated, although the decrease started slightly later in the life of the worm. This suggests that, after a certain point in the animal's life, mtDNA is degraded and is not replaced (i.e., mitochondrial biogenesis is halted). Interestingly, this data also implies that EtBr does not induce the degradation of mtDNA, even though it blocks replication. Importantly, these are whole animal measurements, and it is likely that degradation and turnover rates differ by cell or tissue type.

The potential ability of mtDNA damage to induce mtDNA degradation or turnover is an important question, as mtDNA depletion is linked to many human diseases [20]. Previously published work from our lab indicates that a 50 J/m² dose of Ultraviolet-C radiation (UVC) results in approximately 0.6 lesions per 10 Kb of mtDNA, that is reduced to approximately 0.4 lesions per 10 Kb over 72 hours [34]. Bulky DNA lesions such as those caused by UVC are not repaired in mtDNA, as the nucleotide excision repair pathway is not present in mitochondria [60, 61], they are however, slowly removed [34]. Based on these removal kinetics, the estimated half-life of UVC damaged mtDNA is approximately 5.1 days. While this is somewhat shorter than without damage, the difference is not striking, and suggests that bulky lesions in mtDNA may not dramatically induce mtDNA degradation.

Strain differences

Throughout our experiments we made note of a number of FUdR-dependent and -independent differences between the two experimental strains used, JK1 107 *glp-1(q244)* and PE255 *glp-4(bn2)*. The most striking of differences were copy number and ATP levels. While we do not have a good explanation for the copy number differences, it is worth noting that we have seen the same steady increase in both genome copy numbers over time in other work with the PE255 *glp-4(bn2)* strain (unpublished). Golden *et al.* have also observed large increases in nucDNA copy number with age in wild type nematodes due to the accumulation of masses of nucleic acids, though they reported substantially higher copy numbers than we report here [40]. Furthermore, the PE255 *glp-4(bn2)* nematodes have more mtDNA copies per nucDNA at day 0 than the JK1 107 *glp-1(q244)* nematodes do at their peak on day 4, and this ratio does not decrease with age regardless of treatment.

The difference in steady state ATP levels may be explained somewhat by the different normalizations used between the strains. ATP in the JK1 107 *glp-1(q244)* nematodes is normalized to total protein concentration in a sub-sample removed during the ATP preparation, and therefore accounts for animal size differences between samples quite well. ATP levels are reported per unit protein, not per animal. The PE255 *glp-4(bn2)* strain contains a luciferase-GFP fusion protein, and GFP fluorescence is used to normalize expression of the enzyme between samples. Measured ATP levels are relative as no standard

curve can be used, and are reported per animal. GFP normalization does account for some size difference, but in the case of large size differences may do so less well than total protein normalization. Furthermore, we have observed that GFP fluorescence levels decline with age at a greater rate than does size. When we extracted ATP from the PE255 *glp-4(bn2)* nematodes and used the same measurement and normalization methods as with JK1 107 *glp-1(q244)*, the FUDR effect on ATP levels became much less drastic. Our data do, however, still indicate a main effect of FUDR on steady state ATP levels in the PE255 *glp-4(bn2)* strain. It is possible that this effect was amplified by the GFP normalization process, which may not be easily applicable in aging worms. We believe that both methods have merit, but care should be taken when interpreting results and especially when comparing results across methods.

Lifespan

We found small but significant effects of FUDR treatment on the lifespan of JK1 107 *glp-1(q244)* nematodes. Interestingly, the lower FUDR concentration used extended lifespan, whereas the high concentration reduced it. However, previous research has shown that FUDR can have dramatic effects on lifespan in the contexts of genetic mutations. The normally short lived *gas-1* mutant lives twice as long on 100 μ M FUDR [14], and the lifespan of the *tub-1* mutant is also extended on FUDR [13]. Taken together, the research published to date suggests that FUDR has small effects on lifespan at most commonly used doses in a wildtype genetic background, but the large confounding effects in non-wildtype genetic backgrounds. Such “gene-environment interactions” are common in toxicology, and this observation raises concerns for employing FUDR in lifespan studies of mutant strains. Another concern is that FUDR may interact in unexpected ways with other chemicals (eg., antioxidants and lifespan-extending agents) and stressors, particularly those affecting mitochondria and mitochondrial DNA [62]. The fact that our lifespan results are generally in line with those previously published, however, suggests that our mitochondria-related results may also be generally applicable.

Conclusions

Overall, our data suggest that FUDR exposure has little effect on mtDNA:nucDNA copy number ratio or steady state ATP levels. This may be a result of the long half-life of mtDNA in adult nematodes, which we report here to be between 8.2 and 13.2 days. This is encouraging with respect to the use of FUDR in lifespan and other studies. The utilization of germline-deficient strains, an alternate approach for studies in which proliferating cells are a confounder including DNA repair studies [58, 63] involves difficulties as well since the presence of germ cells clearly affects lifespan, immunity, mitochondrial biology, and fat metabolism [50, 64–67] (and our own results discussed above). Nonetheless, caution is still warranted when utilizing FUDR, because strain background, dose of FUDR, timing of exposure, and co-exposure to other chemicals or stressors are critical and may influence results. Finally, our work also highlights the fact that different strains of *C. elegans* have substantially different mtDNA biology for reasons we do not yet understand.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

mtDNA	mitochondrial DNA
nucDNA	nuclear DNA
FuDR	5-fluoro-2'-deoxyuridine
MRC	mitochondrial respiratory chain

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Highlights

- We investigated the effects of FUdR on mitochondrial function and mitochondrial DNA
- FUdR did not alter mitochondrial/nuclear DNA ratio or cause detectable DNA damage
- FUdR did not alter steady state ATP levels
- FUdR reduced nematode size, mitochondrial DNA, and nuclear DNA copy numbers
- We measured mtDNA half-life in adult *C. elegans* to be between 8 and 13 days.

Effects of FUDR on mt and nuc DNA Copy Numbers in JK1107 *glp-1(q244)*

Figure 1.

FUDR reduces both mitochondrial and nuclear copy number per worm in JK1 107 *glp-1* (Q244) mutants, but does not alter mt/ nuc DNA ratio. (A) mtDNA copy number and (B) nucDNA copy number per worm at 0,4; 8 and 12 days after beginning FUDR exposure. (C) mtDNA / nucDNA ratio at 0,4,8 and 12 days of FUDR exposure. All data are means $\pm$ SEM from 3 separate experiments. Points with asterisks are significantly ($p < 0.05$) different from others by post-hoc Tukey's HSD test.

Steady State ATP Levels in JK1107 *glp-1(q244)*

Figure 2.

ATP levels are unchanged as a result of exposure to either 25 μ M or 400 μ M FUDR, ATP was measured with a luciferase based assay and normalized to total protein from a matched sample. Data are means $\pm$ SEM from 3 separate experiments.

Effects of FUdR on Area and Length in JK1107 *gtp-1(q244)*

Figure 3.

FUdR treatment reduces both area (**A**) and length (**B**) of JK1107 *gtp-1* (Q244) worms. Bars connected by asterisks are significantly different ($p < 0.05$) by post-hoc Tukey's HSD test. Comparisons at each time point were not permitted for area as there was not a significant interaction between time and treatment ($p = 0.26$).

Determination of mtDNA Half-life in JK1107 *glp-1(q244)*

Figure 4. mtDNA half-life was determined by measuring the rate of decrease of mtDNA in adult nematodes in the presence of ethidium bromide. (A) mtDNA copy number per worm, (B) nucDNA copy number per worm, and (C) mtDNA / nucDNA ratio. Based on the rate of decline of mtDNA / nucDNA, the mtDNA half-life was calculated to be between 8.2 and 13.2 days.