Resistance of mitochondrial DNA to chemical-induced germline mutations is independent of mitophagy in *C. elegans*

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ABSTRACT

Mitochondrial DNA (mtDNA) is prone to mutation in aging and over evolutionary time, yet the processes that regulate the accumulation of de novo mtDNA mutations and modulate mtDNA heteroplasmy are not fully elucidated. Mitochondria lack certain DNA repair processes, which could contribute to polymerase error-induced mutations and increase susceptibility to chemical-induced mtDNA mutagenesis. We conducted error-corrected, ultra-sensitive Duplex Sequencing to investigate the effects of two known nuclear genome mutagens, cadmium and Aflatoxin B₁, on germline mtDNA mutagenesis in *Caenorhabditis elegans*. After 1,750 generations, we detected 2,270 single nucleotide mutations. Heteroplasmy is pervasive in *C. elegans* and mtDNA mutagenesis is dominated by C:G → A:T mutations generally attributed to oxidative damage, yet there was no effect of either exposure on mtDNA mutation frequency, spectrum, or trinucleotide context signature. Mitophagy may play a role in eliminating mtDNA damage or deleterious mutations, and mitophagy-deficient mutants *pink-1* and *dct-1* accumulated significantly higher levels of mtDNA damage compared to wild-type *C. elegans* after exposures. However, there were only small differences in overall mutation frequency, spectrum, or trinucleotide context signature compared to wild-type after 3,050 generations, across all treatments. These findings suggest mitochondria harbor additional previously uncharacterized mechanisms that regulate mtDNA mutational processes across generations.

INTRODUCTION

Mitochondria are vital organelles that provide energy and important signaling molecules for almost every form of eukaryotic life on earth. Phylogenetic evidence strongly suggests that mitochondria share ancestry with alpha-proteobacteria and arose by endosymbiosis. Over the course of 1.45 billion years of coevolution, most genes encoding mitochondrial proteins have translocated into the nuclear genome. Nevertheless, mitochondria still maintain a unique genome that encodes proteins essential for mitochondrial function.

mtDNA mutations are implicated in diseases affecting at least one person in 5,000 (1), in addition to potential roles in many neurological and metabolic disorders, various cancers, and aging (2–4). MtDNA mutation rates are often 100 times higher than nuclear DNA mutation rates in aging and over evolutionary time (5–7). Currently, replication errors by the sole mitochondrial DNA polymerase, Pol γ, rather than oxidative stress, are thought to be the main source of mtDNA mutations (8–10). Pol γ contains 3’-5’-exonuclease and 5’-deoxyribose lyase activities which allows for proofreading and base excision repair during mtDNA replication (11). Pol γ has limited translesion synthesis capabilities, potentially rendering mtDNA sensitive to damage-induced mutations or copy number changes due to polymerase stalling at bulky adducts (12). Mitochondria have efficient base excision
repair mechanisms, but lack nucleotide excision repair and mismatch repair machinery, which may also contribute to higher mutation rates (13).

Chemical exposure can contribute to nuclear DNA mutagenesis, but few studies have investigated the role of genotoxicants in mtDNA mutagenesis. Evidence is limited regarding the effects of exogenous stress on mtDNA damage and somatic mutagenesis (14), and to our knowledge, the effects of chemical exposure on germline mtDNA mutagenesis have yet to be investigated. This is critical to understand because the basic biology of mitochondria renders mtDNA particularly susceptible to the harmful effects of environmental toxicants and stress (15–17).

Though mitochondria are limited in DNA repair capacity, organelle dynamics play a significant role in eliminating irreparable damage and maintaining mitochondrial homeostasis. The importance of mitochondrial fission, fusion, and mitophagy in regulating mitochondrial quality are highlighted through manifestation of disease phenotypes from mutations in nuclear encoded genes (18). For example, mutations in nuclear mitophagy genes PARK2 and PINK1 are associated with Parkinson’s disease. Mitochondrial dynamics are also important for mediating response to mitochondrial stress, whether metabolic or chemical, as we have reviewed in the context of chemical stress (19). A damaged organelle can fuse with a healthy organelle, and subsequently undergo fission to create healthy daughter mitochondria. Alternatively, damaged mitochondria may also be selectively targeted for degradation via mitophagy. We have previously demonstrated that genetic disruption of these pathways in *C. elegans* results in increased sensitivity to various stressors and inability to remove ultraviolet C radiation-induced mtDNA damage (20–23). Until this study, variation in accumulation of mtDNA damage associated with mitophagy function after chemical exposure had not been investigated.

Mitophagy is also thought to regulate the selective inheritance of mitochondrial genomes in the germline, where mtDNA is, in most metazoans, uniparentally inherited. Despite the high mtDNA copy number in oocytes, only a few mtDNA genome copies are distributed into primordial germ cells, an event that is referred to as the germline mitochondrial “bottleneck” (24). Evolutionary models predict that the bottleneck increases the probability of removal of deleterious mtDNA variants (25). Whether transmission of mutant mitochondrial genomes is stochastic, permitting genetic drift, or targeted via purifying selection, is controversial (26–29).

Previous studies in *C. elegans* have demonstrated that mitophagy can play an important role in regulating the germline transmission of mtDNA variants. For example, progeny of *C. elegans* that lack functional Parkin (*pdr-1*) accumulate higher frequencies of the 3.1kb *uaDf5* mtDNA deletion compared to wild-type (30). However, the role of mitophagy in regulating the transmission of *de novo* single nucleotide mutations through the germline remains an exciting area of investigation, particularly in the context of exposure to chemicals that cause mtDNA damage. A recent study by Haroon *et al.* suggests that mitophagy may play a role in mtDNA single nucleotide mutagenesis, as *C. elegans* deficient in *pdr-1* accumulate higher frequencies of mtDNA point mutations than wild-type, though only in a Pol γ exonuclease mutant background (31). Therefore, we hypothesized that exposure to mtDNA-damaging chemicals would result in higher rates of mtDNA mutation accumulation, and that this would be exacerbated in mitophagy-deficient *C. elegans*. 
C. elegans is a well-established organism in which fundamental knowledge of spontaneous mtDNA mutational processes and other evolutionary insights (including genotoxin-induced nuclear mutation studies) has been achieved through classical mutation accumulation line experiments (32). Two independent mutation accumulation line (MA) experiments have been conducted in wild-type C. elegans, though only Konrad et al. conducted next-generation Illumina sequencing (7, 33). A third MA experiment was conducted in the C. elegans mutant, gas-1, which renders complex I of the ETC dysfunctional, thereby resulting in increased reactive oxygen species production and reduced ATP production (34). However, no other MA approach has investigated the effect of either chemical stressors or mitophagy on mtDNA mutagenesis in C. elegans.

A limitation of previous MA studies in C. elegans is that only a few mtDNA variants that arose to high frequencies or fixation were detected, with one very recent exception (35). However, it is now known that most mtDNA variants are not only heteroplasmic, but exist at very low frequencies that are lower than the error rate of standard next-generation Illumina sequencing assays. Therefore, we used a novel sequencing approach, Duplex Sequencing, that permits accurate detection of mtDNA variants at frequencies as low as one mutation in ~10^8 base pairs (36). Using this ultra-sensitive, mtDNA-targeted sequencing approach, we were able to detect more heteroplasmic mtDNA mutations at lower frequencies than ever before reported in C. elegans, and also investigate the mutational rates, spectrum, context, and genomic sites of mutagenesis after 50 generations of mutation accumulation.

To investigate the effects of chemical stress and the role of mitophagy in the origin and transmission of mtDNA mutagenesis in C. elegans, we performed mutation accumulation experiments in wild-type and two mitophagy mutant strains, pink-1 and dct-1, under various environmental conditions. All three strains were bottlenecked for 50 generations in control conditions, in addition to chronic exposure for 50 generations to the heavy metal pollutant, cadmium (Cd, in the form of cadmium chloride, CdCl_2) or the mycotoxin aflatoxin B_1 (AfB_1), both known nuclear mutagens and human carcinogens. Cd inhibits many DNA repair enzymes and interferes with antioxidant enzymes, resulting in increased levels of ROS that can damage mtDNA (37, 38). AfB_1 metabolites form bulky DNA adducts, which can inhibit replication and result in somatic mutations in the nuclear genome (39). The accumulation of AfB_1 in mitochondria, in addition to lack of nucleotide excision repair (NER), results in higher levels and greater persistence of mtDNA lesions compared to nuclear DNA damage, as we and others have previously measured (40–42). Therefore, we used these two toxicants as models to investigate how two different mechanisms of mtDNA damage may affect mtDNA mutational processes.

Duplex Sequencing of C. elegans mtDNA revealed a strong signature of oxidative damage. Exposure to levels of CdCl_2 and AfB_1 that caused mtDNA damage did not increase the frequency of mtDNA single nucleotide mutations (SNMs) in wild-type C. elegans. Surprisingly, inhibiting mitophagy did not result in an increase in mtDNA mutations in control conditions or after exposure to CdCl_2 or AfB_1. These results suggest that the mitochondrial genome harbors robust mechanisms of avoiding chemical-induced mtDNA mutagenesis that are independent of mitophagy.

MATERIAL AND METHODS
**C. elegans** strains and maintenance

This work used the *C. elegans* wild-type Bristol N2 strain and two mitophagy deficient strains, *dct-1* and *pink-1*. The *dct-1*(tm376) mutant harbors a 912 bp deletion in the promoter region of *dct-1* (DAF-16/FOXO Controlled, germline Tumour affecting-1, putative orthologue to mammalian mitophagy receptor, BNIP3) and has been characterized previously (43). The *pink-1*(1779) mutant harbors a 350 bp deletion in *pink-1* (PTEN-induced kinase 1) and exhibits altered mitochondrial morphology (Luz 2015). These deletion strains were acquired from the National Bioresource Project (Tokyo, Japan), genotyped, and backcrossed into N2 six times (*dct-1*) and eight times (*pink-1*) prior to any experiments. All *C. elegans* were maintained following standard procedures (44). We replaced the potassium phosphate in traditional nematode growth medium (NMG plates) with KCl ("K-agar plates") in order to prevent buffering which reduces the bioavailability of CdCl$_2$ (45, 46). All strains were maintained at 20°C on OP50 *E. coli*.

**CdCl$_2$ and AfB$_1$ treatment**

Stocks of CdCl$_2$ and AfB$_1$ (Sigma-Aldrich, St. Louis, MO) were made in ddH$_2$O and DMSO vehicles, respectively, and stored at 4°C. Treatment plates were always made fresh: OP50 was spiked with either CdCl$_2$ and AfB$_1$ immediately prior to seeding, and plates were prepared two days prior to experiments. 100µL of OP50 was seeded on 6cm plates (containing 8mL of K-Agar) and 300µL of OP50 on 10cm plates (containing 20mL K-agar).

**Mitochondrial morphology**

To assess effects of exposure on mitochondrial morphology, wild-type *C. elegans* harboring an extrachromosomal array P*myo-3::GFP*; which expresses GFP in the mitochondrial matrix of body wall muscle cells, were exposed to 10µM and 50µM of CdCl$_2$ and 2µM and 10µM AfB$_1$. Images were taken on a Keyence BZ-X700, and analyzed in ImageJ using the Blinder software (47). Blinded images were analyzed qualitatively by the established classification system as previously described (48), with Class 1 indicating highly networked, fused mitochondrial morphology, and Class 5 indicating extremely fragmented mitochondrial morphology. Two experimental replicates were conducted, and the total number of individuals analyzed is displayed above each stacked bar plot in Supplementary Figure S5. Statistical differences in the distribution of classes of mitochondrial morphology were determined by Fisher's Exact Test followed by Bonferroni correction for multiple comparisons.

**Mitochondrial respiration**

Age-synchronized L1 *C. elegans* were transferred to plates seeded with OP50 containing 50µM and 100µM CdCl$_2$, or 10µM and 25µM AfB$_1$. Approximately 48 hours later, L4 *C. elegans* were washed off the plates and respiration parameters were quantified using the Seahorse Extracellular Flux Bioanalyzer, as previously described (49). The number of individual nematodes per well were counted, such that the Oxygen Consumption Rate (OCR) measurements were normalized per worm. The mean of technical replicates per plate was then determined. Two to six independent experiments
were conducted per strain per treatment. Two-way ANOVAs were run for each chemical in order to compare Cd to control and AfB1 to control across three strains.

Life history trait analyses

Growth was measured in three independent experiments in the ancestral (G0) wild-type, dct-1, and pink-1 strains on control and treatment plates. About 300 age-synchronized L1s were plated on each 10cm plate with the following conditions: control, 50, 200, 1000µM CdCl2, and 10, 50, and 200µM AfB1. After 48 hours, L4 C. elegans were washed off the treatment plates and transferred to unseeded plates. Images of each plate were then captured with a Keyence BZ-X700 using brightfield, and analyzed in ImageJ with the Fiji plug-in WormSizer (51). Length data were normalized to the mean control length of each strain for each biological replicate. Box and whisker plots of length distributions were plotted in R and the means of biological replicates were used for statistical analysis (2-way ANOVA, Tukey’s HSD post-hoc analysis). Total brood size was also determined in all three strains on control, 50µM CdCl2 and 10µM AfB1 plates (N = 9-10 individuals per strain per treatment).

On day 1 of adulthood, each individual worm was transferred to a new 6cm plate with the respective treatment until reproduction ceased. Plates containing offspring were stored at 20°C for two days, and then counted.

Population growth rate was measured on all MA lines at G0 and G50 to measure the effects of mutation accumulation on fitness. Population growth rate was determined by an eating race experiment as originally described by Hodgkin and Barnes (52). Each plate was monitored hourly up to the time when all bacteria had been consumed and the population dispersed.

As an additional fitness measurement, total reproduction and lifespan were also determined on a subset of 20 MA lines per strain per treatment at G50. The 20 MA lines were determined with a random number generator. Of these 20 sublines, three L4 individuals (G50 sisters) were picked onto individual control plates. Founders were transferred to a new plate daily until cessation of egg-laying, and total brood was counted as described above. Biological replicates were then averaged per MA line. Individuals that were sterile were included in our analysis. Lifespan was determined by scoring individuals every day until they stopped responding to touch. Reproduction and lifespan results were censored if the animal was lost, dehydrated, or bagged. This only occurred in four of the 180 MA lines.

mtDNA copy number and damage

OP50 was spiked with either CdCl2 or AfB1, such that the final concentrations of chemicals in the bacterial lawn were 50µM of CdCl2 and 10µM of AfB1. Plates were seeded with 100µL of each treatment, and control, and allowed to dry for two days. 20-30 gravid adults of each strain, wild-type, pink-1, and dct-1, were then picked onto each treatment plate. Adults were left on each plate for a 2-hour egg-lay, after which all of the adults were removed from the plate. After 56 hours, synchronized L4 C. elegans were then analyzed for mtDNA CN and damage, as described (53). Two to three biological replicates of six individual L4 C. elegans per strain per treatment were picked into 90µL of lysis buffer, placed at -80°C for at least overnight, then lysed at 65°C for one hour, followed by 95°C
for 15 minutes to inactivate Proteinase K. Long-amplicon PCR was then performed (53), with one modification that the final reaction volume was halved (25µL instead of 50µL). mtDNA CN was measured using a plasmid-based standard curve and real-time PCR as described (54). Long-amplicon PCR products were normalized to mtDNA copy number and mtDNA lesions were calculated relative to the control within each strain. Each biological sample was run in duplicate or triplicate. This experiment was performed twice. A two-way ANOVA followed by Tukey’s HSD post-hoc analysis was used to determine statistical differences in mtDNA lesions between strains and treatment. Results of mtDNA lesions following exposure to additional concentrations of CdCl₂ and AfB₁ that were not used in the mutation accumulation line experiment are included in Supplementary Materials (Supplementary Figure S1). The effects of exposure on mtDNA CN are described in Supplementary Figure S2.

Mutation Accumulation Lines

Our criteria to determine a single concentration of CdCl₂ and AfB₁ to use for mutagenesis experiments were: 1) the concentration caused significant mtDNA damage, but 2) did not have significant effects on organismal fitness (growth, fecundity, and mitochondrial toxicity). Once we determined a concentration for each exposure, we performed a mutation accumulation line experiment in wild-type, pink-1, and dct-1 C. elegans, in control conditions, 50µM CdCl₂, or 10µM AfB₁ (Figure 2). One random young adult individual of each strain was isolated (G0) and allowed to reproduce. Once the offspring reached gravid adulthood, about 20 individuals were picked onto a plate. After a 2-hour egg lay, the adults were picked off the plate. When these offspring reached L4, one individual (G1) was randomly picked to an individual plate to begin the mutation accumulation experiment. The MA experiment began with 50 individual lines per strain per treatment. All lines were maintained at 20°C. Every four days, one individual L4 was randomly picked and transferred to a new plate to propagate the next generation. This population bottlenecking was conducted for 50 generations per strain per treatment, resulting in a total of 2,500 generations of mutation accumulation per strain per treatment. The previous generation was always maintained at 15°C, such that if an individual was sterile or dead, a back-up “sister” individual could be transferred to a new plate to continue the MA line. As in previous C. elegans MA experiments, if three attempts were not viable, the line was then considered extinct (55). At the end of the experiment, the average percentage of extinctions per strain per treatment was 4.5% (Supplementary Figure S3). All 450 MA lines were cryopreserved every 5 generations for 50 generations.

DNA isolation, Duplex Sequencing library preparation, and sequencing

After 50 generations of mutation accumulation, an individual L4 from each MA line was transferred onto a control 6cm plate. As has been previously described in other MA experiments, as soon as all of the OP50 had been consumed and the population was composed largely of synchronized L1s, the C. elegans were immediately washed off of the plate with ddH₂O, transferred to a 1.7mL Eppendorf tube, pelleted, and flash frozen in liquid nitrogen (33–35). Total DNA was isolated with the DNeasy Blood & Tissue kit (Qiagen, Germany) following the manufacturer’s instructions. DNA
quantity and purity was analyzed using a Thermo Scientific™ NanoDrop™ One Microvolume UV-Vis Spectrophotometer (Thermo Fisher Scientific) and Qubit 2.0 (Perkin Elmer Victor x2).

Illumina Sequencing libraries were prepared following the original Duplex Sequencing (DS) protocol with slight modifications (56, 57). An input of 50 ng of total DNA was used for library preparation. Total DNA was sheared (Covaris E210), followed by preparation and repair of DNA fragments using the NEBNext Ultra II Library prep kit, in which 2µL of 15µM of the unique DS adapters were added directly to the Ultra II Ligation Master Mix, according to manufacturer’s instructions (New England Biolabs). Relative mtDNA copy number of the post-adapter ligated sample was then determined via RT-qPCR (Applied biosystems StepOnePlus). This critical step determined the amount of post-ligated DNA to use as input for the pre-capture indexing PCR to optimize the number of Duplex Consensus Sequences formed for each Unique Molecular Identifier (57). The optimal family size was determined by earlier sequencing of a C. elegans wild-type sample compared to a known reference standard in order to determine volume of post-adapter-ligated for pre-capture indexing PCR. On average, 0.338µL of template was required (range of 0.164 – 0.632). After the pre-capture indexing-PCR, the entire product was lyophilized with the addition of uniquely designed blocking oligos. An enrichment of the C. elegans mitochondrial genome was then performed following the IDT xGen Hybridization Capture of DNA libraries for NGS target enrichment protocol with a custom designed Discovery Pool probe panel that covered 13,091bp of the 13,991bp C. elegans mitochondrial genome, capturing 93.5% of the genome. The AT-rich, highly repetitive non-coding region of the genome was excluded in order to minimize sequencing fragments that would not accurately map to the reference genome.

Post-capture PCR and clean-up were then performed using according to manufacturer’s instructions. Libraries were sequenced on an Illumina NovaSeq 6000 platform to obtain 150bp paired-end reads (20 million per library). The DS adapter sequences, qPCR and Illumina primer sequences, blocking oligo sequences, and the C. elegans mitochondrial genome custom probe panel oligonucleotide sequences are provided in the Supplementary Data File.

Bioinformatics processing and analysis

Sequencing data were processed on the Duke Computer Cluster using the custom Duplex-Seq Pipeline (v1.1.4) workflow developed by the Kennedy Lab (github.com/Kennedy-Lab-UW/Duplex-Seq-Pipeline) and described in detail in Supplementary Figure 3 of Kennedy et al. (56) and again in Sanchez-Contreras et al. (10). Only reads mapping to the C. elegans mitochondrial reference genome were analyzed, and only single nucleotide mutations were analyzed, while insertions and deletions were ignored. Possible artifacts were removed by 12-bp end clipping at the beginning of each read. A minimum of three reads were required to call a variant, with a minimum heteroplasmia cutoff of 70% per Duplex Consensus Sequence (DCS) compared to the reference genome. Other input parameters are included in the Configuration File (Supplementary Data File). A summary output file of mutations, sequencing depth, and genome coverage per library is included (Supplementary Data File). Sequencing data is uploaded and can be accessed at SRA SRP350474 (PRJNA787252). We detected three polymorphisms that were completely fixed in our C. elegans strains compared to the...
reference genome (NC_001328.1), two of which have been previously detected (35). These polymorphisms were not included in our analysis and are listed in Supplementary Table S1.

**Mutation frequency and spectrum analysis**

Each mutation was only called once at each genomic position. Overall mutation frequency was calculated for each library with the following equation: \([\text{total number of unique mutations}] / [\text{total number of error-corrected nucleotides sequenced}]\). For mutational signature calculations, the total number of each type of the six possible transition and transversion mutations was divided by the coverage of the reference nucleotides sequenced (i.e., \([\text{total # C:G} \rightarrow A:T \text{ mutations}] / [\text{total number of cytosines + guanines sequenced}]\)). Mean mutation frequencies were calculated for each strain and treatment, followed by parametric statistical analysis and appropriate multiple comparisons corrections, as described in Results.

**Trinucleotide context mutational signature**

Trinucleotide context mutations were calculated using the Bioconductor Package *MutationalPatterns* (v1.1.0) (58) after forging a *C. elegans* mitochondrial reference genome (*BSgenome* v1.58.0). The number of mutations in each of the 96 possible trinucleotide contexts were determined in reference to pyrimidines C and T, hence six possible mutation types instead of 12. The number of each trinucleotide context mutation was normalized to the average number of wild-type control mutations in each context. A Welch Two Sample T-test was performed on a specific mutation only after a significant ANOVA was determined.

**Synonymous and nonsynonymous mutation analysis**

The dNdS ratio was calculated in order to determine if mtDNA evolution departed from neutrality in our MA experimental design. We used the *R* package *dNdScv*, a statistical modeling approach that allows for normalization to depth of coverage as a covariate, and incorporation of the invertebrate mitochondrial genetic code (translation table 5) (59).

**Statistical analysis**

All statistical analysis and data visualization were conducted in RStudio (v1.1.463; *R* v4.0.3). Values are conveyed as means and standard error, unless otherwise indicated. We performed Welch Two Sample T-test to determine significance between two groups, and ANOVA (one way if more than two groups and two way if multiple factors, as indicated in further detail in the figure legends) followed by Tukey’s HSD to correct for multiple comparisons. Fisher’s Exact Test was performed to determine variation in the proportion of each type of mutation (Supplementary Figure S4).

**RESULTS**

CdCl₂ and AFB₁ exposure causes mtDNA damage in wild-type *C. elegans*
We have previously shown that exposure to CdCl₂ and AfB₁ results in significantly higher levels of mtDNA damage than nuclear DNA damage in wild-type *C. elegans* (40). In order to determine a single dose of CdCl₂ and AfB₁ to use for mutation accumulation lines and sequencing where there was detectable mtDNA damage but no evident effect on fitness, we first conducted a dose response and quantified mtDNA damage (Supplementary Figure S1), followed by measuring growth, reproduction, mitochondrial morphology, and mitochondrial respiration. Exposure to 50µM CdCl₂ induced 0.92 lesions/10kb, and 10µM AfB₁ induced 0.25 lesions/10kb relative to control in wild-type *C. elegans* (*P* < 0.01, Figure 1A). These concentrations of CdCl₂ and AfB₁ did not result in statistically significant growth delay (8.3% and 6.3% growth inhibition, respectively, (Figure 1B) or fecundity (Figure 1C)). We also observed little effect on mitochondrial morphology (Supplementary Figure S5) and no decrease in mitochondrial function (Supplementary Figure S6). Therefore, these concentrations were used for the mutation accumulation experiments in order to avoid any effects of fitness on mutation rate calculations.

**Wild-type *C. elegans* exhibit resistance to mtDNA damage-induced single nucleotide mutations**

The strong increase in mtDNA damage suggested that mutations should be correspondingly increased. To test this hypothesis, we conducted Duplex Sequencing after 50 generations of mutation accumulation to determine the frequency and spectrum of mtDNA single nucleotide mutations on wild-type *C. elegans* in control and genotoxicant-treated conditions. To verify the Duplex Sequencing method in *C. elegans* (which had not previously been conducted before, prior to the recent work by Waneka et al. (35)) we first optimized the protocol in wild-type *C. elegans* after exposure to 7 J/m² ultraviolet C radiation, where we observed a 1.6-fold increase in mtDNA SNMs compared to a control sample (4.89 versus 3.07 x 10⁻⁵ mutations per base pair) (Supplemental Figure S7). This confirmed that Duplex Sequencing is not only feasible in *C. elegans*, but has the sensitivity to detect effects of mtDNA damage on mutagenesis.

After confirming the utility of Duplex Sequencing to detect rare variants and exposure differences in *C. elegans*, we sequenced 11 wild-type MA lines and identified a total of 760 SNMs (an average of 70 mutations/MA line) under control conditions. The overall mtDNA mutation frequency of wild-type *C. elegans* was 10.1 x 10⁻⁷ SNMs/bp. Despite significant mtDNA lesions (Figure 1A), there was no effect of CdCl₂ (14 MA lines, 874 total SNMs) or AfB₁ (10 MA lines, 636 total SNMs) on the overall mtDNA SNM frequency in wild-type *C. elegans* (9.75 x 10⁻⁷ and 9.47 x 10⁻⁷, respectively) (Figure 3A, Supplementary Table S2). The mutation spectrum of *C. elegans* was dominated by C:G → A:T and C:G → G:C transition mutations, and C:G → T:A mutations. There was no effect of CdCl₂ or AfB₁ -induced mtDNA damage on mutation spectrum (Figure 3B; Welch Two Sample T-test). Wild-type *C. elegans* exhibited no strand asymmetry in mtDNA mutation accumulation in control conditions (Figure 3C, top panel). There was no strand asymmetry after exposure to CdCl₂ (Figure 3C, middle panel). We did observe a trend of a slightly higher frequency of C → T over G → A transversions after exposure to AfB₁ compared to control conditions (P = 0.08; two-way ANOVA) (Figure 3C, bottom panel).

**Mitophagy deficiency exacerbates mtDNA damage but not point mutations**
Given the surprising finding that exposure to well-known mutagens, despite the increase in damage, did not result in mtDNA mutations, we hypothesized that mitophagy may be working to remove the damaged mitochondria before having a chance to form mutations. To test this hypothesis, we conducted a similar MA exposure experiment in mitophagy deficient strains. We chose dct-1 and pink-1 deficient strains because dct-1 and pink-1 (BNIP-3 and PINK1 human homologs) are involved in two independent mitophagy pathways in C. elegans. Mitophagy-deficient dct-1 mutants did not accumulate significant mtDNA lesions compared to control after exposure to CdCl2 (0.21 lesions/10kb), but did after exposure to AfB1 (1.33 lesions/10kb, \( P < 0.01 \); two-way ANOVA, Tukey HSD) (Figure 4A). pink-1 mutants accumulated significantly higher levels of mtDNA damage after exposure to CdCl2 and AfB1 respectively compared to control (1.03 and 0.72 lesions/10kb, \( P < 0.01 \); two-way ANOVA, Tukey HSD) (Figure 4A). Neither dct-1 or pink-1 mutants accumulated higher levels of mtDNA damage after exposure to CdCl2 compared to wild-type (two-way ANOVA). dct-1 mutants did accumulate significantly higher levels of mtDNA damage compared to wild-type after exposure to AfB1 (\( P < 0.05 \); two-way ANOVA, Tukey HSD) (Figure 4A). pink-1 mutants may accumulate higher levels of mtDNA damage compared to wild-type after AfB1 exposure; this trended towards, but did not reach, significance (\( P = 0.08 \); two-way ANOVA, Tukey HSD) (Figure 4A).

We sequenced dct-1 and pink-1 MA lines after 50 generations of mutation accumulation in control, CdCl2, and AfB1-treated conditions. A total of 491, 727, and 574 total SNMs were detected after sequencing 11 control, 11 CdCl2, and 9 AfB1 dct-1 MA lines, respectively. A total of 530, 696, and 752 total SNMs were detected after sequencing 9 control, 11 CdCl2, and 10 AfB1 pink-1 MA lines, respectively. There was no effect of mitophagy deficiency or exposure on overall mtDNA mutation frequency (Supplementary Table S2, Supplementary Figure S8; two-way ANOVA, \( P = 0.3 \)). The mutational spectrum of dct-1 and pink-1 was also dominated by C:G \( \rightarrow \) A:T and C:G \( \rightarrow \) G:C transitions, and C:G \( \rightarrow \) T:A transversions (Figure 4B). There was no effect of CdCl2 or AfB1 on the mutational spectrum in either dct-1 or pink-1 mutants (two-way ANOVAs).

**Trinucleotide context mutagenesis**

Mammalian mtDNA has been reported to have a distinctive mutational signature (60). Therefore, we analyzed the identity of the 5’ and 3’ neighboring nucleotides to investigate possible enrichment of SNMs in specific sequence contexts. Overall, the C. elegans mitochondrial genome does have a distinct trinucleotide mutational signature (Supplementary Figure S9). Specifically, C:G \( \rightarrow \) T:A mutations occur in a G[C\( \rightarrow \)T]C context, while C:G \( \rightarrow \) A:T mutations occur mainly at A[C/G]A, X[C/G]T, T[C/G]A, and T[C/G]T sites. In order to determine the effect of chemical-induced mtDNA damage on trinucleotide context mutagenesis, we normalized the relative contribution of each mutation to the mean wild-type control contribution (Figure 5). In wild-type exposed to CdCl2, there were more C \( \rightarrow \) G mutations in a C[C/G]A context compared to control (\( P < 0.05 \); Welch Two Sample T-test). There was also a trend of more T \( \rightarrow \) C mutations in a C[T/A]T context compared to control (\( P = 0.06 \); one-way ANOVA, Welch Two Sample T-test). In AfB1 MA lines, we observed fewer C \( \rightarrow \) G and C \( \rightarrow \) T mutations in a C[C/G]C context, and more T \( \rightarrow \) C mutations in a C[T/A]A context compared to control (\( P < 0.05 \); one-way ANOVA, Welch Two Sample T-test). In dct-1 mutants, we observed more
T → C mutations in CdCl₂ MA lines in a C[T/A]A context compared to control (p < 0.05; one-way ANOVA, Welch Two Sample T-test), and more C → T in a A[C/G]T context and T → C in A[T/A]T and G[T/A]A contexts after exposure to AfB₁ compared to control (P < 0.05; one-way ANOVA, Welch Two Sample T-test). In the pink-1 mutants, we observed lower relative contributions of mutations at specific trinucleotide contexts compared to wild-type control, with the exception of more C → A mutations in a A[C/G]C context in AfB₁ compared to pink-1 control (P < 0.05; one-way ANOVA, Welch Two Sample T-test).

We used the software MutationalPatterns to determine the cosine similarities of trinucleotide context mutational signatures between strain and treatment. As there were very few effects in the enrichment of trinucleotide mutations due to treatment as described above, it was unsurprising that the trinucleotide context mutational signatures were highly similar between strains and treatment (Supplementary Figure S9). Indeed, the cosine similarity values across the three strains and three conditions were all close to 1 (Supplementary Table S3). We next determined the similarity of these mutational signatures to known mutational processes as described in the COSMIC database (Supplementary Table S4). It is critical to note that the COSMIC signatures are derived from somatic nuclear mutagenesis from human cancer genomes, and C. elegans germline mitochondrial mutational processes are likely very different. Taking this into consideration, it was striking that across all of our samples we detected high cosine similarity to the single base substitution (SBS) mutational signature that is associated with nucleotide excision repair deficiency (SBS24), which is absent in mitochondria. There was also high similarity with signatures associated with tobacco exposures (SBS4, SBS29). Tobacco products contain high levels of cadmium and smoke contains high levels of benzo[a]pyrene, which results in the formation of DNA adducts similar to those caused by AfB₁. There also exists high similarity between signatures in which high levels of ROS is the proposed etiology (SBS18), in addition to defective base excision repair due to ROS-induced DNA damage (SBS36) and replication error across abasic sites (SBS13a). The similarities of C. elegans mtDNA signatures with certain COSMIC signatures are likely due to the high C:G → A:T mutational bias in the C. elegans mitochondrial genome. Cosine similarity values are located in Supplementary Table S4 and S5.

DISCUSSION

Mutation accumulation experiments in C. elegans and other organisms have determined that chemical exposures contribute to mutagenesis in the nuclear genome (61, 62). However, the role of chemicals in mtDNA mutagenesis is not well studied (63). There is some evidence that mtDNA is resistant to single nucleotide mutations in somatic tissues after exposure to the polycyclic aromatic hydrocarbon benzo[a]pyrene and hydrogen peroxide (8, 14), and in germ cells after exposure to hydrogen peroxide (64). As mitophagy is responsible for degrading organelles with damaged mtDNA (22) and purifying selection against highly deleterious mutations in the germline (30), a widely accepted theory in the field is that mitophagy regulates mtDNA mutational processes. Indeed, in various models including a study in C. elegans, placing a Pol γ mutator strain in a mitophagy-deficient background resulted in increased mtDNA mutation frequency (65, 66). However, to our knowledge, empirical evidence investigating the role of mitophagy in ameliorating chemical-induced mtDNA
damage, or mutations caused by damage, had not yet been investigated. Therefore, we conducted a mutation accumulation line experiment to determine the role of mitophagy in chemical-induced germline mtDNA mutagenesis (Figure 2). We found that *C. elegans* mtDNA is resistant to germline mutagenesis from exposure to known nuclear mutagens Cd and AIB1, despite accumulating high levels of mtDNA damage, extending the results observed previously with somatic mtDNA mutagenesis. Unexpectedly, we found that this resistance appears to be independent of mitophagy.

*C. elegans* continues to advance fundamental understanding of mitochondrial structure and function pertaining to human health and disease due to the conservation of mtDNA genes and various pathways (67). *C. elegans* is also a key research organism in the fields of evolutionary biology (32) and toxicology (68, 69), and has provided many insights into our knowledge of mtDNA mutagenesis and mitochondrial toxicity. For example, previous mutation accumulation line experiments with *C. elegans* have been instrumental in our understanding of mtDNA mutation rates (7, 33). However, various intricacies of mitochondrial genomics in *C. elegans* and other species render the study of mtDNA mutagenesis highly complex (70). The mitochondrial genome exists in multiple copies per organelle and cell, meaning that multiple genomes can pass through the germline bottleneck (~60 copies in *C. elegans*) (33), potentially allowing selection to still act on the organelle and cellular level even in a neutral mutation accumulation design (71). Because mtDNA is polyploid, the frequency of each *de novo* single nucleotide mutation is often lower than the limit of detection of traditional next-generation sequencing (error rate of 1 in $10^3$ base pairs). Furthermore, mutations that result from DNA damage are likely even more rare than spontaneous mutagenesis (72). Therefore, we employed Duplex Sequencing in order to accurately detect *de novo* variants as low as 1 in $10^7$ bases, and hypothesized that inhibiting mitophagy would prevent purifying selection from acting within an individual during a mutation accumulation line experiment.

We sequenced 96 MA lines after about 50 generations of mutation accumulation each, resulting in a total of ~4,800 generations of mutation accumulation. Overcoming the technical limitations of conventional next-generation sequencing by using Duplex Sequencing allowed us to detect a total of 6,040 SNMs, which is about 250-fold higher than any previous *C. elegans* mitochondrial mutation accumulation line experiment to date. The results of this Duplex Seq and MA approach were not biased by selection, as dN/dS ratios were ~1 across all MA lines (Supplementary Table S6), as has been a potential artifact and concern in previous studies (73, 74). Although proportionally similar across transition and transversion mutations, we observed roughly a 5-fold increase in mutation frequency compared to a recent study that conducted Duplex Sequencing to investigate mtDNA mutagenesis in wild-type *C. elegans*, which could possibly be attributed to the higher number of generation bottlenecks in our experiment (50 generations of one individual versus three generations of passaging 10 individuals) or other subtle differences in methodological and computational parameters (35). However, we did observe a mutation spectrum that is highly consistent with Waneka et al. (35). High frequencies of C:G → A:T and C:G → G:C transversion mutations support the conclusion that oxidative damage may drive mtDNA mutagenesis in *C. elegans*, though the mechanism underlying this spectrum remains an exciting area of future study. *C. elegans* appear to lack some homologs of *E. coli* 7,8-dihydro-8-oxoguanine (8-oxo-G) repair enzymes (MutY and MutM),
as well as OGG1, which may explain the preponderance of transversion mutations (75–77). An earlier mutation accumulation study demonstrated that under extreme drift, the intracellular oxidizing environment increased in C. elegans and resulted in oxidative damage to the nuclear genome (78). This has not been measured in the mitochondrial genome, but could explain the high C:G → A:T mutation frequency in our MA experiment. We observed a dominant G[C→T]C trinucleotide mutation across all strains and treatments (Supplemental Figure S6). This signature has been detected in various studies including human mitochondrial studies in aged populations, and has also been observed in some cancers, suggesting that either deoxycytidine deamination readily occurs in the C. elegans mitochondrial genome, or oxidized cytosines contribute to this mutational pattern (79).

Cytosine deamination due to oxidative stress results in an excess of C → T over G → A mutations on the mtDNA heavy strand in many different organisms including Drosophila (8), mouse (80), and human mtDNA (81), and is likely the primary source of Polγ error-induced mtDNA mutagenesis (10). However, our results did not reveal any strand asymmetry in mutation frequencies in wild-type C. elegans as would be predicted, particularly since oxidative damage is the prevailing model of mtDNA mutagenesis in C. elegans (35). Lack of strand-asymmetric mutagenesis could be because the mechanism of mtDNA replication in C. elegans may deviate from the single-strand displacement theory of other organisms (82), perhaps restricting exposure of damage-prone single-stranded mtDNA to oxidative stress and thus leading to no observable strand bias. This remains an intriguing area of future study – especially the compensatory mechanisms that have evolved to evade high rates of oxidative damage-induced mutagenesis. An earlier C. elegans MA study determined that elevated ROS levels due to an electron transport chain complex I deficiency (gas-1 mutant) resulted in a 3-fold increase in mtDNA copy number after almost 50 generations of mutation accumulation (34). Wernick et al. speculate that this is perhaps a compensatory mechanism to avoid the accumulation of potentially deleterious mtDNA variants when under extreme drift. It is important to note however that in the gas-1 study, only five MA lines were sequenced after 50 generations and only five SNVs were detected among all five MA lines (three of which were already present in the gas-1 progenitor). We did not investigate mtDNA copy number over generations, although it is possible that an increase in mtDNA replication under stress or other compensatory mechanisms contributed to our results (83, 84). However, the concentrations of CdCl₂ and AlB₃ at which the MA line experiments were conducted did not affect mtDNA copy number levels initially at G0 (Supplementary Figure S2). Alternatively, it is possible that high levels of endogenous oxidative damage and sequential spontaneous mutagenesis could overwhelm our ability to detect other forms of rare, damage-induced mutagenesis.

To our knowledge, no mutation accumulation experiment has investigated the effects of Cd exposure on mtDNA or in C. elegans. Cd is a ubiquitous heavy metal that is released from a range of sources, including, but not limited to, industrial processes such as mining and smelting. Combustion and wastewater contamination pollute terrestrial and aquatic environments and contaminate air, crops, and water. The most significant sources of human exposure to Cd are from contaminated food, smoking, e-waste sites, and even in products such as toys, jewelry, and plastics (85). Cd is a known carcinogen (86) and inhibits DNA repair mechanisms such as mismatch repair (37, 87–89), base
excision repair (90), and many other zinc-dependent enzymes, and also induces oxidative stress, likely via antioxidant depletion mechanisms. Cd preferentially targets cellular components, including mitochondria (91). However, the genotoxic and mutagenic effects of Cd on mtDNA are unknown. Ultimately, this led us to investigate the effects of chronic, low-level exposure to CdCl₂ on mtDNA mutagenesis in C. elegans.

To our surprise, we observed no effect of exposure to 50 µM CdCl₂ on C. elegans mtDNA mutational spectrum across all genotypes, even though this exposure results in high levels of mtDNA damage (Figure 1A, Figure 4A). Overall, there was almost no effect on the trinucleotide context mutational signature after exposure to CdCl₂, except for an enrichment of a C → G transversion mutation in a C[N]A context compared to control in wild-type C. elegans, which may be indicative of oxidative damage. 2,5-diamino-4H-imidazole-4-one (Iz) lesions contribute to C → G transversions in E. coli (92). Intriguingly, a recent study determined that human Pol γ is more prone to preferentially misincorporate a G opposite 2,2,4-triamino-5(2 H)-oxazolone (Oz), the common hydrolyzed product of Iz (93, 94). Though we did not have the capability to identify the specific type of mtDNA lesions that are a result of Cd exposure (the long-amplification quantitative PCR assay used in this study cannot differentiate between different types of damage to mtDNA such as breaks, oxidized bases, or adducts), the preponderance of this C → G transversion mutation could suggest that Cd causes a variety of oxidation damage, possibly not just 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) lesions which are likely repaired in the mitochondria and contribute to C → A/G → T transversions. The mechanism is which oxidative damage and repair contributes to mtDNA mutational spectrum in C. elegans mtDNA mutagenesis is unclear, but there is evidence that C. elegans is able to repair at least some forms of oxidative mtDNA damage (77, 95–98), despite the aforementioned absence of clear homologues of some BER enzymes. Neither of the mitophagy mutant strains accumulated significantly higher levels of mtDNA damage after exposure to 50µM CdCl₂ compared to exposed wild-type. This suggests that mitophagy is not the primary mechanism responsible for removal of damaged mtDNA or mtDNA mutations, at least at this level of mtDNA damage and frequency of mtDNA mutations. dct-1 mutants do have an enrichment of one T → C transition mutation in a C[N]A context in Cd conditions compared to control, but the process underlying this mutation is unknown. It is possible that the quantity of lesions formed at this concentration of Cd was not enough to contribute to mtDNA mutagenesis. However, the internal body burden of Cd in C. elegans after exposure to 50µM CdCl₂ (Supplementary Table S7) is equivalent to levels detected in human blood (86), suggesting that this concentration is relevant to human exposure.

This study contributes to evidence suggesting that C. elegans mtDNA mutagenesis may be driven by endogenous oxidative damage; however, as mentioned, C. elegans can repair at least some oxidative mtDNA damage. Therefore, to investigate the effect of irreparable mtDNA damage, we investigated mtDNA mutational spectrum and signatures after exposure to AFB₁. AFB₁ is a mycotoxin produced by the fungus Aspergillus flavus, and is one of the leading causes of hepatocellular carcinoma worldwide. A. flavus grows on staple grains and legumes, and when consumed, is metabolized to an epoxide that intercalates 5' to guanine targets, resulting in stable AFB₁-N'-G adducts that cause G → T mutations in the nuclear genome, primarily in CGC trinucleotide contexts.
AfB1 exposure results in a similar mutational spectrum in the C. elegans nuclear genome that has been observed in human and mammalian studies (62, 99). However, to our knowledge, no study has investigated effects on mtDNA mutagenesis, even though it has been known for decades that AfB1 preferentially attacks mtDNA over nuclear DNA (42).

We measured very small effects of 10μM AfB1 on mtDNA mutational spectrum in wild-type or mitophagy-deficient C. elegans after 50 generations of exposure, even though AfB1 exposure resulted in significant levels of mtDNA damage which were exacerbated in the dct-1 mitophagy mutant. We do observe a trend towards an increase in C:G → A:T transition mutations in AfB1-exposed pink-1 mutants compared to wild-type (1.4-fold greater mutation frequency, \( P = 0.07 \), two-way ANOVA, Tukey HSD). This suggests that perhaps pink-1 may play a role in ameliorating mtDNA damage or mediating purifying selection against deleterious mutations. In fact, we observe that over 50 generations of mutation accumulation, pink-1 mutants have a greater fitness decline than wild-type and dct-1 mutants, suggesting that they are accumulating more deleterious mutations (Supplementary Figure S10). Of course, we cannot rule out that this is due to mutations in the nuclear genome or nongenetic effects. However, it is not obvious why a mitophagy deficiency would elevate nuclear DNA mutagenesis, although it is conceivable that inhibiting mitophagy may attenuate mitochondria-nuclear DNA repair crosstalk mechanisms (100).

The trinucleotide context mutational signature in AfB1 MA lines does not reveal the clear signature that has been observed in nuclear genome studies. There is a significant but small enrichment of C → A mutations in a A[C/G]C context in AfB1 MA lines compared to control in pink-1 mutants, which varies slightly from the known nuclear C[G]C context, suggesting a subtle but unique mechanism of aflatoxin damage-induced mutagenesis in the C. elegans mitochondrial genome. The unique AfB1 signature may result from the fact that damage removal processes are different for the two genomes; AfB1–induced damage in the nuclear genome is repaired by the enzyme NEIL1 as well as NER (101), but NER does not operate in the mitochondria and C. elegans does not appear to have a NEIL1 gene.

The lack of an effect of mitophagy deficiency on mutation accumulation in these studies appears initially to be at odds with reports of a role for mitophagy in regulating selection against inheritance of mtDNA mutations in germ cells. A few possibilities may explain this. First, mitophagy may work efficiently only in the case of high-frequency or highly-deleterious mutations. It is possible that de novo mutations do not have functional consequences on organelle function, and therefore evade targeted degradation via mitophagy. Second, it is possible that the levels of chemical-induced mtDNA damage may not be high enough to contribute significantly to mtDNA mutagenesis in this study. We consider this to be unlikely however, as we used an exposure concentration of AfB1 that is considerably higher than a previous C. elegans study that detected nuclear mutational signatures after exposure to AfB1 at concentrations 33 and three-fold lower than this study (0.3μM and 3μM AfB1). Volkova et al. detected a clear mutational signature that shared high similarity to the AfB1 mutational signature observed in humans (C → A/G → T mutations) after only one generation of exposure (62, 99). Because we conducted targeted capture sequencing of just mtDNA, we were unable to analyze effects of exposure on nuclear genome mutagenesis. However, given those results, it is likely that a significant amount of de novo variants arose in the nuclear genome in our MA study.
after 50 generations of exposure to a higher dose of AFB1. Third, with Duplex Sequencing, we were only able to measure single nucleotide point mutations, not large insertions or deletions. Recent work indicates that mtDNA in somatic cells accumulates a large number of individually uncommon deletions with age, likely as a result of polymerase error (102). It is possible that chemical-induced adducts (in the case of AFB1) or oxidative damage (CdCl2) may exacerbate polymerase error-mediated deletion accumulation.

Overall, our study suggests that in *C. elegans*, mitochondria harbor important quality control processes that are perhaps complementary or redundant to the two mitophagy pathways investigated in this study, resulting in a remarkable resistance to chemical-induced mutagenesis. A recent study in *Drosophila* found that unhealthy organelles failed to import nuclear-encoded factors essential for mtDNA replication and biogenesis. This resulted in the replication of wild-type mtDNA in healthy organelles, and not mutant mtDNA (103). Perhaps a “replication-competition” model remains to be discovered in *C. elegans*. Direct degradation of damaged or mutant mtDNA may also play a protective role (104), though to our knowledge, *C. elegans* lacks a homolog for mitochondrial genome maintenance exonuclease (MGME-1) that degrades linear and damaged mtDNA at the mtDNA replisome. Mechanisms that target paternal mitochondria and mtDNA for degradation in *C. elegans* are known, and an intriguing possibility is that similar pathways that target damaged or mutant mtDNA are present the germline, which could suppress accumulation of deleterious mtDNA variants (105, 106). On the organelle level, mitochondrial fusion and fission play a significant role in mediating mitochondrial quality control and are prerequisites for mitophagy (20, 23). At 50µM CdCl2 and 10µM AFB1 exposure, we did not observe fragmentation of the *C. elegans* body wall muscle mitochondrial network in G0 wild-type *C. elegans* compared to control. We observed increased mitochondrial fusion at lower levels of Cd and AFB1 exposure, with recovery at higher doses, which suggests that mitochondrial dynamics are responding to Cd and AFB1-induced toxicity (Supplementary Figure S5). Additionally, we do not observe any effects on mitochondrial respiration in all three strains, so it is not likely that these exposures severely compromised mitochondrial function (Supplementary Figure S6). However, we propose that future studies investigate the role of mitochondrial dynamics in organelle and mtDNA turnover and biogenesis in the context of other chemical exposures. Lastly, another potential mechanism of ameliorating the removal of damaged mtDNA in the germline on the organismal level is germline apoptosis, which has previously been reported to increase after Cd and AFB1 exposure in *C. elegans*, though not at the concentrations that were used in our study (107, 108).

The use of chemicals in society has resulted in over 86,000 chemicals of unknown toxicity in production (109), in addition to the hundreds of thousands of tons of pharmaceutical and industrial waste, increases in air pollution, and other sources of pollution that impact the health of hundreds of millions of people daily around the globe (110). Many chemicals can cause genome instability, which drives cancer development and other diseases; therefore it is necessary to better understand how low-level exposures to environmental agents can promote mutagenesis (111, 112). mtDNA mutagenesis contributes to cancer and other diseases (60, 113–116), yet is far less understood than nuclear DNA mutagenesis. Mitochondria are vital organelles that are highly susceptible to toxicity and genomic damage (16, 117, 118). MtDNA mutations accumulate with age, yet there remains conflicting
evidence in the literature for the role of genotoxicant exposure in mtDNA mutagenesis (63). Surprisingly, we found that in *C. elegans*, after thousands of generations of continuous chemical exposure, with thousands of SNMs detected, and in the context of loss of two key mitophagy genes, mtDNA is resistant to damage-induced single nucleotide mutations. Future studies to investigate the effect of more chemicals in various genetic backgrounds may elucidate mechanisms in which mitochondria resist chemical-induced point mutations.

**AVAILABILITY**

The Duplex-Seq-Pipeline is written in Python and R, but has dependencies written in other languages. The Duplex-Seq-Pipeline software has been tested to run on Linux, Windows WSL1, Windows WSL2 and Apple OSX. The software can be obtained at [https://github.com/Kennedy-Lab-UW/Duplex-Seq-Pipeline](https://github.com/Kennedy-Lab-UW/Duplex-Seq-Pipeline) under the BSD license.

**ACCESSION NUMBERS**

The data are available as raw reads under SRA Accession SRP350474 (BioProject PRJNA787252).

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR online.

**ACKNOWLEDGEMENT**

We would like to thank members of the Duke Center for Genomics and Computational Biology, particularly Dr. Nicolas Devos and Nicholas Hoang for their help with library preparation and sequencing. We thank Inigo Martincorena for his help with the *dNdScv* package. We would also like to thank members of the Meyer, Baugh, Wernegreen, and Jayasundara labs at Duke University for their thoughtful feedback throughout the design, implementation, and analysis stages of this project, and feedback on this manuscript.

**FUNDING**

This work was supported by the National Institutes of Health [(F31 ES030588 to TCL, P42ES010356 to JNM, IRM supported by T32ES021432); Triangle Center for Evolutionary Medicine (TriCEM) Graduate Student Award to TCL; Duke University School of Medicine Sequencing and Genomics Technologies Core Facility Voucher to TCL and JNM; Congressionally Directed Medical Research Programs [W81XWH-16-1-0579, W81XWH-18-1-0339]; National Human Genome Research Institute [R21 HG011229] to SRK. Funding for open access charge: National Institutes of Health.

**CONFLICT OF INTEREST**

S.R.K. is an equity holder and paid consultant for Twinstrand Biosciences, a for-profit company commercializing Duplex Sequencing. No Twinstrand products were used in the generation of the data.

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Figure 1. 50µM CdCl₂ and 10µM AfB₁ exposure induces mtDNA lesions in wild-type *C. elegans*, but has no effect on growth or reproduction. (A) mtDNA damage from pools of six individual age-synchronized L4 *C. elegans* was quantified after chronic exposure to control (N = 8), 50µM CdCl₂ (N = 8), or 10µM AfB₁ (N = 4). (**) *P* < 0.01, one-way ANOVA) (B) A dose-response was conducted to measure effects on growth after 48 hours of exposure (L1-L4). Each individual worm length was first normalized to the mean control length for each experiment. Dots represent technical replicates (individual nematodes, total N displayed) and the boxplots display the median and upper and lower quartiles. The mean normalized length was then calculated within each experimental replicate. There was no effect of growth after exposure to 50µM or 200µM CdCl₂, but we observed 22% growth inhibition at 1,000µM CdCl₂ compared to control (*P* = 0.15, *P* = 0.2, *P* = 0.009, respectively; one-way ANOVA, Tukey HSD). We did not observe growth inhibition after 10µM or 50µM AfB₁ compared to control, but did observe 11% growth inhibition at 200µM AfB₁, though trending (*P* = 0.27, *P* = 0.59, *P* = 0.09, respectively; one-way ANOVA, Tukey HSD). (C) There is no effect of either 50µM CdCl₂ or 10µM AfB₁ on total brood size compared to control (N = 9-10, *P* = 0.9, *P* = 0.63, respectively; one-way ANOVA, Tukey HSD). Error bars indicate standard error (* *P* < 0.05; ** *P* < 0.01).
Figure 2. Schematic of Mutation Accumulation line experimental design. Offspring of a single founding ancestor (G0) were isolated onto individual plates. Every t = 4 days, a single L4 nematode was randomly selected and transferred to a new plate. This was repeated every generation (G) for 50 generations. We conducted MA lines on control plates, and plates that contained OP50 that was spiked with a final concentration of 50µM CdCl₂ and 10µM AFB₁. We conducted MA line experiments in wild-type C. elegans and two mitophagy mutant strains, dct-1 and pink-1. 50 replicates were passaged for each strain*treatment. MA lines were randomly selected after 50 generations (9-14 MA lines/strain/treatment) for life history analysis and targeted mtDNA Duplex-Sequencing. Image created with BioRender.com.
Figure 3. The mtDNA single nucleotide mutation signature in wild-type *C. elegans* is consistent with oxidative damage and demonstrates resistance to point mutations caused by CdCl₂ and AfB₁ mtDNA lesions. (A) Overall SNM frequency was determined by Duplex Sequencing after 50 generations of mutation accumulation in wild-type *C. elegans* in control conditions (gray dots; N = 11), as well as after 50 generations of exposure to 50µM CdCl₂ (gold dots; N = 14) and 10µM AfB₁ (green dots, N = 10). There was no effect of either 50µM CdCl₂ or 10µM AfB₁ on overall mtDNA mutation frequency.
compared to control ($P = 0.96$, $P = 0.90$, respectively); one-way ANOVA, Tukey HSD). Horizontal lines indicate mean values. (B) Mutation spectrum of control, CdCl$_2$, and AfB$_1$-treated MA lines. Each dot represents a single MA line. The wild-type *C. elegans* mtDNA mutational signature was dominated by C:G $\rightarrow$ A:T and C:G $\rightarrow$ G:C transversion mutations, and there was no effect of Cd or AfB$_1$ exposure on mtDNA mutational signature (Welch Two Sample T-test). Error bars represent standard error of the mean. (C) mtDNA lesions may accumulate disproportionately on mtDNA strands, resulting in mtDNA mutation strand bias. We observed a trend towards an increase in C $\rightarrow$ T over G $\rightarrow$ A mutations after exposure to AfB$_1$ compared to control ($P = 0.08$; two-way ANOVA, Tukey HSD).
Figure 4. Mitophagy deficient mutants accumulate higher levels of mtDNA damage compared to wild-type, but exhibit no differences in mtDNA mutation frequencies after exposures. (A) mtDNA damage from pools of six individual age-synchronized L4 C. elegans after exposure to control (N = 8), 50µM CdCl$_2$ (N = 8), or 10µM AfB$_1$ (N = 8). pink-1 mutants accumulated mtDNA damage after exposure to CdCl$_2$, but not more than wild-type, while dct-1 mutants did not accumulate mtDNA damage. Both mutants accumulated high levels of mtDNA damage after exposure to AfB$_1$, and dct-1 accumulated significantly higher levels compared to wild-type exposed C. elegans. Error bars indicate standard error of the mean (* $P < 0.05$; ** $P < 0.01$; two-way ANOVA, Tukey HSD). (B) Mutation spectrum of control (gray), CdCl$_2$ (gold), and AfB$_1$ (green) treated MA lines in wild-type, dct-1, and pink-1 strains. The mitophagy mutant C. elegans mtDNA mutational signatures were also dominated by C:G $\rightarrow$ A:T
and C:G → G:C transversion mutations, and there was no effect of CdCl₂ or AfB₁ exposure on dct-1 or pink-1 mtDNA mutational signature (two-way ANOVA). Error bars indicate standard error of the mean.
Figure 5. Trinucleotide context mutational signature of wild-type, dct-1, and pink-1 C. elegans after exposure to CdCl₂ or AfB₁. Contributions of each of the 96 possible trinucleotide mutations were determined for each MA line, and then normalized to the mean wild-type control contribution (show as a dashed line) to determine effects relative to wild-type control. Bar graphs show the relative mean and standard error of each trinucleotide context mutation, with each mutation represented by a different color and control, CdCl₂, and AfB₁ treatment as increasing color hues. Potential effects of
each exposure compared to control were determined within each mutation type (Welch Two Sample T-test) only after a significant ANOVA was determined. * $P < 0.05$. 