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## Homeostatic responses regulate selfish mitochondrial genome dynamics in *C. elegans*

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### Summary

Mutant mitochondrial genomes (mtDNA) can be viewed as selfish genetic elements that persist in a state of heteroplasmy despite having potentially deleterious metabolic consequences. We sought to study regulation of selfish mtDNA dynamics. We establish that the large 3.1kb deletion-bearing mtDNA variant *uaDf5* is a selfish genome in *Caenorhabditis elegans*. Next, we show that *uaDf5* mutant mtDNA replicates in addition to, not at the expense of, wildtype mtDNA. These data suggest existence of homeostatic copy number control that is exploited by *uaDf5* to ‘hitchhike’ to high frequency. We also observe activation of the mitochondrial unfolded protein response (UPR<sup>mt</sup>) in *uaDf5* animals. Loss of UPR<sup>mt</sup> causes a decrease in *uaDf5* frequency whereas its constitutive activation increases *uaDf5* levels. UPR<sup>mt</sup> activation protects *uaDf5* from mitophagy. Taken together, we propose that mtDNA copy number control and UPR<sup>mt</sup> represent two homeostatic response mechanisms that play important roles in regulating selfish mitochondrial genome dynamics.

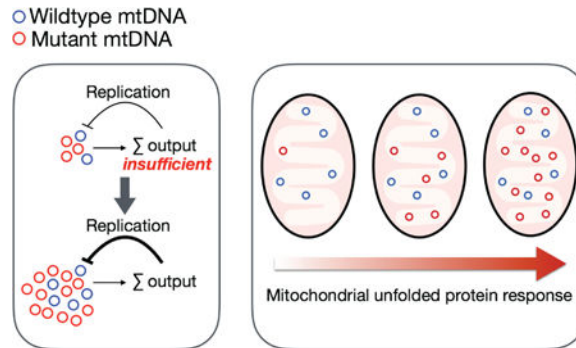
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**Author contributions:** MRP and BLG conceived and designed the study. BLG and MRP carried out the experiments. CSK carried out experiments with *mptDf1* mtDNA heteroplasmy. BLG and RDG performed ddPCR experiments in the laboratory of SAM. MRP, BLG, and DCS analyzed the data. BLG and MRP prepared the manuscript.

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## Graphical abstract

Using genetic and molecular approaches in *C. elegans*, Gitschlag et al. show that mutant mtDNA exploits copy number control to achieve high levels. At high levels, mutant mtDNA causes mitochondrial stress and activates UPR<sup>mt</sup>. By alleviating this stress, UPR<sup>mt</sup> promotes proliferation of mutant mtDNA.



## Introduction

Many ecological systems are predicated upon mutualistic interactions between different organisms (Momeni et al., 2011). Similar interactions between different genetic entities can underlie fundamental aspects of cell biology. For instance, the nuclear and the mitochondrial genome (mtDNA) work in a coordinated fashion to ensure optimal cellular fitness. However, just like in ecology, mutualistic interactions between these genomes are fraught with the risk that selfish mtDNA could arise. Selfish mtDNA can be defined as mutant mtDNA variants that have a transmission advantage over wildtype mtDNA despite jeopardizing cellular fitness and even organismal survival (Clark et al., 2012). Such selfish mtDNA variants often replicate and are co-transmitted along with wildtype mtDNA in a state of heteroplasmy (Taylor et al., 2002). The presence of such mutant mtDNA at frequencies above a critical threshold can be pathogenic and is thought to be one of the causes underlying inherited and age-related degeneration and metabolic diseases (Stewart and Chinnery, 2015, Wallace and Chalkia, 2013).

Selfish mtDNA are most studied in yeast, where they arise at very high rates and result in formation of ‘petite’ colonies with mitochondrial respiration failure (Bernardi, 2005, Williamson, 2002). Despite harboring major deletions and rearrangements, and causing severe cell growth defects, many of these mutant mtDNA are able to outcompete wildtype mtDNA in yeast cells and are hence dubbed ‘hypersuppressive’ mtDNA (MacAlpine et al., 2001, Jasmin and Zeyl, 2014, Harrison et al., 2014). While these selfish mtDNA were at first thought to outcompete wildtype mtDNA by virtue of possessing more origins of replication, this interpretation has been questioned in light of new data (Williamson, 2002). In metazoans, mutant mtDNA are largely characterized by deletions rather than the massive genomic rearrangements observed in hypersuppressive mtDNA. These data suggest that the mechanisms employed by selfish mtDNA in yeast are fundamentally different than those in metazoans. A deletion-harboring mtDNA was recently discovered in natural populations of the nematode species *C. briggsae*. Interestingly, this mutant mtDNA variant is found across

*C. briggsae* strains of diverse geographic origins, suggesting that it has stably persisted on evolutionary time-scales (Howe and Denver, 2008, Clark et al., 2012). The presence of this mtDNA variant decreases fecundity and pharyngeal pumping rates, suggesting that it negatively impacts organismal fitness (Estes et al., 2011). While this mutant mtDNA was shown to have a transmission advantage over wildtype mtDNA in mutation accumulation lines and in small populations, the cellular and molecular bases underlying its competitive success are not well understood (Clark et al., 2012, Phillips et al., 2015).

In this study, we establish the mutant mtDNA variant called *uaDf5* as a selfish genetic element in *C. elegans*. *uaDf5* coexists as a heteroplasmy with wildtype mtDNA despite being slightly deleterious. Next, using droplet digital PCR (ddPCR) to quantify mtDNA copy number dynamics, we show the existence of homeostatic copy number control for wildtype mtDNA. Our data on *uaDf5* mtDNA copy number dynamics are most consistent with *uaDf5* exploiting this mtDNA copy number control to ‘hitchhike’ to high frequency. Finally, we observe activation of the mitochondrial unfolded protein response (UPR<sup>mt</sup>) in *uaDf5* heteroplasmic animals. Loss of UPR<sup>mt</sup> results in a decrease in *uaDf5* levels, while constitutive activation leads to its increase. Hence, besides mtDNA copy number control, *uaDf5* also exploits UPR<sup>mt</sup>, together suggesting that homeostatic cellular processes are important determinants of selfish mtDNA dynamics.

## Results

### A bona fide ‘selfish’ mtDNA in *C. elegans*

We focused on identifying potentially ‘selfish’ mtDNA in a genetically tractable metazoan. *C. elegans* is an ideal model system to study mtDNA dynamics in a multicellular organism. Besides offering a powerful genetic toolkit, *C. elegans* mtDNA shares many conserved features with its mammalian counterpart (Okimoto et al., 1992). First, like mammalian mtDNA, mtDNA in *C. elegans* is uniparentally inherited through the oocyte (Tsang and Lemire, 2002, Sato and Sato, 2011, Al Rawi et al., 2011). Second, *C. elegans* mtDNA encodes 12 of the 13 proteins encoded by mammalian mtDNA. Finally, like in mammals but in contrast to yeast, most large-scale mutations in *C. elegans* mtDNA are deletions rather than genomic rearrangements (Denver et al., 2000).

Previous studies have identified a *C. elegans* strain harboring mutant mtDNA called *uaDf5*, which has a 3.1kb deletion that removes four protein-coding genes and seven tRNAs (Fig. 1A) (Tsang and Lemire, 2002). Due to this large deletion, individuals carrying only *uaDf5* mtDNA would not be expected to be viable. Indeed, animals homoplasmic for *uaDf5* have not been reported (Tsang and Lemire, 2002, Liao et al., 2007). Remarkably however, animals that have lost *uaDf5* have also not been previously observed, even after passaging over hundreds of generations (Tsang and Lemire, 2002, Liao et al., 2007). Moreover, *uaDf5* levels steadily increase in individuals that inherit it at a low frequency (Tsang and Lemire, 2002).

One explanation for the stable maintenance of *uaDf5* over many generations is balanced heteroplasmy, in which two mtDNA haplotypes possess lethal but non-overlapping mutations. In this scenario, neither mtDNA type can be lost because neither mtDNA is

capable of fully supporting viability. In order to test this hypothesis, we sought to sequence the entire non-*uaDf5* mtDNA in the heteroplasmic animals. By designing primers inside the *uaDf5* deletion, we were able to specifically amplify the entire genic region of non-*uaDf5* mtDNA as two large PCR products (Fig. S1). Under the ‘balanced heteroplasmy’ hypothesis, we expected to find deleterious mutations in the non-*uaDf5* mtDNA in the *uaDf5* heteroplasmic individuals. In contrast, upon sequencing, we found that the non-*uaDf5* mtDNA in *uaDf5* heteroplasmic animals is wildtype. Next, to sequence an approximately 500bp highly AT-rich non-coding region that was not captured within the two PCR products, we amplified just the non-coding region of the mtDNA using primers that are common to both *uaDf5* and non-*uaDf5* mtDNA. Upon sequencing however, we did not observe any apparent heteroplasmic mutations, as we might expect if there were any mutations specific to the non-*uaDf5* mtDNA. Thus, our sequencing data do not support balanced heteroplasmy as an explanation for *uaDf5* persistence since the non-*uaDf5* mtDNA is wildtype, suggesting that *uaDf5* is not critical for organismal viability.

Our sequencing data are instead consistent with *uaDf5* behaving like a selfish genetic element. We reasoned that if *uaDf5* is not critical for viability, then we should be able to recover healthy individuals that have lost it. In order to test this hypothesis, we carried out additional experiments in which we selected individuals with progressively lower *uaDf5* levels across multiple generations (red boxes in schematic in Fig. 1B). Under this ‘selection’ regime, we were able to recover healthy individuals homoplasmic for wildtype mtDNA that had undetectable levels of *uaDf5* (Fig. 1C), suggesting that these individuals have lost *uaDf5* mutant mtDNA. To confirm complete loss of *uaDf5*, we conducted droplet digital PCR (ddPCR) with *uaDf5* specific primers. ddPCR relies on performing thousands of independent PCR reactions simultaneously in small droplets that are designed to hold an average of one template per droplet (Hindson et al., 2011). Hence, it provides a highly sensitized way to detect rare variants. We observed complete loss of *uaDf5* using ddPCR (Fig. 1D). Together with our sequencing results, our finding that *uaDf5* can be eliminated rules out balanced heteroplasmy as an explanation for *uaDf5* maintenance. Instead, together with the previously published data that *uaDf5* has a transmission advantage when inherited at low frequencies (Tsang and Lemire, 2002) and is associated with decreased organismal fitness (Liau et al., 2007), we consider the second alternative that *uaDf5* is a selfish mtDNA.

### ***uaDf5* has ‘runaway’ copy number dynamics while wildtype mtDNA levels are tightly controlled**

Although *uaDf5* mtDNA levels are variable across individuals, previous reports have suggested they can reach high frequency in individuals (Tsang and Lemire, 2002). Indeed, we were able to stably maintain laboratory populations in which individual worms are heteroplasmic for *uaDf5* mtDNA, where *uaDf5* comprises 60-80% of overall mtDNA present in each worm (Fig. 2A). To ascertain mechanisms that regulate *uaDf5* levels, we next examined how levels of mutant *uaDf5* mtDNA affected the levels of wildtype mtDNA. One simple ‘direct competition’ model suggests that wildtype mtDNA levels should be inversely proportional to *uaDf5* mtDNA levels, while total mtDNA levels remain consistent. Alternatively, we might find that the copy number of wildtype mtDNA is maintained independent of *uaDf5* levels.

Here, we sought to distinguish between these possibilities of maintenance of total mtDNA copies versus wildtype mtDNA copies. Using ddPCR, we quantified mtDNA copy number from individual day 4 adults. mtDNA replication in *C. elegans* does not start until the L4 stage, after which it greatly increases during the first few days of adulthood and reaches steady state levels by day 4 (Fig. S2) (Bratic et al., 2009). mtDNA copy number measurements in day 4 adults reflect germline mtDNA since more than 95% of the mtDNA content in an adult hermaphrodite is in the germline syncytium, the shared cytoplasm of germ cells (Bratic et al., 2009). The ‘direct competition’ model predicts relatively constant levels of total mtDNA amongst individuals, with a trade-off between the amount of wildtype and *uaDf5* mtDNA copies. In contrast, our results show that wildtype mtDNA levels are remarkably consistent across individuals while *uaDf5* levels span a wide range. These findings are especially pronounced when animals are rank-ordered from lowest to highest *uaDf5* levels (Fig. 2B). Analysis of the range of wildtype and *uaDf5* mtDNA copy number shows substantially wider range in *uaDf5* mtDNA copy number compared to wildtype mtDNA (Fig. 2C). Taken together, our data show that *uaDf5* mtDNA levels increase in addition to, not at the expense of, wildtype mtDNA levels. These data also provide an explain for previously published data showing that total mtDNA levels in *uaDf5* heteroplasmic animals are higher than in homoplasmic wildtype animals (Tsang and Lemire, 2002). In conclusion, our data are consistent with the hypothesis that wildtype mtDNA levels, but not the total mtDNA levels, are well regulated.

### Small deletion bearing *mptDf1* mtDNA also has ‘runaway’ copy number dynamics

One attractive hypothesis for maintenance of mutant mtDNA with large deletions like *uaDf5* invokes replicative advantage over wildtype mtDNA because of their smaller genome size (Wallace, 1992). The replicative advantage of a smaller genome has been shown under limited physiological contexts (Moraes et al., 1999, Diaz et al., 2002); nevertheless, this advantage could potentially give rise to the ‘runaway’ dynamics that we observe for *uaDf5*. If this hypothesis were correct, we would expect to see different mtDNA dynamics in mtDNA harboring smaller deletions. To address this possibility, we investigated mtDNA copy number dynamics in animals that are heteroplasmic for *mptDf1*, a mutant mtDNA carrying a 179bp deletion (Fig. 1A), significantly smaller than the 3.1kb *uaDf5* deletion. We identified *mptDf1* deletion in a heteroplasmic strain from whole genome sequencing data of mutagenized worms from the Million Mutation Project (Thompson et al., 2013). In contrast to the *uaDf5* deletion, which reduces mtDNA genome size by ~20%, the *mptDf1* deletion removes only ~1% of the genome. Interestingly, despite backcrossing to N2 reference strain five times, we noticed that high levels of *mptDf1* cause gonadal defects, and likely explain the lower average heteroplasmy frequency of *mptDf1* compared to *uaDf5* (Fig. S3). Despite this lower heteroplasmy frequency, and the fact that we had to use L4 stage individuals given germline defects in adults, we find that the mtDNA copy number dynamics in *uaDf5* and *mptDf1* heteroplasmies are remarkably similar, that is, wildtype mtDNA levels were relatively steady across individuals despite increasing levels of mutant mtDNA (Fig. 2D-F). While not ruling out replicative advantage, the data suggest that other mechanisms contribute to regulation of *uaDf5* and *mptDf1* copy number dynamics.

### mtDNA transcriptional imbalance in *uaDf5* heteroplasmic animals

Since heteroplasmic animals carry *uaDf5* mtDNA in addition to wildtype mtDNA, we ascertained whether the *uaDf5* mtDNA contributed to the mtDNA transcripts. We reasoned that expression of all genes from the wildtype mtDNA but only some from the *uaDf5* genome would result in a stoichiometric imbalance in mtDNA-encoded transcript levels (see schematic Fig. 3A). We found that *CYTB* and *ND1*, genes deleted from *uaDf5*, were expressed at the same levels in homoplasmic wildtype animals as in *uaDf5* heteroplasmic animals (Fig. 3B). Transcript levels of the nuclear-encoded electron transport chain component *NUO2*, as well as actin, were also unaltered in the heteroplasmic animals (Fig. 3B). However, compared to homoplasmic wildtype animals, transcript levels of *COI*, *COII*, *COIII*, *ND4*, *ND5*, and *ND6*, which are still encoded by *uaDf5* mtDNA, were significantly elevated in the *uaDf5* heteroplasmic population (Fig. 3B). This implies that *uaDf5* mtDNA are transcriptionally active and their expression contributes to substantial transcriptional imbalances in mtDNA-encoded genes.

### Mitochondrial perturbations in *uaDf5* heteroplasmic animals

*uaDf5* affects organismal fitness; *uaDf5* animals have decreased egg-laying and defecation rates, reduced lifespan, and decreased sperm motility (Liau et al., 2007). At the molecular level, we observe overexpression of *uaDf5* mtDNA-encoded transcripts, resulting in transcriptional imbalance. Given these effects, we sought to determine whether *uaDf5* has cellular consequences. Mitochondrially targeted green fluorescence protein (GFP<sup>mt</sup>) has previously been used as a model matrix protein to assess mitochondrial proteostasis (Yoneda et al., 2004, Benedetti et al., 2006). We reasoned that if *uaDf5* animals have altered proteostasis, it might result in decreased fluorescence of GFP<sup>mt</sup>. Indeed, we observe a significant decrease in fluorescence in *uaDf5* animals that express GFP<sup>mt</sup> in the intestinal cells under the control of the *ges-1* promoter (Fig. 3C) (Benedetti et al., 2006). No such decrease is observed in fluorescence of cytoplasmic GFP (GFP<sup>cyt</sup>) in *uaDf5* animals (Fig. 3C). These data suggest that the fluorescence of mitochondrially targeted GFP is specifically affected in *uaDf5* animals. Consistent with fluorescence data, western blot analysis shows decreased GFP<sup>mt</sup> protein levels in *uaDf5* animals (Fig. 3D). In contrast to decreased GFP<sup>mt</sup>, we observe increased staining in *uaDf5* animals with fluorescent dye MitoTracker Green FM, which localizes to mitochondria independent of the membrane potential (Dingley et al., 2014, Hicks et al., 2012), as well as with tetramethyl rhodamine ethyl ester (TMRE) (Dingley et al., 2014, Yoneda et al., 2004, Billing et al., 2011, Palikaras et al., 2015), which localizes to mitochondria in a membrane potential dependent manner (Fig. 3E-F). These data suggest an increase in mitochondrial organelle mass in *uaDf5* animals, correlating with an increase in total mtDNA levels. These data also suggest that the decreased GFP<sup>mt</sup> levels and fluorescence in *uaDf5* animals are due to alterations in mitochondrial proteostasis rather than due to decreased mitochondrial organelle mass. This altered proteostasis might reflect decreased GFP<sup>mt</sup> import efficiency into mitochondria or a compromised protein-folding environment inside the mitochondrial matrix. Either scenario might result in degradation of the unfolded/misfolded GFP<sup>mt</sup>. Taken together, these data suggest mitochondrial alterations in *uaDf5* animals.

## High levels of *uaDf5* activate the mitochondrial unfolded protein response

The mitochondrial unfolded protein response (UPR<sup>mt</sup>) has emerged as an important protective stress response that is activated under a variety of conditions that affect mitochondrial proteostasis (Houtkooper et al., 2013, Yoneda et al., 2004, Haynes and Ron, 2010, Runkel et al., 2013, Baker et al., 2012). This homeostatic response involves expression of hundreds of target genes including chaperones and proteases that eliminate misfolded and nonfunctional complexes inside mitochondria (Nargund et al., 2015, Nargund et al., 2012). To determine whether UPR<sup>mt</sup> is induced in *uaDf5* animals, we quantified *hsp-6* and *hsp-60* transcript levels in *uaDf5* animals. HSP-6 and HSP-60 are mitochondrial chaperones induced by UPR<sup>mt</sup> (Yoneda et al., 2004, Nargund et al., 2012). We observed significant increase in *hsp-6* and *hsp-60* transcript levels in *uaDf5* animals (Fig. 4A). We were able to confirm this induction with a transgenic fluorescence reporter in which the *hsp-60* promoter drives GFP expression, although UPR<sup>mt</sup> induction is variable across individuals (Fig. 4B-C) (Yoneda et al., 2004). These data suggest that UPR<sup>mt</sup> might be appreciably induced in animals harboring *uaDf5* levels above a certain threshold. Consistent with this notion, we were able to observe a weak but positive relationship between UPR<sup>mt</sup> activation and *uaDf5* levels (Fig. 4D). The lack of a stronger correlation might be a product of the fact that most of the animals we analyzed have *uaDf5* levels within a very narrow range of 60-80%. Taken together, these results suggest that presence of the selfish mtDNA *uaDf5* induces UPR<sup>mt</sup>.

## UPR<sup>mt</sup> modulates *uaDf5* heteroplasmy levels

UPR<sup>mt</sup> plays a protective role under conditions that affect mitochondria (Nargund et al., 2012, Baker et al., 2012, Runkel et al., 2013). Might the protective role for UPR<sup>mt</sup> inadvertently create the conditions that allow *uaDf5* to sustain high levels in certain individuals? According to this hypothesis, we predict that loss of UPR<sup>mt</sup> would result in a decrease in *uaDf5* levels. To test this hypothesis, we conducted RNAi-mediated knockdown of *atfs-1*, a gene that encodes a transcription factor central to UPR<sup>mt</sup> induction (Nargund et al., 2012). We observed a significant decrease in the average *uaDf5* frequency in *atfs-1* knockdown animals (Fig. 5A). We similarly observe a decrease in *uaDf5* levels in *atfs-1* loss-of-function mutants (Fig. 5B). Quantification of mtDNA dynamics shows that wildtype mtDNA levels are well regulated in *atfs-1* loss-of-function mutants, thus ruling out loss of mtDNA copy number control as a potential explanation for the decrease in *uaDf5* levels (Fig. 5C-D, compare Fig. 2B-C). Given that mutant mtDNA molecules typically disrupt mitochondrial function and become pathogenic only when their levels are high enough to pass a critical threshold (Rossignol et al., 2003, Stewart and Chinnery, 2015), cells may become tolerant to mutant mtDNA, even with a relatively modest reduction in mutant levels, relaxing further selection against mutant mtDNA. Moreover, because UPR<sup>mt</sup> is likely induced in animals harboring *uaDf5* levels above a certain threshold, and given the hypothesis that *uaDf5* may exploit additional mechanisms to persist, such as mtDNA copy number control, we predict that although *uaDf5* levels decrease in absence of UPR<sup>mt</sup>, the mutation would still be able to persist. Indeed, *uaDf5* is still present, albeit at low levels, in *atfs-1* loss-of-function animals that we have continuously maintained for more than 30 generations (Fig. 5E). These data are consistent with the hypothesis that loss of UPR<sup>mt</sup> exposes *uaDf5* to more stringent selection, which causes a decrease in *uaDf5* levels but not its complete elimination.

If UPR<sup>mt</sup> activation allows for tolerance to high levels of *uaDf5*, then we predict that restoring *atfs-1* will allow *uaDf5* levels to recover after *atfs-1* RNAi treatment. Indeed, *uaDf5* levels recover within a single generation when *atfs-1* expression is restored in *uaDf5* animals after growing for several generations on *atfs-1* RNAi conditions (Fig. 6A). These data suggest that UPR<sup>mt</sup> is required for *uaDf5* to attain high levels. We next tested if the converse was also true, *i.e.*, whether constitutive activation of UPR<sup>mt</sup> decreases selection against *uaDf5*, thereby driving *uaDf5* to higher frequency. For this, we tested animals heterozygous for an *atfs-1* gain-of-function allele to constitutively activate UPR<sup>mt</sup> (Rauthan et al., 2013). We did not observe any significant increase in the *uaDf5* levels in animals heterozygous for the *atfs-1* gain-of-function allele (Fig. 6B). However, given that the starting *uaDf5* levels in our *uaDf5* strain are already very high (approximately 80%), we speculated that they might not be able to increase substantially due to an upper threshold effect. It is also possible that given the induction of UPR<sup>mt</sup> in individuals with high levels of *uaDf5*, the *atfs-1* gain-of-function allele might not induce significant further UPR<sup>mt</sup> activation in these animals. To overcome these limitations, we tested whether *uaDf5* levels can rise in *atfs-1* gain-of-function heterozygotes in a population with lower starting *uaDf5* frequency (approximately 30%). In this case, we observed a significant increase in *uaDf5* frequency in *atfs-1* gain-of-function heterozygotes relative to animals homozygous for wildtype *atfs-1* (Fig. 6C). Together, our results show that *atfs-1* can modulate *uaDf5* levels.

### Loss of UPR<sup>mt</sup> does not select against *uaDf5* at the organismal level

Increased sensitivity of animals with high *uaDf5* levels to *atfs-1* loss at the organismal level provides one potential explanation for the observed decrease in *uaDf5* levels. RNAi knockdown of *atfs-1* enhances developmental delay in animals exposed to paraquat (Runkel et al., 2013). Mitochondrial-stressed *isp-1(qm150)* and *clk-1(qm30)* mutants similarly fail to develop under *atfs-1* RNAi conditions (Nargund et al., 2012). We sought to determine whether knockdown of *atfs-1* RNAi causes similar developmental delay in *uaDf5* animals. While we observe a mild developmental delay in *uaDf5* animals, it is not enhanced by *atfs-1* knockdown (Fig. 7A). We also did not observe appreciable levels of embryonic lethality in *uaDf5* animals raised on *atfs-1* RNAi (Fig. 7B). Finally, there is no increase in lethality up to day 4 of adulthood (Fig. 7C), which is when we assess and observe a decrease in *uaDf5* levels on *atfs-1* RNAi. The absence of an *atfs-1* knockdown-dependent effect on reproduction suggests that selection against high *uaDf5* levels is unlikely to operate at the organismal level.

### UPR<sup>mt</sup> modulates *uaDf5* levels via mitophagy

Organelle level selection provides an alternate possibility for the observed decrease in *uaDf5* levels in absence of *atfs-1*. According to this hypothesis, *uaDf5* might be more susceptible to mitophagy in absence of UPR<sup>mt</sup>. Mitophagy is initiated by stabilization of Pink-1 on dysfunctional mitochondria, which in turn recruits parkin to mediate mitophagy (Randow and Youle, 2014). Here, we observe an increase in Pink-1::GFP fluorescence in *uaDf5* animals with RNAi knockdown of *atfs-1* compared to control (Fig. 7D; these experiments were conducted in parkin homolog *pdr-1* RNAi background to stabilize Pink-1::GFP localization to mitochondria). The increase in Pink-1::GFP fluorescence suggests that loss of UPR<sup>mt</sup> promotes mitophagy in *uaDf5* animals. If mitophagy mediates decrease in *uaDf5*

levels in absence of UPR<sup>mt</sup>, then we predict that *uaDf5* levels should recover in animals without UPR<sup>mt</sup> that also have compromised mitophagy. In order to test this prediction, we quantified *uaDf5* levels in *atfs-1;pdr-1* double mutants (Fig. 7E-F). We observed significant recovery of *uaDf5* levels in *atfs-1;pdr-1* double mutant animals compared to *atfs-1* single mutants. Relative to the wildtype nuclear background, the highest *uaDf5* levels were observed in *pdr-1* single mutants (Fig. 7F), consistent with the recently published observation that *uaDf5* frequency increases in *pdr-1* mutants (Valenci et al., 2015). In summary, our genetic analyses indicate that UPR<sup>mt</sup> facilitates high levels of *uaDf5* by protecting the mutant genome from mitophagy, with loss of UPR<sup>mt</sup> exposing it to mitophagy.

## Discussion

Mechanisms that regulate selfish mutant mtDNA dynamics are poorly understood. Here, we address this issue by establishing that an mtDNA mutation in *C. elegans*, *uaDf5*, behaves as a selfish genetic element. We show that the ‘runaway’ copy number dynamics of *uaDf5* are consistent with it exploiting the host's homeostatic mtDNA copy number control mechanism to achieve high frequency. We further show that the UPR<sup>mt</sup>, a mitochondrial stress response system, is activated in animals with high *uaDf5* levels. *uaDf5* frequency decreases in absence of UPR<sup>mt</sup>, suggesting that the *uaDf5* mutant also exploits UPR<sup>mt</sup> to persist at high levels. Thus, *uaDf5* can be viewed as exploiting the very homeostatic responses that its presence activates to persist and proliferate (Fig. S4). Such exploitation of endogenous pathways provides a potential explanation for how mutant mtDNA are able to achieve high frequency to cause disease in humans.

### mtDNA copy number control

Because mitochondrial respiration depends on the expression of mtDNA-encoded proteins, mtDNA copy number can serve as a proxy indicator of metabolic capacity. Furthermore, altered mtDNA copy number is implicated in numerous human diseases and prognosis (Clay Montier et al., 2009, Reznik et al., 2016). Thus, maintenance of mtDNA copy number is a subject of great research interest, and several models have been proposed. One study found that mtDNA levels in cultured human cells depend on the number of replication origins in mtDNA molecules, with replication favoring mutant molecules that contain a larger number of replication origins (Tang et al., 2000a).

Conversely, comparison of mutant and wildtype mtDNA content in homoplasmic cell lines revealed that total mtDNA mass was constant even when mutant and wildtype genomes differed in size and in number of replication origins, suggesting a possible role for the availability of nucleotides in regulating mtDNA copy number (Tang et al., 2000b). More recently, computational modeling and experimental observation support a previously proposed “maintenance of wild type” model, whereby the cell induces mtDNA replication to establish optimal mtDNA levels, which can stochastically proliferate mutant mtDNA in a heteroplasmy (Chinnery and Samuels, 1999, Capps et al., 2003, Durham et al., 2007, Tam et al., 2015).

Our investigation of mtDNA copy number dynamics in a *uaDf5* heteroplasmic *C. elegans* strain shows regulation of wildtype mtDNA levels but ‘runaway’ dynamics of *uaDf5* levels. We find that our data are most consistent with the “maintenance of wild type” model, which requires a feedback mechanism whereby mtDNA replication is inversely related to wildtype mtDNA levels, allowing cells to maintain mtDNA at optimal levels (Fig S5). When mutant mtDNA fail to support normal mitochondrial function, insufficient levels of wildtype mtDNA in a heteroplasmy induce mtDNA replication. However, random sampling by the replication machinery can lead to replication of mutant mtDNA, requiring additional rounds of replication and, in turn, production of more mutant mtDNA. Thus, this negative feedback model of copy number homeostasis predicts ‘runaway’ dynamics of mutant mtDNA, exactly like those observed for *uaDf5*. Based on these data, *uaDf5* can be viewed as taking advantage of homeostatic mtDNA copy number control to hitchhike to high frequency. Similar over-proliferation of mtDNA is observed in individuals with heteroplasmic mtDNA diseases (Durham et al., 2007). Taken together, these data suggest that exploitation of homeostatic mtDNA copy number control by mutant mtDNA might be a conserved and widespread strategy. Ours is the first report to show that this strategy can operate in the germline to regulate heteroplasmy transmission dynamics.

Studies in mammalian cell culture have shown that mutant mtDNA with large deletions like *uaDf5* may out-compete wildtype mtDNA for replication due to their smaller genome size (Wallace, 1992, Moraes et al., 1999, Diaz et al., 2002). However, mtDNA dynamics in *mptDf1* heteroplasmic animals, which harbors a very small deletion, are also like those of *uaDf5*. These data suggest that this mutant mtDNA also exploits mtDNA copy number control. While these data suggest that it is not necessary to invoke replicative advantage to explain *uaDf5* and *mptDf1* dynamics, our data do not rule out a role for such mechanisms in contributing to persistence of selfish mtDNA. Another model that ascribes an inherent advantage to mutant mtDNA was recently proposed (Kowald and Kirkwood, 2014). According to this model, the production of the output sensed to ‘count’ mtDNA might be coupled to inhibition of replication of that specific mtDNA molecule (Kowald and Kirkwood, 2014). This feature would have the effect of hastening runaway dynamics of mutant mtDNA. Our data does not exclude this possibility and it would be interesting to investigate the role of such coupling in the future.

What is the mtDNA output that is sensed to achieve mtDNA copy number control? Given that ND1 is the only gene deleted from both *uaDf5* and *mptDf1* mtDNA, it is possible that some output that is dependent on ND1 function is required to ‘count’ mtDNA. However, ND1 is one subunit of the large electron transport chain complex I. Hence, it is also possible that the output is produced by this complex and mutations in any one of the mtDNA encoded complex I subunits will result in the same runaway copy number dynamics. Consistent with a role of complex I, analysis of mtDNA deletions in mammals implicates components of complex I in regulating mtDNA copy number (Kowald and Kirkwood, 2014). Finally, it is also possible that production of the sensed output is dependent on the entire electron transport chain. In this instance, mutations in any of the mtDNA-encoded genes will result in failure of the mutant mtDNA to contribute to the output. Future experiments with additional heteroplasmies with mutations or deletions in different mtDNA-encoded genes will shed

light on this issue. Failure to contribute to the sensed output used for mtDNA copy number control might be a common feature of selfish mtDNA.

mtDNA copy number control has to occur during periods of mtDNA replication. Previous work has shown that mtDNA replication starts in L4 stage and continues throughout adulthood (Bratic et al., 2009). This replication coincides with germline proliferation. Furthermore, there is almost no increase in mtDNA copy number during these stages of development in germline free animals, suggesting that almost all mtDNA replication occurs in the developing germline (Bratic et al., 2009). Based on this data, we suggest that mtDNA copy number control occurs in the germline syncytium either continuously from L4 stage onwards, or during adulthood after mtDNA has reached steady state levels.

Interestingly, we observe a slight increase in wildtype mtDNA levels with increasing *uaDf5* levels. These data might be reflective of overcompensation in wildtype mtDNA replication. For instance, interference with wildtype mtDNA function by *uaDf5*-encoded products might result in an effective decrease in the number of functional wildtype mitochondria. Such interference might result in a cellular effort to increase levels of wildtype mtDNA above baseline.

### UPR<sup>mt</sup> in mtDNA heteroplasmy

We observed UPR<sup>mt</sup> activation in animals with high *uaDf5* levels as determined using a transgenic reporter of the *hsp-60* promoter driving GFP expression. We confirmed this UPR<sup>mt</sup> activation by measuring *hsp-6* and *hsp-60* transcript levels. How does *uaDf5* induce UPR<sup>mt</sup>? Defective protein handling inside mitochondria is believed to trigger UPR<sup>mt</sup> (Yoneda et al., 2004, Houtkooper et al., 2013, Mouchiroud et al., 2013). Here, we observe an overexpression of mtDNA-encoded transcripts that are still intact in *uaDf5* mtDNA. If these transcripts are translated, they might overwhelm the protein-handling environment in the mitochondria. Alternatively, they might result in formation of stoichiometrically imbalanced non-functional electron transport chain complexes. Decrease in GFP<sup>mt</sup> levels and fluorescence in *uaDf5* animals is consistent with the hypothesis that either import of GFP<sup>mt</sup> into mitochondria or its folding inside the matrix are compromised. Future experiments aimed at characterizing the details of protein-handling environment in *uaDf5* animals will help determine the mechanistic basis of UPR<sup>mt</sup> induction.

UPR<sup>mt</sup> activation relies on decreased import efficiency of ATFS-1 into mitochondria. Active protein import into mitochondria is known to rely on the mitochondrial membrane potential. In *uaDf5* animals, although we see UPR<sup>mt</sup> induction and a role for ATFS-1 in modulating *uaDf5* heteroplasmy levels, mitochondrial membrane potential is likely not compromised. Indeed, we actually see an increase in staining of *uaDf5* animals with TMRE, a mitochondrial membrane potential-dependent dye. This increase might not be reflective of an actual increase in mitochondrial membrane potential but probably results from an overall increase in mitochondrial organelle mass since we also observe an increase in staining with MitoTracker Green FM, a dye that localizes to mitochondria independent of the membrane potential. Overall, these data suggest a different molecular basis for decreased ATFS-1 import efficiency than decreased mitochondrial membrane potential. This conclusion is

perhaps not surprising given that ATFS-1 import efficiency has not been linked to decreased membrane potential (Nargund et al., 2012).

Our data show that  $UPR^{mt}$  is an important regulator of *uaDf5* levels. Why does loss of  $UPR^{mt}$  result in reduced *uaDf5* levels? We suggest that  $UPR^{mt}$  protects *uaDf5* against selection, with loss of  $UPR^{mt}$  sensitizing the cell and exposing *uaDf5* to selection. Selection can operate at the organismal level, which would result in elimination of animals with high *uaDf5* levels either through developmental delay or lethality. While we observe a slight developmental delay in *uaDf5* animals, this delay is not enhanced by *atfs-1* RNAi. We also did not observe enhancement in embryonic lethality or increased death up till day 4 of adulthood when we assessed decreased *uaDf5* levels in *atfs-1* RNAi. These data suggest that  $UPR^{mt}$  protects *uaDf5* against selection, perhaps at the organelle level. Under this hypothesis, mitochondria harboring high levels of *uaDf5* might be more susceptible to mitophagy in absence of  $UPR^{mt}$ . In support of this hypothesis, we observe an increase in Pink-1::GFP fluorescence in *uaDf5* animals without the ability to induce  $UPR^{mt}$ . We also observed recovery of *uaDf5* levels in *atfs-1;pdr-1* double mutants that lack the ability to induce  $UPR^{mt}$  and mitophagy. Interestingly, *pdr-1* mutants exhibit *uaDf5* levels higher than either the wildtype controls or the *atfs-1;pdr-1* double mutants, consistent with the recently published data that loss of the parkin homolog *pdr-1* in *C. elegans* results in up-regulation of *uaDf5* frequency (Valenci et al., 2015). Furthermore, overexpression of parkin is reported to result in selection against mutant mtDNA in a mammalian cell line (Suen et al., 2010). Together, the observation that *uaDf5* levels recover in *atfs-1;pdr-1* double mutants suggests that  $UPR^{mt}$  protects selfish mtDNA like *uaDf5* from organelle level selection, while the increased *uaDf5* frequency in *pdr-1* single mutants with intact  $UPR^{mt}$  likely suggests additional roles for  $UPR^{mt}$  in promoting the propagation of mutant mtDNA. These findings are consistent with recent evidence suggesting an *atfs-1*-dependent role for mitochondrial biogenesis, as well as organelle fusion-fission dynamics, in modulating mutant mtDNA levels (Lin et al., 2016).

Many mitochondrial stressors, such as exposure to paraquat or disruption of electron transport chain function, induce  $UPR^{mt}$  (Runkel et al., 2013, Durieux et al., 2011). It will be interesting to determine whether *uaDf5* levels are modulated under these conditions. Severe mitochondrial stressors might sensitize *uaDf5* animals, or trigger additional stress response mechanisms such as mitophagy (Palikaras et al., 2015). In this instance, *uaDf5* levels might decrease due to selection against high levels of *uaDf5* either at the organismal or organelle level. In contrast, *uaDf5* levels might be able to increase under mild mitochondrial stress conditions that activate  $UPR^{mt}$  without inducing significant mitophagy or significantly compromising organismal fitness. An elegant series of experiments using RNAi dilution series show that the level of mitochondrial stress is an important determinant of aging (Rea et al., 2007). These studies show that mild mitochondrial stress extends lifespan while severe mitochondrial stress can shorten lifespan. Similarly, it will be interesting to test whether varying levels of mitochondrial stress result in differential modulation of *uaDf5* levels, the prediction being that *uaDf5* levels will increase with mild mitochondrial stress but decrease with severe stress.

Given that high levels of mutant mtDNA are required to disrupt mitochondrial function and activate the UPR<sup>mt</sup>, we anticipate that as mutant mtDNA levels decrease, they would become less phenotypically consequential and hence exposed to less stringent selection. Indeed, *uaDf5* mtDNA persists, albeit at lower levels, in *afts-1* loss-of-function mutants even after continuously propagating these animals for five months. These data suggest that first, while UPR<sup>mt</sup> allows *uaDf5* to rise to high levels, it is not required for *uaDf5* persistence at lower frequency. This interpretation is consistent with our observations that UPR<sup>mt</sup> activation generally correlates with *uaDf5* levels, is likely not induced at appreciable levels in animals with *uaDf5* below a certain threshold, and that loss of UPR<sup>mt</sup> fails to impact *uaDf5* levels when they drop below this threshold. In support of this view, relatively modest shifts in heteroplasmic frequency of a pathogenic mtDNA point mutation were recently shown to coincide with abrupt changes in transcriptional profiles and phenotypic consequences in human patients (Picard et al., 2014). Such findings indicate that the cellular response to mutant mtDNA can vary considerably with even small changes in the frequency of the mutation. Accordingly, the abrupt but modest reduction (~15%) that we observe in *uaDf5* frequency in the absence of UPR<sup>mt</sup> may reflect a shift to levels at which the mutation is under more relaxed selection. Second, the persistence of *uaDf5* mtDNA in *afts-1* loss-of-function mutants suggests roles for additional mechanisms such as exploitation of mtDNA copy number control (see above). In this instance, loss of mtDNA copy number control and UPR<sup>mt</sup> might be required to force permanent loss of *uaDf5*.

As with the “maintenance of wild type” model of mtDNA copy number control, successful proliferation of mutant mtDNA via UPR<sup>mt</sup> activation does not invoke a replicative advantage of the mutant genome. In both cases, the proliferation of mutant mtDNA is linked to a deleterious effect on mitochondrial function, underscoring their behavior as selfish genomes. Consequently, we can expect that an array of mtDNA mutations of varying sizes, including small deletions and point mutations, would proliferate selfishly as long as they induce UPR<sup>mt</sup> similarly to *uaDf5*.

Exploitation of homeostatic processes might also underlie persistence of naturally occurring selfish mtDNA. Many natural isolates of *C. briggsae* are heteroplasmic for mutant mtDNA (*nad5* mtDNA) with a ~900bp deletion that disrupts an essential gene (Howe and Denver, 2008). This *nad5* mtDNA is widespread in *C. briggsae* natural populations despite its deleterious organismal effects (Estes et al., 2011). It has also been shown to have a strong drive to increase in frequency when bottlenecked through small populations (Clark et al., 2012). It will be interesting to determine in the future whether homeostatic mtDNA copy number control and UPR<sup>mt</sup> activation contribute to persistence of *nad5* mtDNA in *C. briggsae*.

Mitochondrial stress is known to promote longevity (Dillin et al., 2002, Rea et al., 2007). In particular, UPR<sup>mt</sup> is thought to mediate this extension in lifespan (Durieux et al., 2011, Houtkooper et al., 2013). Given the role of UPR<sup>mt</sup> in alleviating proteotoxic stress in mitochondria (Schulz and Haynes, 2015), it has been proposed to protect mitochondria from age-related decline in proteostasis (Taylor and Dillin, 2011, Jensen and Jasper, 2014). Here we show that UPR<sup>mt</sup> is activated in the context of high mutant mtDNA frequency, which works to restore mitochondrial function that can be affected by mutant mtDNA. However,

this indicates that the longevity-promoting protective role of UPR<sup>mt</sup> can also be viewed as a double-edged sword, by creating the conditions that allow the mutant mtDNA to propagate. Consistent with this interpretation, *uaDf5* animals expressing an *atfs-1* gain-of-function mutation that constitutively activates UPR<sup>mt</sup> exhibit reduced metabolic activity compared to *uaDf5* animals with wildtype *atfs-1* (Lin et al., 2016).

Although UPR<sup>mt</sup> is less characterized in mammals, previous reporting suggests that the mechanism by which UPR<sup>mt</sup> modulates heteroplasmy levels in *C. elegans* reported here may be widely conserved. Specifically, the accumulation of unfolded protein was shown to result in the up-regulation of the chaperone HSPD1 (HSP-60) and the mitochondrial protease CLPP in mammalian cells (Zhao et al., 2002). More recently, pharmacologically induced mitochondrial stress from rapamycin treatment was reported to correlate with UPR<sup>mt</sup> activation and increased longevity in both *C. elegans* and mice (Harrison et al., 2009, Houtkooper et al., 2013). Finally, numerous UPR<sup>mt</sup> factors, including HSPD1 and CLPP, are transcriptionally regulated by the CHOP-C/EBP $\beta$  heterodimer in mammalian cells (Aldridge et al., 2007), providing an attractive target for future studies characterizing the cellular mechanisms underlying mutant mtDNA dynamics in the context of human disease. Overall, the data presented here suggests that exploitation of homeostatic responses may represent a general strategy underlying the proliferation of pathogenic mtDNA mutations.

## Materials & Methods

### Strains

Worm strains were maintained on nematode growth medium (NGM) seeded with OP50 *E. coli* at 20°C under standard laboratory conditions.

### Mutants

LGIII, *pdr-1(gk448)*; LGV, *atfs-1(tm4525)*, *atfs-1(et15)*; mtDNA, *uaDf5*, *mptDf1*.

### Transgenic lines

zcIs9 [*hsp-60::GFP* + *lin-15(+)*], zcIs17 [*ges-1::GFP(mit)*], zcIs18 [*ges-1::GFP(cyt)*], byEx655 [*pink-1::Pink-1:GFP* + *myo-2::mcherry* + herring sperm DNA].

### Droplet digital PCR

Day 4 adults were lysed in 50 $\mu$ L lysis buffer. Lysates were diluted 1:50 or 1:100 in water (same dilution factor across all samples in an experiment) before using 2 $\mu$ L for a ddPCR reaction. For ddPCR quantification of wildtype and *uaDf5* mtDNA copy number and heteroplasmy frequency, we amplified product specifically off of wildtype mtDNA as well as a product common to wildtype and mutant templates; *uaDf5* copy number was then determined by subtracting wildtype from total mtDNA copy number. Quantification of *mptDf1* mtDNA was accomplished in a similar manner but from L4 animals. For quantification of actin, we used lysates diluted 1:5 fold.

### Gene expression quantification

Worms were lysed by briefly incubating them at 65°C for 10 minutes to generate RNA, which was then used to synthesize cDNA according to manufacturer's protocol (ThermoFisher). Quantification of gene expression was performed using ddPCR, using primers listed in supplementary material.

### Fluorescence microscopy

Worms were imaged using Zeiss Axio Zoom V16 stereo zoom microscope. Fluorescence intensities were quantified using Image J. 25 animals were used to calculate average fluorescence intensity for each group. To correlate *hsp-60::gfp* fluorescence with *uaDf5* levels, worms heteroplasmic for *uaDf5* mtDNA and expressing the *hsp-60::gfp* marker were individually picked as day 4 adults and immobilized on unseeded NGM plates treated with 250µL 10mM levamisole. Worms were individually imaged, followed by lysis and ddPCR quantification of mtDNA heteroplasmy as described above.

### TMRE and Mitotracker green FM staining

Adult animals were grown overnight on 250µL of 10µM TMRE or 50µM Mitotracker Green FM (Molecular Probes). They were allowed to recover on new plates without the dye for 1 hr before imaging.

### RNAi

RNAi bacterial cultures were grown at 37°C overnight. 750µL of the overnight culture was diluted in 75mL LB with ampicillin and grown at 37°C until  $OD_{550-600} > 0.8$ . An additional 75mL LB was added to the culture along with 1M IPTG to induce RNAi expression. Cultures were incubated an additional 3.5-4 hours at 37°C before pelleting and resuspending in M9 buffer with IPTG. These RNAi cultures were seeded onto IPTG-containing plates. L4 worms were grown on these RNAi plates and their progeny used for experimentation.

### Fitness assays

Three adults picked from population of animals growing on RNAi plates since L4 stage were allowed to lay eggs for 3 hours on corresponding fresh RNAi plates. Number of unhatched and hatched embryos were counted one day later to determine fraction of unhatched embryos. After additional two days, total number of larva and adults were counted to determine fraction of animals with delayed growth. Subsequently, all animals were transferred to fresh RNAi plates every other day until day four of adulthood. Total number of dead animals were counted until day four of adulthood to determine fraction of dead animals.

### Western blot analysis

One hundred worms in 10µL M9 were boiled in 10µL 2× SDS sample buffer for 10 minutes. SDS PAGE gel and transfer were performed according to standard protocol. Mouse monoclonal anti-beta-actin (sc-47778, Santa Cruz Biotechnology) or mouse monoclonal anti-GFP (sc-9996) were used at 1:500 dilution overnight at 4°C. HRP-conjugated goat anti-mouse antibody (sc-2005) was used at 1:5000 dilution for 90 minutes at room temperature.

SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher) was used to detect HRP.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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*uaDf5* and *mptDf1* heteroplasmic strains, *pdr-1(gk448)* and *atfs-1(e15)* mutant strain, and *zcls9*, *zcls17*, *zcls18*, and *byEx655* transgenic lines were kindly provided by Caenorhabditis Genetics Center (CGC). *atfs-1(tm4525)* mutant strain was kindly provided by the Mitani Lab through the National Bio-Resource Project of the MEXT, Japan. We would like to thank Sarah Sturgeon for technical assistance. We would like to thank O. Thompson and R. Waterston (University of Washington) for identifying *mptDf1* deletion from the Million Mutation Project worm collection. We would like to thank Harmit Malik (HHMI/Fred Hutchinson Cancer Research Center) for valuable advice and support. We thank Harmit Malik, Nitin Phadnis, Janet Young, and Sarah Zanders for providing critical comments on the manuscript. Funding for this work was provided in part by Helen Hay Whitney Foundation Fellowship (MRP), by grants from the Mathers Foundation and HHMI (to Harmit Malik), startup funds from Vanderbilt University (MRP), the NIH-sponsored Cellular, Biochemical and Molecular Sciences Training Program (5T32GM008554-18) (BLG), and the NIH-funded Tennessee Center for AIDS Research (P30 AI110527) (to SAM).

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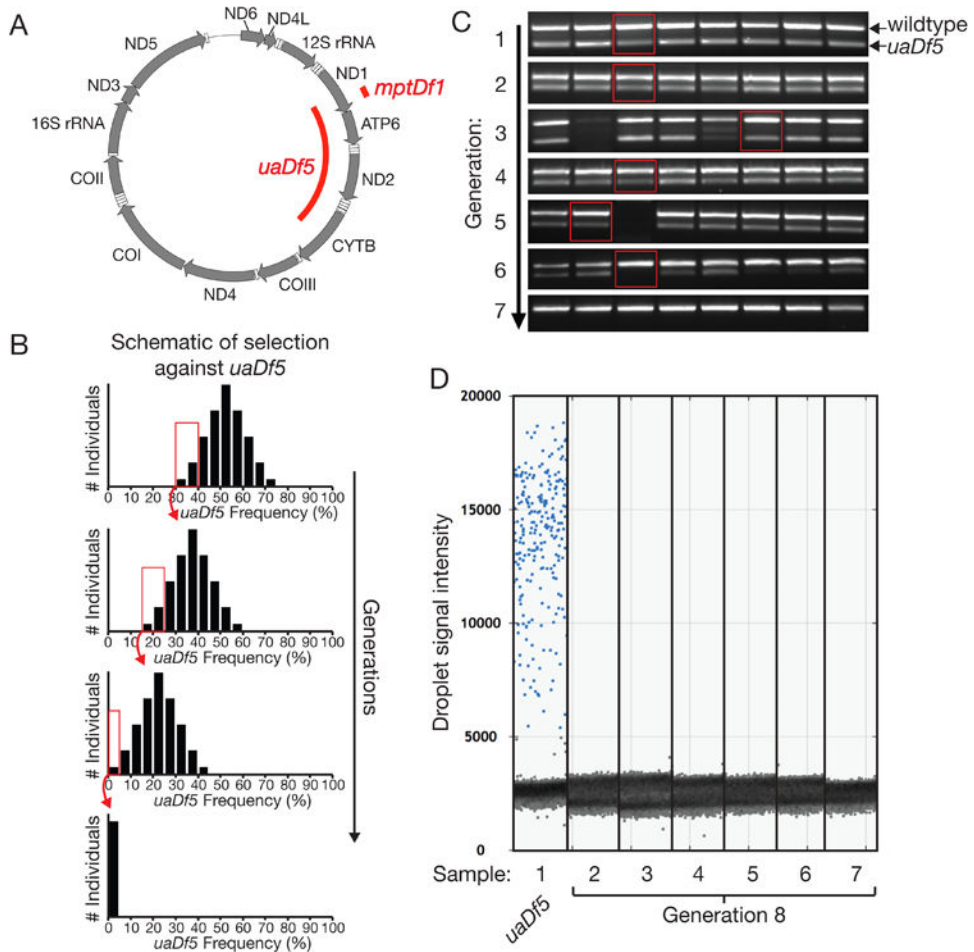
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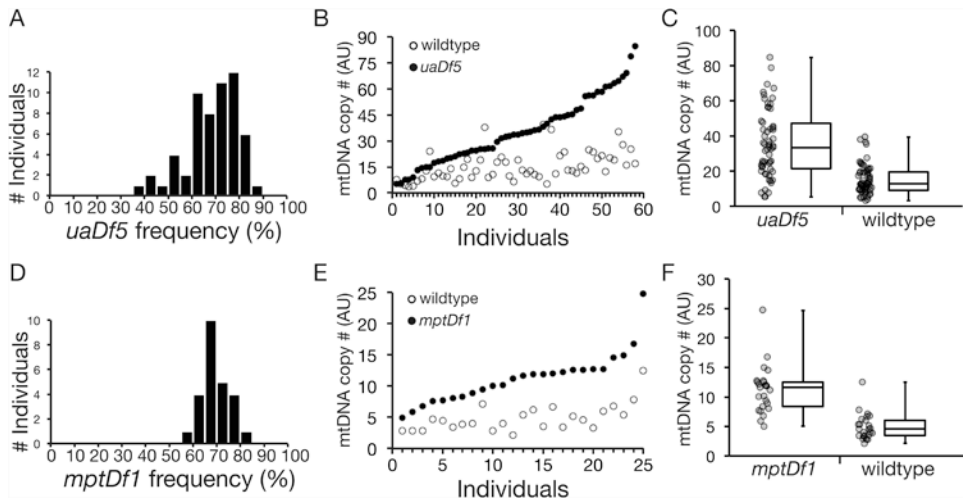
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**Highlights**

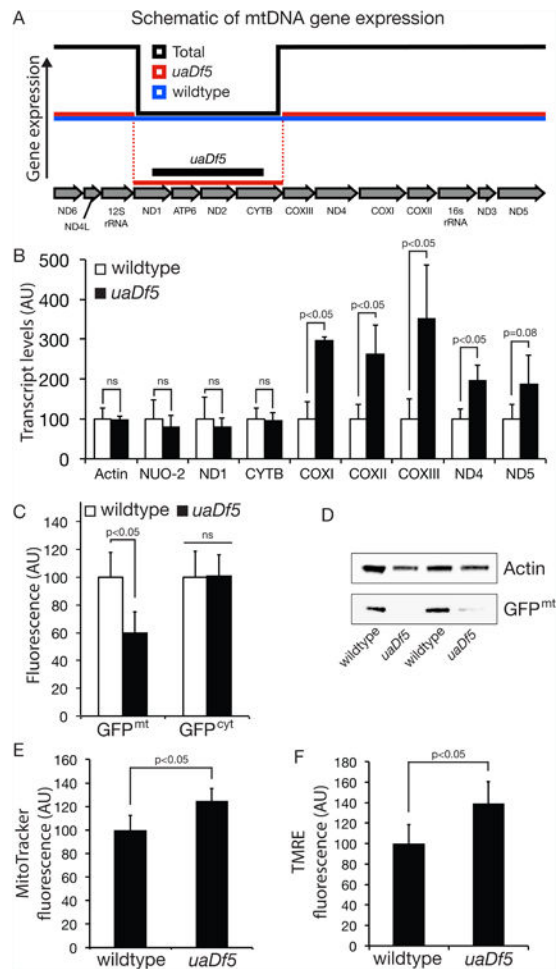
- Mutant mtDNA *uaDf5* behaves as a selfish genetic element in *C. elegans*
- Mutant mtDNA exploit mtDNA copy number homeostasis to hitchhike to high frequency
- Mitochondrial proteostasis defects in heteroplasmic animals induce UPR<sup>mt</sup>
- UPR<sup>mt</sup> protects mutant mtDNA from parkin-mediated mitophagy



**Figure 1. Mutant mtDNA *uaDf5* can be forced out from a stably persisting heteroplasmity in *C. elegans***  
 (A) Schematic of *C. elegans* mtDNA showing the *uaDf5* and *mptDf1* deletions (long and short red bars, respectively). Grey arrows show protein and rRNA-encoding genes and their orientation. White boxes show genes encoding tRNAs. (B) Schematic illustrating the selection strategy to force loss of *uaDf5* mtDNA from a heteroplasmic *C. elegans* line. Each generation, the progeny of individuals with the lowest *uaDf5* levels were selected for subsequent propagation. (C) Single worm PCR of wildtype and *uaDf5* mtDNA. Successive propagation of individual worms with low *uaDf5* levels (red boxes) results in complete loss of *uaDf5* mtDNA from the population over multiple generations. (D) ddPCR data from single worms confirming complete loss of *uaDf5*. Positive droplets containing *uaDf5*-specific PCR product exhibit increased fluorescence intensity (blue) compared to negative droplets that contain no *uaDf5* mtDNA (gray). For each droplet, the droplet reader detects droplet size, shape, and fluorescence intensity, and automatically distinguishes positive from negative droplets on the basis of these criteria. Sample 1, control containing *uaDf5*.

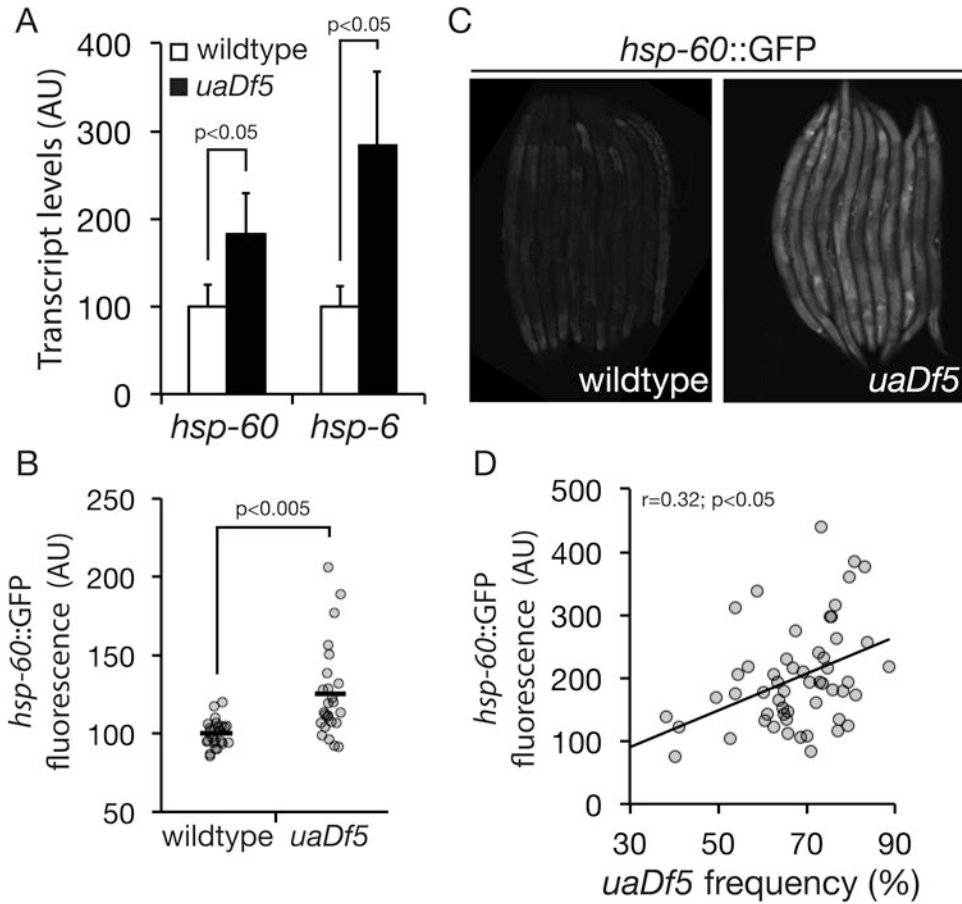


**Figure 2. Quantification of mtDNA copy number dynamics reveals mtDNA copy number control** (A) Histogram showing *uaDf5* frequency (%) distribution in individuals from a population stably maintaining *uaDf5* mtDNA. Heteroplasmy frequency was determined using ddPCR to quantify wildtype and *uaDf5* mtDNA copy number in single individuals. (B) mtDNA levels in individual day 4 adult worms, normalized to actin and rank-ordered by *uaDf5* mtDNA copy number. (C) Wider variation in *uaDf5* relative to wildtype copy number ( $p < 0.05$ ) suggests that wildtype mtDNA, but not *uaDf5* mtDNA, is subject to homeostatic copy number control. Grey data points show mtDNA copy number from single individuals. Box and whisker plot shows the median, lower and upper quartile (boxes), and minimum and maximum (error bars) mtDNA copy number. (D) *mptDf1* frequency distribution obtained from single individuals from a population stably maintaining *mptDf1* heteroplasmy. (E) mtDNA levels in individual L4 worms, normalized to actin and rank-ordered by *mptDf1* copy number. (F) Similar to *uaDf5*, wider variation in *mptDf1* relative to wildtype copy number ( $p < 0.05$ ) suggests that wildtype mtDNA, but not *mptDf1* mtDNA, is subject to homeostatic copy number control. AU, arbitrary units.



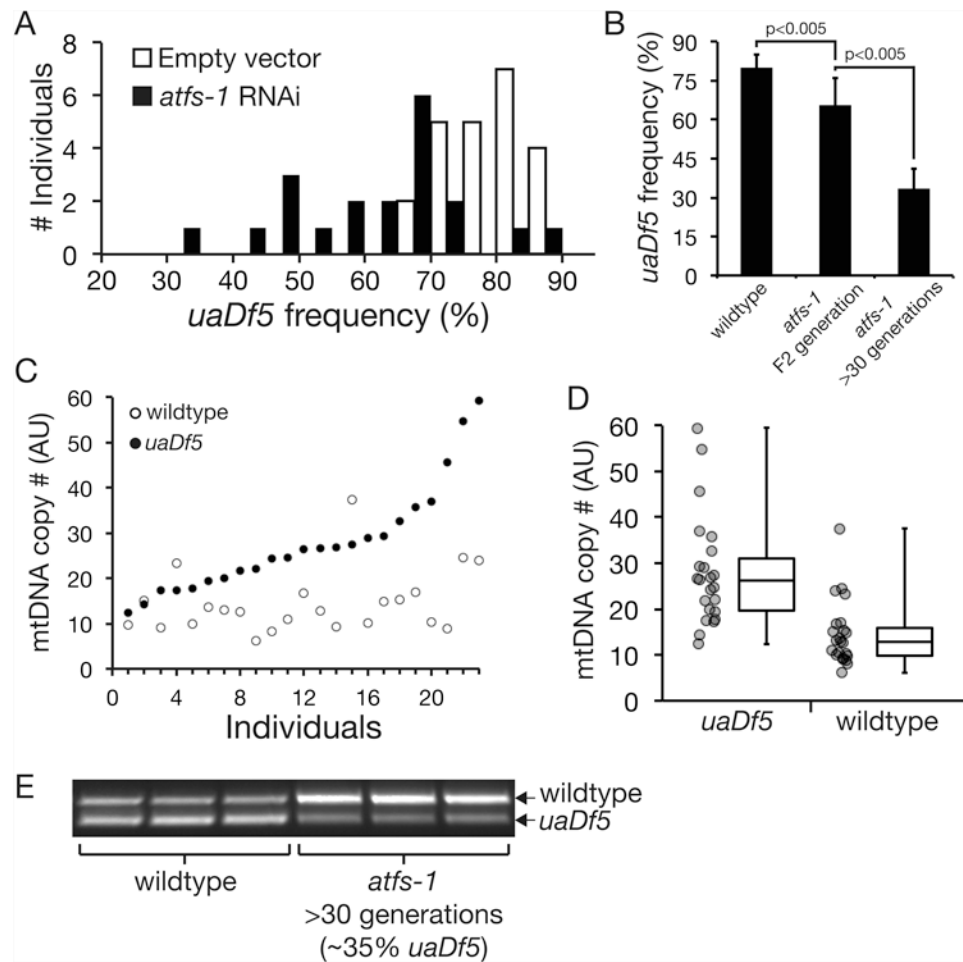
### Figure 3. Mitochondrial function is perturbed in *uaDf5* animals

(A) Schematic showing expected expression of mtDNA-encoded transcripts. The presence of *uaDf5* mtDNA is expected to result in stoichiometric imbalance of gene expression, as the expression of *uaDf5* and wildtype mtDNA copies (red and blue lines, respectively) combine to generate total expression (black line) at elevated levels for genes located outside the deletion but at wildtype levels for genes missing from the *uaDf5* mtDNA. (B) Animals heteroplasmic for *uaDf5* exhibit expression levels similar to that of wildtype animals for mtDNA-encoded genes affected by the deletion (*CYTB* and *ND1*), as well as a nuclear-encoded mitochondrial gene (*NUO2*) and actin. However, *uaDf5* heteroplasmy results in overexpression for mtDNA-encoded genes located outside the *uaDf5* deletion (*COXI*, *COXII*, *COXIII*, *ND4*, and *ND5*). All transcript levels are normalized to wildtype. Error bars represent standard deviation. (C) Mitochondrially targeted GFP (GFP<sup>mt</sup>), but not cytosolic GFP (cGFP<sup>cyt</sup>), is significantly reduced in *uaDf5* heteroplasmic individuals. (D) Western blot analysis of wildtype and *uaDf5* heteroplasmic animals expressing GFP<sup>mt</sup> reveals reduced levels in *uaDf5* heteroplasmic individuals relative to actin. Data are shown from two biological replicates each for wildtype and *uaDf5* strain. (E) Fluorescence increase in *uaDf5* animals stained with mitochondrial membrane potential independent dye MitoTracker Green FM and (F) membrane potential dependent dye TMRE. Error bars represent standard deviation. AU, arbitrary units.



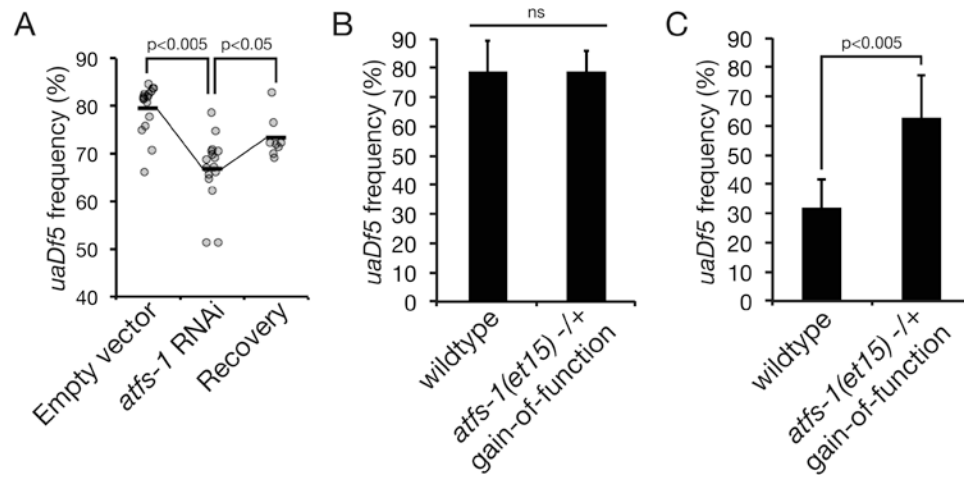
**Figure 4. UPR<sup>mt</sup> is activated in heteroplasmic animals carrying *uaDf5* mtDNA**

(A) Transcription of two UPR<sup>mt</sup>-activated molecular chaperones (*hsp-60* and *hsp-6*) is increased in individuals with *uaDf5* compared to wildtype individuals. (B) Quantification of fluorescence between wildtype homoplasmic and *uaDf5* heteroplasmic animals shows increased activation of the UPR<sup>mt</sup> marker *hsp-60::GFP* in the presence of *uaDf5* mtDNA. Each data point is from a single individual picked randomly from a population. (C) Visual comparison of GFP fluorescence between *uaDf5* and wildtype animals, each expressing *hsp-60::GFP*. Wildtype animals were picked at random from a population but only *uaDf5* animals with apparent fluorescence were picked to show UPR<sup>mt</sup> activation. (D) Positive relationship between *uaDf5* frequency and *hsp-60::GFP* fluorescence (trendline) indicates that UPR<sup>mt</sup> activation increases at higher *uaDf5* frequency. Each data point corresponds to a single individual. Error bars represent standard deviation. AU, arbitrary units.

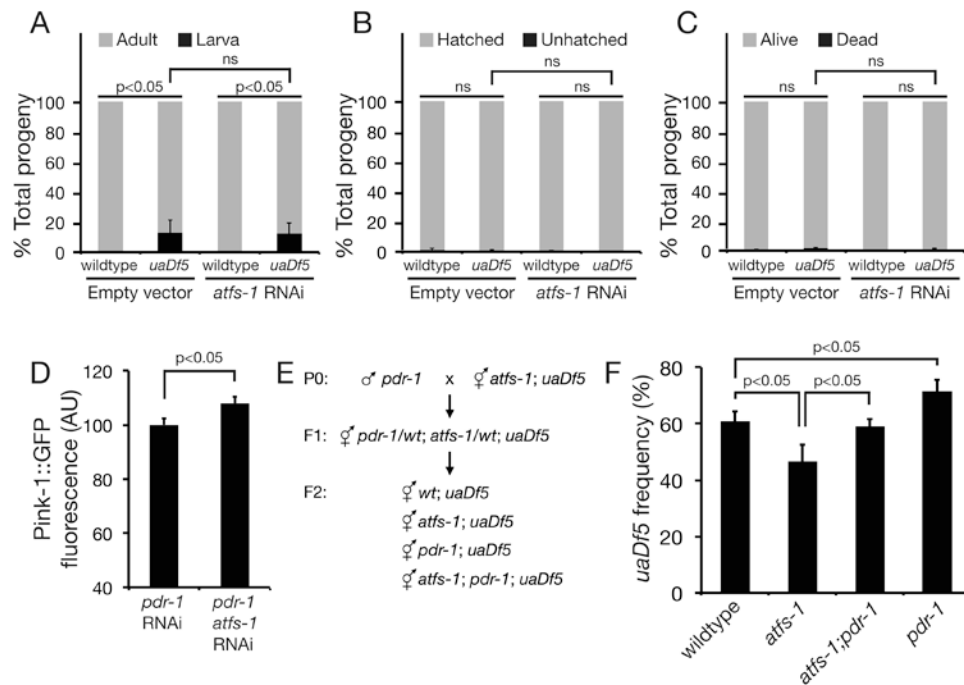


**Figure 5. Loss of UPR<sup>mt</sup> activation results in decreased *uaDf5* levels but does not affect mtDNA copy number control**

(A) Growth under RNAi-mediated knockdown of *atfs-1*, required for UPR<sup>mt</sup> activation, results in a shift to lower *uaDf5* frequency relative to growth under control conditions ( $p < 0.05$ ). (B) *uaDf5* frequency decreases in heteroplasmic animals homozygous for the *atfs-1(tm4525)* loss-of-function allele compared to heteroplasmic animals that express wildtype *atfs-1*, in which high *uaDf5* levels are stably maintained. *uaDf5* frequency decreases further in the *atfs-1* null animals after multiple generations but is not lost completely. (C) Quantification of mtDNA copy number in individual day 4 adult animals homozygous for the *atfs-1* loss-of-function allele, normalized to actin and rank-ordered by *uaDf5* mtDNA copy number. (D) Wider variation in *uaDf5* relative to wildtype copy number ( $p < 0.05$ ) in *atfs-1* null animals suggests that mtDNA copy number control persists in absence of UPR<sup>mt</sup>. (E) PCR of single heteroplasmic individuals against the *atfs-1* wildtype or *atfs-1* null nuclear background shows that *uaDf5* is retained in both lines but is at lower levels in the null animals after about 30 generations. Note that because mutant and wildtype templates compete for amplification, the wildtype band appears fainter when *uaDf5* levels are high but does not actually reflect reduced wildtype mtDNA levels (see Fig. 2B). Error bars represent standard deviation. AU, arbitrary units.



**Figure 6. Persistence of *uaDf5* at high frequency depends in part on UPR<sup>mt</sup> activation**  
 (A) Growth under RNAi-mediated knockdown of *atfs-1* across seven generations reduces average *uaDf5* frequency. However, restoration of *atfs-1* expression by returning *atfs-1* knockdown animals to control conditions results in recovery of elevated *uaDf5* frequency in a single generation. (B) When starting *uaDf5* frequency is high (75-80%), constitutive UPR<sup>mt</sup> activation in individuals heterozygous for an *atfs-1* gain-of-function allele causes no further rise in average *uaDf5* frequency; (C) however, *uaDf5* frequency rises when the *atfs-1* gain-of-function allele is crossed into a strain harboring lower *uaDf5* levels (~30%). Error bars represent standard deviation.



### Figure 7. UPR<sup>mt</sup> protects *uaDf5* from mitophagy

(A) Heteroplasmic individuals exhibit delayed growth: as 100% of progeny from wildtype parents reach adulthood in three days, approximately 10% of *uaDf5* progeny remain in the larval stage. Knockdown of *atfs-1* showed no effect on development in homoplasmic wildtype animals and did not further enhance developmental delay in *uaDf5* heteroplasmic animals. (B) No significant difference was observed between *uaDf5* and wildtype animals, or between *atfs-1* knockdown and control conditions, on the percentage of embryos that remain unhatched after one day or (C) on the percentage of lethality among day 4 adults. (D) Quantification of Pink-1::GFP fluorescence shows increased mitophagy in *uaDf5* animals upon *pdr-1;atfs-1* double knockdown compared to knockdown of *pdr-1* alone. AU, arbitrary units. (E) Crossing scheme employed to isolate *uaDf5* animals in wildtype, *atfs-1* null, *pdr-1* null, and *atfs-1;pdr-1* double mutant backgrounds. (F) Quantification of *uaDf5* levels shows recovery of *uaDf5* levels in *atfs-1;pdr-1* double mutants compared to *atfs-1* single mutant animals. *uaDf5* recovers to the highest levels in *pdr-1* single mutants. Error bars represent standard deviation. AU, arbitrary units.