Loss of flavin adenine dinucleotide (FAD) impairs sperm function and male reproductive advantage in *C. elegans.*

Chia-An Yen[1,2], Dana L. Ruter[1,2], Christian D. Turner[1,2], Shanshan Pang[3], and Sean P. Curran[1,2,4*]

1. Leonard Davis School of Gerontology, University of Southern California, Los Angeles, CA 90089
2. Department of Molecular and Computation Biology, Dornsife College of Letters, Arts, and Sciences, University of Southern California, Los Angeles, CA 90089
3. School of Life Sciences, Chongqing University, Chongqing 401331, China.
4. Norris Comprehensive Cancer Center, Keck School of Medicine, University of Southern California, Los Angeles, CA 90089

Correspondence to: spcurran@usc.edu

KEYWORDS

spermatogenesis, mitochondria, germ cells, reproduction, proline catabolism, alh-6/ALDH4A1, C. elegans, senescence, aging, male-specific, FAD, flavin cofactor, riboflavin

ABSTRACT

Exposure to environmental stress is clinically established to influence male reproductive health, but the impact of normal cellular metabolism on sperm quality is less well-defined. Here we show that impaired mitochondrial proline catabolism, reduces energy-storing flavin adenine dinucleotide (FAD) levels, alters mitochondrial dynamics toward fusion, and leads to age-related loss of sperm quality (size and activity), which reduces competitive fitness. Loss of the 1-pyrroline-5-carboxylate dehydrogenase enzyme *alh-6* that catalyzes the second step in mitochondrial proline catabolism, leads to premature male reproductive senescence. Reducing the expression of the proline catabolism enzyme *alh-6* or FAD biosynthesis pathway genes in the germline is sufficient to recapitulate the sperm-related phenotypes observed in *alh-6* loss-of-function mutants. These sperm-specific defects are suppressed by feeding diets that restore FAD levels. Our results define a cell autonomous role for mitochondrial proline catabolism and FAD homeostasis on sperm function and specify strategies to pharmacologically reverse these defects.

INTRODUCTION

As individuals wait longer to have families, reproductive senescence has become an increasingly prudent topic (Lemaitre & Gaillard; Mills et al.). Decline in oocyte quality is well-documented with age and can result in fertility issues when older couples try to conceive (Baird et al.). Furthermore, pregnancies at an older age pose risks for higher incidences of birth defects and miscarriages. In humans, female reproduction ceases at an average age of 41-60, with the onset of menopause (Treloar). The *Caenorhabditis elegans* "wild type" is hermaphroditic and self-fertilizing; however, they are capable of making and maintaining Mendelian ratios of male (sperm-only) animals in their populations. Like humans, *C. elegans* experience a decline in fecundity with age by halting oocyte production at roughly one-third of their lifespan (Kadandale & Singson). In addition, regulators of reproductive aging, such as insulin/IGF-1 and *sma*-2/TGF-β signaling, are conserved regulators of reproductive aging from worms to humans (Luo, Kleemann, Ashraf, Shaw, & Murphy). While the majority of studies in reproductive senescence have focused on maternal effects, male factors contribute to a large portion of fertility complications with increasing evidence of an inverse relationship between paternal age and sperm health (Lemaitre & Gaillard). In fact, studies in mammals have shown an age-related decline in sperm quality with increased incidences of DNA damage, reduced motility, abnormal morphology, and decreased semen volume (Cocuzza et al.; Kidd, Eskenazi, & Wyrobek; Ozkosem, Feinstein, Fisher, & O'Flaherty).

Flavin adenine dinucleotide (FAD) is an important cofactor that participates in enzymatic redox reactions that are used in cellular metabolism and homeostasis. FAD is synthesized from riboflavin by the concerted actions of FAD synthetase and riboflavin kinase. Like humans, *C. elegans* cannot synthesize riboflavin, and therefore requires dietary intake (Braeckman, Houthoofd, & Vanfleteren). Disruption of flavin homeostasis in humans and animal models has been associated with several diseases, including: cardiovascular diseases, cancer, anemia, abnormal fetal development, and neuromuscular and neurological disorders (Barile et al.).; however, a link between FAD homeostasis and fertility is undefined.

Several studies have documented fertility defects in *C. elegans* mitochondrial mutants. Mutation in *nuo-1*, a complex I component of the mitochondria respiratory chain, results in reduced brood size caused by impaired germline development (Grad & Lemire). Similarly, *clk-1* mutation affects the timing of egg laying, resulting in reduced brood size (Jonassen, Marbois, Faull, Clarke, & Larsen). Both of these mitochondrial mutations impact fertility, but their role(s) in spermatogenesis are unclear. *alh-6*, the *C. elegans* ortholog of human *ALDH4A1*, is a nuclear-encoded mitochondrial enzyme that functions in the second step of the proline metabolism pathway, converting 1-pyrroline-5-carboxylate (P5C) to glutamate (Adams & Frank). We previously revealed that *alh-6(lax105)* loss-of-function mutants display altered mitochondrial structure in the muscle accompanied by increased level of ROS in adult animals (Pang & Curran). Furthermore, mutation in *alh-6* results in the activation of SKN-1/NRF2 (Pang, Lynn, Lo, Paek, & Curran), an established regulator of oxidative stress response, likely through the accumulation of toxic P5C disrupting mitochondrial homeostasis (Deuschle et al.; Miller et al.; Nomura & Takagi; Pang & Curran; Pang et al.). Interestingly, SKN-1 was recently shown to respond to accumulation of damaged mitochondria by inducing their biogenesis and degradation through autophagy (Palikaras, Lionaki, & Tavernarakis). Here, we identify a genetic pathway for regulating male reproductive decline stemming from perturbation of mitochondrial proline metabolism leading to redox imbalance, cofactor depletion, and altered mitochondria dynamics; all of which play a role in sperm dysfunction.

RESULTS

Mutation in mitochondrial *alh-6* results in diet-independent reduction in fertility

Altered mitochondrial structure and activity have been correlated with sperm dysfunction across different species (Amaral, Lourenco, Marques, & Ramalho-Santos; Liau, Gonzalez-Serricchio, Deshommes, Chin, & LaMunyon; Nakada et al.; Ramalho-Santos & Amaral). In addition, proper sperm function requires low levels of ROS (de Lamirande & Gagnon; Kodama, Kuribayashi, & Gagnon; Leclerc, de Lamirande, & Gagnon), although a specific role for endogenous mitochondrial derived ROS is undefined. ALH-6/ALDH4A1, is a nuclear-encoded mitochondrial enzyme that functions in the second step of proline catabolism, converting 1-pyrroline-5-carboxylate (P5C) to glutamate (**Figure 1A**). We anticipated that mutation of *alh-6* may affect the germline, based on our previous assessment of the premature aging phenotypes in somatic cells in *alh-6* mutants (Pang & Curran). Using a UV-integrated *alh-6::gfp* strain under its endogenous promoter, we saw that ALH-6 localizes to the mitochondria in the germline of both hermaphrodites (Figure S1A-B) and males (Figure S1C-D). We then assessed progeny output of *alh-6(lax105)* hermaphrodites fed the standard OP50/*E. coli* B strain diet and found a reduction in self-fertility brood size (-12.9%) (**Figure 1B**). Since the somatic phenotypes of *alh-6(lax105)* mutants are known to be diet-dependent (Pang & Curran; Pang et al.), we examined self-fertility of animals fed the HT115/*E. coli* K-12 strain diet to determine if the reduced reproductive output is also dependent on the type of bacterial diet ingested. Surprisingly, we found that the self-fertility of *alh-6* animals was markedly reduced (-20.7%), when animals were fed the HT115 diet (**Figure 1C**). *alh-6* mutants have similar timing in their progeny output as compared to wild type animals on both diets (Figures S2A-B). Since *alh-6* mutants display normal development and reproductive timing, the progeny deficit is not a result of an attenuated reproductive span which reveals the differential impact of *alh-6* loss in the soma (diet-dependent) (Pang & Curran) and the germline (diet-independent).

alh-6 fertility defects are sperm-specific

We noted that *alh-6* mutant hermaphrodite animals laid twice as many unfertilized oocytes as wild type animals over their reproductive-span (**Figure 1D)**, suggesting an impairment of sperm function (Argon & Ward; McCarter, Bartlett, Dang, & Schedl; Ward & Miwa)**.** It is notable that *alh-6* mutant hermaphrodites lay very few, if any, dead eggs (**Figure 1D**), suggesting that the loss of ALH-6 activity is not lethal. To determine whether the reduced brood size of *alh-6* mutants are due to a general loss of germ cells or a specific defect in oocytes or sperm, we examined the mated-fertility of these animals by mating wild type young adult (day 0-1) males to either wildtype or *alh-6* mutant virgin hermaphrodites (in wild type *C. elegans*, male sperm outcompetes hermaphrodite sperm >99% of the time (LaMunyon & Ward; Ward & Carrel) (**Figure 1E**). We found that the reduced fertility in *alh-6* mutant hermaphrodites is fully rescued by wild type sperm, which confirmed that oocyte quality is not impaired but rather, *alh-6* hermaphrodite sperm appears to be dysfunctional (**Figure 1F**).

To better assess the quality of *alh-6* mutant sperm, we compared the ability of *alh-6* mutant male sperm to compete against wild type hermaphrodite sperm (Singson, Hill, & L'Hernault). In *C. elegans* wild type animals, male sperm are larger and faster than hermaphrodite sperm, which affords a competitive advantage (LaMunyon & Ward). To differentiate between progeny resulting from mating and progeny that arise from hermaphrodite self-fertilization, we made use of male animals harboring a GFP transgene such that any cross-progeny will express GFP while progeny that arise from hermaphrodite self-sperm will not (**Figure 1E)**. We found that wild type hermaphrodites when mated to *alh-6* mutant males have significantly more self-progeny as compared to wild type hermaphrodites mated to wild type males (**Figure 1G**). This finding indicates a sperm competition deficit of *alh-6* males resulting in an increased proportion of progeny derived from self-fertilization, which is uncommon after mating has occurred (Ward & Carrel). *C. elegans* hermaphrodites produce a set amount of sperm exclusively at the L4

165 developmental stage, before switching exclusively to oogenesis. As such, hermaphrodites eventually
166 deplete their reservoir of sperm (Hirsh, Oppenheim, & Klass; Ward & Carrel). To assess whether *alh-6*
167 mutant sperm are generally dysfunctional, we mated older hermaphrodites that had depleted their
168 complement of self-sperm and found that *alh-6* mutant males are able to produce equal numbers of
169 progeny as wild type males when the need for competition with hermaphrodite sperm is abated (Figure
170 S3A); thus, although *alh-6* mutant sperm are impaired for competition, they remain viable for
171 reproduction. Similarly, older sperm-depleted *alh-6* mutant hermaphrodites produced similar brood sizes
172 when mated to young wild type or *alh-6* mutant males, which further supports a model where sperm, but
173 not oocytes, are defective in *alh-6* mutants (Figure S3B).
174
175 **Defects in mitochondrial proline catabolism impact sperm quality**
176 Similar to mammals, the contribution of sperm to fertility in *C. elegans* is dictated by distinct
177 functional qualities, which include: sperm number, size, and motility (LaMunyon & Ward; Singson et al.).
178 We next sought to define the nature of the sperm competition defect in *alh-6* mutants by measuring sperm
179 number, size, and motility in *alh-6* mutants compared to wild type animals. One day after the on set of
180 spermatogenesis (at the L4 larval stage of development), *alh-6* adult hermaphrodites have a reduced
181 number of sperm in the spermatheca as compared to wild type (Figure S4A), which is correlated with the
182 reduced self-fertility observed (**Figures 1B-C**). In contrast, age-matched *alh-6* mutant virgin males have
183 similar numbers of spermatids as WT virgin males, suggesting that they have a similar rate of production
184 (**Figure 2A**). We next examined sperm size in day 1 adult males and discovered that *alh-6* mutant
185 spermatids are significantly smaller as compared to wild type (**Figure 2B**). To achieve motility, *C. elegans*
186 spermatids must be activated to allow pseudopod development which requires protease activation (Ward,
187 Hogan, & Nelson) (Figure S4B). Sperm activation can be recapitulated *in vitro* by treatment of isolated
188 spermatids with the *Streptomyces griseus* protease Pronase (Shakes & Ward). After 30 minutes of
189 Pronase treatment, 80% of wildtype spermatids are fully activated, while a significantly reduced
190 population of in *alh-6* mutant spermatids mature over the same time period (**Figure 2C**). Interestingly,
191 although sperm number was the same between WT and *alh-6* mutant males on the OP50 diet, sperm
192 number was reduced in *alh-6* mutant males fed HT115 diet compared to age-matched WT males on the
193 same diet (**Figure 2D**). We also noted that spermatids from *alh-6* mutant males raised on the HT115
194 diet were similarly defective in size and activation (**Figure 2E-F).** Taken together, although diet can
195 influence sperm number, the reduction of sperm size and activation are likely contributors to the reduced
196 fertility and competitive fitness in *alh-6* mutant males; which is independent of diet.
197
198 **Transcriptional signatures define temporal phenotypes of *alh-6* mutant animals**
199 We first identified *alh-6* mutant in a screen for activators of the cytoprotective transcription factor
200 SKN-1/NRF2 using *gst-4p::gfp* as a reporter (Pang & Curran; Pang et al.). When activated, SKN-1
201 transcribes a variety of gene targets that collectively act to restore cellular homeostasis. However, this can
202 come with an energetic cost with pleiotropic consequences (An & Blackwell; Blackwell, Steinbaugh,
203 Hourihan, Ewald, & Isik; Glover-Cutter, Lin, & Blackwell; Lynn et al.; Paek et al.; Palikaras, Lionaki, &
204 Tavernarakis; Pang & Curran; Pang et al.). *alh-6* mutants have normal development, but display progeroid
205 phenotypes towards the end of the normal reproductive span (Pang & Curran) indicating a temporal switch
206 in phenotypic outcomes. We reasoned that the temporally controlled phenotypes in the *alh-6* mutants
207 could be leveraged to identify potential mechanisms by which *alh-6* loss drives cellular dysfunction. As
208 SKN-1 is activated in *alh-6* mutants after day 2 of adulthood (Pang & Curran), we defined genes that
209 display differentially altered expression in the L4 developmental stage, when spermatogenesis occurs, as
210 compared to day 3 adults (post SKN-1 activation). We performed RNA-Seq analyses of worms with loss
211 of *alh-6* and identified 1935 genes in L4 stage animals and 456 genes in day 3 adult animals that are
212 differentially expressed (+/- Log_2 (fold change), 0.05 FDR) (**Figure S5**). Notably, the gene expression

213 changes at these two life periods had distinct transcriptional signatures (**Figures 3A-B**). Because the loss
214 of *alh-6* drives compensatory changes in normal cellular metabolism, which later in life results in the
215 activation of SKN-1, we expected to identify significant changes in both metabolic genes and SKN-1
216 target genes. Supporting this hypothesis, the Gene Ontology (GO) terms most enriched include
217 oxidoreductases and metabolic enzymes in L4 stage animals (**Figure 3A**) and SKN-1-dependent targets
218 such as glutathione metabolism pathway genes in day 3 adults (**Figure 3B**). Importantly, our
219 transcriptomic analysis recapitulated the temporally-dependent phenotypic outcomes resulting from *alh-*
220 *6* loss; genes in the pseudopodium and germ plasm GO terms class displayed reduced expression in L4
221 *alh-6* mutant animals (**Figure 3A**), which include many genes in the major sperm protein (MSP) family
222 that comprises 15% of total protein content in *C. elegans* sperm and impact sperm function (Klass &
223 Hirsh). In contrast, genes in the muscle-specific GO term class displayed increased expression in day 3
224 adults (**Figure 3B**), which is when SKN-1 activity is enhanced in the muscle of *alh-6* mutants (Pang et
225 al.). Taken together, the transcriptomic analysis of *alh-6* mutants is diagnostically relevant and informative
226 for defining drivers of organism-level phenotypic changes in animals with altered proline catabolism.
227
228 **FAD mediates sperm functionality and competitive fitness**
229 The strong enrichment of genes whose protein products utilize and/or bind cofactors or co-
230 enzymes was intriguing as the maintenance of metabolic homeostasis and the redox state of the cell
231 requires a sophisticated balance of multiple cofactors (**Figure 4A**). In fact, the proline catabolism pathway
232 utilizes multiple cofactors to generate glutamate from proline; PRDH-1 uses FAD as a co-factor to convert
233 proline to P5C while ALH-6 utilizes the reduction of NAD+ to convert P5C to glutamate. Additionally,
234 in the absence of ALH-6, accumulation of P5C, the toxic metabolic intermediate of proline catabolism,
235 drives the expression of pathways to detoxify P5C (oxidoreductases, P5C reductase, etc.) (**Figure 3,**
236 Figure S6A). We measured FAD and found a significant reduction in *alh-6* mutant animals fed the OP50
237 diet at the L4 stage (**Figure 4B**) and a similar reduction in animals fed HT115 bacteria at L4 stage (**Figure**
238 **4C**). Differences in FAD levels were unremarkable in day 3 adult animals, when spermatogenesis has
239 long since ended (Figure S6B). Based on this finding, we predicted that restoration of FAD levels might
240 alleviate the sperm-specific phenotypes of *alh-6* mutants. Riboflavin is a precursor of FAD (**Figure 4D**)
241 and dietary supplementation of riboflavin has been shown to increase cellular FAD levels in wild-type
242 animals (Burch, Combs, Lowry, & Padilla; Redondo, Menasche, & Le Beau). Similarly, riboflavin
243 supplementation to the OP50 diet of *alh-6* mutants restored FAD levels to wild-type levels (**Figure 4E**).
244 We found that wild type hermaphrodites mated to *alh-6* mutant males fed a riboflavin supplemented diet
245 produced significantly more total progeny than *alh-6* males fed the standard OP50 diet (Figure S6C).
246 Moreover, riboflavin supplementation was sufficient to partially restore male sperm size (**Figure 4F**) and
247 also rescued the impaired activation (**Figure 4G**) of male sperm in *alh-6* mutants. Riboflavin
248 supplementation increases sperm size in WT males, but do not change sperm activation in WT males
249 (Figure S6D-E).
250 We next asked whether FAD metabolism was required for proper sperm function. FAD can be
251 synthesized *de novo* by a two-step enzymatic reaction where riboflavin is converted to FMN by Riboflavin
252 Kinase/R10H10.6, which is subsequently converted to FAD by FAD synthase/FLAD-1 (**Figure 4D**). We
253 used RNA interference (RNAi) against *R10H10.6* or *flad-1* in wild-type male animals and measured sperm
254 quality. Similar to *alh-6* mutant sperm, RNAi reduction of the FAD biosynthetic pathway decreased
255 sperm size (**Figure 4H, 4I,** Figure S6F, S6G) and impaired sperm activation (**Figure 4J, 4K,** Figure S6F,
256 S6G)**.**
257 NAD+ and NADH are also central adenine dinucleotide cofactors that play critical roles in
258 metabolism and have received recent attention as a method to combat the decline seen in biological
259 function with age(Guarente). As such, we also measured NAD and NADH levels, but found the ratio

unremarkable between wild-type and *alh-6* mutant animals (Figure S6F-H). Taken together, these data suggest that loss of *alh-6* leads to a specific decrease in cellular FAD levels and that FAD is a critical cofactor that drives proper sperm function.

Mitochondrial dynamics regulate spermatid function

Although there is a clear and documented role for mitophagy in the clearance of paternal mitochondria post-fertilization in *C. elegans*, the role(s) for mitochondrial dynamics and turnover in sperm function prior to zygote formation are unclear. We first examined mitochondrial dynamics in wild type spermatids by staining with the fluorescent mitochondrial-specific dye JC-1 (ThermoFisher), and noted that each spermatid on average contained multiple discernable spherical mitochondria that are mostly not fused (**Figures 5A, 5B, 5E**). Previous studies in yeast and cultured mammalian cells have shown that when cells are exposed to mild stress, the initial response of mitochondria is to fuse in order to dilute damage (Gomes, Di Benedetto, & Scorrano; Rambold, Kostelecky, Elia, & Lippincott-Schwartz; Tondera et al.). Indeed *alh-6* mutant spermatids had mitochondria that were more interconnected (**Figures 5C-E**) as compared to wild type spermatids, which suggests that *alh-6* sperm are under metabolic stress. The increase in fused mitochondria in spermatids was also present in animals fed the HT115 diet, which further supports a diet-independent role for *alh-6* in the germline (Figure S7A).

The JC-1 dye accumulates in mitochondria in a membrane potential-dependent manner, and as concentration exceeds threshold, its fluorescence switches from green to red emission; thus, a higher red-to-green fluorescence ratio is indicative of healthier mitochondria. *alh-6* mutant spermatids have reduced red:green JC-1 fluorescence that indicates a lower mitochondrial membrane potential, and an accumulation of unhealthy mitochondria (**Figure 5F**).

A connection between mitochondrial dynamics (fusion and fission) and FAD homeostasis has not been previously described. To understand this, we perturbed FAD biosynthesis pathway and then examined mitochondrial connectivity in spermatids. We first reduced FAD biosynthesis with RNAi targeting *R10H10.6* or *flad-1*, which resulted in more connected mitochondria that resembles the increased fusion in *alh-6* mutant spermatids that are under metabolic stress (**Figure 5G-H,** Figure S6A). In addition, increasing FAD levels by dietary supplementation of riboflavin, restored mitochondria in spermatids of *alh-6* animals to more wild-type-like distributions (**Figure 5I**), but did not change mitochondrial morphology in WT male spermatids (Figure S7B). Thus, the reduction of FAD in *alh-6* mutants, alters mitochondrial dynamics to a more fused and less punctate state. Therefore, the homeostatic control of FAD level is critical to maintain proper mitochondrial dynamics in sperm.

The role of mitochondrial dynamics in the maturation of sperm has not been studied; however recent work has revealed that the mitochondrial fusion and fission machinery are important for the elimination of paternal mitochondria post-fertilization (Wang et al.). FZO-1 is required for proper fusion of the mitochondrial outer membrane while EAT-3/OPA1 regulates inner membrane fusion. In opposition to the activities of FZO-1 and EAT-3, DRP-1 is required for mitochondrial fission (Lima et al.; Smirnova, Griparic, Shurland, & van der Bliek). The balance of this fusion and fission machinery in the upkeep of mitochondrial homeostasis allows cells to respond to changes in metabolic needs and external stress (Shaw & Nunnari; van der Bliek, Sedensky, & Morgan). RNAi of *fzo-1* or *eat-3* reduced mitochondrial fusion in wild-type male sperm (**Figure 5J** and Figure S7C) and suppressed the enhanced fusion observed in *alh-6* mutant spermatid mitochondria (**Figure 5K**); indicating mitochondrial fusion of both membranes is active in spermatids with impaired proline catabolism. We next examined spermatids from *drp-1* mutant animals and observed a greater level of mitochondrial fusion as compared to wild type and *alh-6* mutant spermatids (**Figure 5L**). We observed a synergistic level of mitochondrial fusion in spermatids derived from *alh-6; drp-1* double mutants. This finding is consistent with previous studies in yeast which reveal that defects in fusion can be compensated for by changes in the rates of fission and vice versa (Shaw & Nunnari; van der Bliek et al.). In support of our model where mitochondrial dynamics act as a major driver of the sperm-specific defects in *alh-6* mutants, we discovered that loss of *drp-1*, which results in increased mitochondrial fusion (like that observed in *alh-6* mutants), also reduces sperm

activation (**Figure 5M**). Moreover, reducing *fzo-1* or *eat-3* does not alter activation in wild type sperm, while *fzo-1* but not *eat-3* RNAi restores activation in *alh-6* sperm (**Figure 5N-O** and Figure S7C), suggesting increased fusion mediated predominantly by *fzo-1* in *alh-6* sperm mitochondria is impairing proper function. We noted that *alh-6* mutant animals have an increased expression of *fzo-1* transcripts that is suggestive of a retrograde signaling response from the mitochondria (Figure S7D). Taken together, these data support a model where loss of mitochondrial proline catabolism induces mitochondrial stress, activating mitochondrial fusion, in order to dilute damage to preserve functional mitochondria at the cost of sperm function. These data also reveal a functional role for mitochondrial fusion and fission in spermatid development and sperm function.

***alh-6* and FAD are cell autonomous regulators of sperm function**

Signaling between germ and somatic cells can alter function in each cell type (J. Berman & C. Kenyon; J. R. Berman & C. Kenyon; Curtis, O'Connor, & DiStefano; Ghazi, Henis-Korenblit, & Kenyon; Greenwald; Hsin & Kenyon; Libina, Berman, & Kenyon; Lin, Hsin, Libina, & Kenyon). In light of the differences between somatic and germline phenotypes observed in *alh-6* mutant animals, we performed germline specific RNAi targeting *alh-6* to deduce whether the sperm defects observed were cell autonomous. Germline specific RNAi of *alh-6* in wild-type males was not sufficient to alter sperm size (**Figure 6A),** but did result in diminished sperm activation (**Figure 6B,**) and increased mitochondrial fusion in sperm (**Figure 6C**). We conclude that the effects of loss of *alh-6* on sperm function are cell autonomous because germline specific RNAi could phenocopy the sperm defects observed in whole animal loss of *alh-6*, (while the effect of *alh-6* on sperm size is non-cell autonomous and requires somatic input (Figure S8A-D). Since FAD functions in a variety of essential cellular processes, we next asked if proper sperm function required FAD homeostasis in germ cells. Similarly, we reduced *R10H10.6* or *flad-1* only in the germline, which phenocopies germline knockdown *of alh-6* on sperm size (**Figure 6D,G**), sperm activation (**Figure 6E, H**), and mitochondrial fusion in sperm (**Figure 6F, I**), as observed in whole animal RNAi of *flad-1* or *R10H10.6* (**Figure 4J-K** and **Figure 5G-H**).These results suggest that FAD functions similarly to *alh-6* in cell autonomously regulating sperm function (activation and mitochondrial dynamics), while affecting sperm size in a cell non-autonomous manner (**Figure 4H-I**). Lastly, we restored wild-type *alh-6* expression, only in the germline, in *alh-6* mutant animals, which restored sperm size in one of the two transgenic lines (**Figure 6J**), activation (**Figure 6K**) and mitochondrial dynamics (**Figure 6L**), as compared to non-transgenic siblings. Taken together these data identify the importance of proline catabolism and FAD homeostasis in germ cells to maintain proper sperm function. In conclusion, our studies define mitochondrial proline catabolism as a critical metabolic pathway for male reproductive health.

345 **DISCUSSION**

346 Here we investigate the effects of disrupting mitochondrial proline catabolism through the loss of
347 the mitochondrial enzyme gene *alh-6* and the resulting changes in FAD homeostasis, mitochondrial
348 dynamics, and male fertility (**Figure 7**). We found that *alh-6* mutants show a reduction in brood size that
349 is sexually dimorphic; defects in sperm function but not oocytes contribute to reduced hermaphrodite
350 fertility. As societal factors continue to push individuals to wait longer to have children, the increase in
351 paternal age is inversely correlated with proper sperm function and can give rise to fertility issues.
352 Consequently, it is incumbent on future studies to elucidate how restoring and maintaining functional
353 amino acid catabolism during aging in order to promote reproductive success.

354 Although *C. elegans* is a well-established organism for studying aging and reproduction, with
355 several studies describing hermaphrodite reproductive senescence, many questions regarding the basis of
356 male reproductive decline remain unanswered. Decades of work have shown that exposure to pollution,
357 toxins, xenobiotics, and other ROS-inducing compounds can prematurely drive the loss of sperm function
358 across species (Agarwal, Virk, Ong, & du Plessis; Cocuzza, Sikka, Athayde, & Agarwal; Wagner, Cheng,
359 & Ko), but the impact that normal cellular metabolism plays on sperm function and the identification of
360 specific molecules that can mediate sperm quality are not well-defined. In this study we characterized a
361 new role for mitochondrial proline catabolism and FAD homeostasis in the maintenance of proper sperm
362 function. Perturbation of this pathway, through mutation of *alh-6/ALDH4A1*, causes metabolic stress.
363 Consequently, this perturbation leads to reduction of cellular FAD level and increases mitochondrial
364 fusion in spermatids, which results in impaired sperm function and premature reproductive senescence.

365 Mutation in proline dehydrogenase (*PRODH*) in humans results in hyperprolinemia type I (HPI),
366 while mutation in delta-1-pyrroline-5-carboxylate dehydrogenase (*ALDH4A1/P5CDH*) results in
367 hyperprolinemia type II (HPII). This study reveals that in *C. elegans*, proline catabolism impacts several
368 functional qualities of male sperm. Loss of proline catabolism results in smaller sperm with impaired
369 activation, two qualities that directly impact competitive advantage. As such, proline biosynthesis,
370 catabolism, and steady state concentrations must be tightly regulated, and the importance of proline in
371 cellular homeostasis may help explain the transcriptional responses measured in animals with
372 dysfunctional *alh-6*. Our data support a cell autonomous role for proline catabolism in sperm. However,
373 although whole animal RNAi of *alh-6* closely phenocopies the *alh-6* mutant including reduced spermatid
374 size, germline specific RNAi of *alh-6* did not significantly reduce the size of spermatids; perhaps
375 suggesting a partial role for ALH-6 in somatic tissues for spermatid development, which is in line with
376 recent studies in *C. elegans* describing soma to germline signaling in sperm activation (Chavez, Snow,
377 Smith, & Stanfield) . Intriguingly, the impact of loss of *alh-6* is mostly independent of diet source, unlike
378 the somatic phenotypes which are diet-dependent (Pang & Curran). The exception is sperm number in
379 *alh-6* mutant animals on the HT115 diet, which appears to be diet-dependent (**Figure 2D**). WT males have
380 more spermatids when fed the HT115 diet, as compared to WT animals fed OP50 diet, while *alh-6* mutants
381 have the same number of spermatids on both diets.

382 Our previous work defined the age-dependent decline in function of somatic tissues, particularly
383 muscle in animals lacking functional ALH-6 (Pang & Curran; Pang et al.), which does not manifest until
384 day 3 of adulthood. Our current study reveals that although somatic phenotypes in *alh-6* mutants are
385 observed post-developmentally, the germline, or more specifically spermatids, are sensitive to loss of *alh-*
386 *6* much earlier in development (phenotypes assayed at L4 or Day 1 of adulthood). Reproductive
387 senescence is a field of growing significance as the number of couples that choose to delay having children
388 increases. Importantly, although *alh-6* mutant sperm are impaired for competition, they remain viable for
389 reproduction. This is similar to recent study on *comp-1,* a mutation which results in context-dependent
390 competition deficit in *C. elegans* sperm (Hansen, Chavez, & Stanfield).

391 Recent studies have focused on the role of NAD+ metabolism in cellular health, while the impact
392 of FAD has received less attention. FAD levels are diminished in *alh-6* animals specifically at the L4 stage
393 when spermatogenesis is occurring. Riboflavin (Vitamin B_2) is a precursor to the FAD and FMN cofactors
394 that are needed for metabolic reactions in order to maintain proper cellular function, like proline
395 catabolism and mitochondrial oxidative phosphorylation. Despite its importance, humans, like *C. elegans*,
396 lack a riboflavin biosynthetic pathway and therefore require riboflavin from exogenous sources (Powers).
397 Insufficient intake can lead to impairment of flavin homeostasis, which is associated with cancer,
398 cardiovascular diseases, anemia, neurological disorders, impaired fetal development, etc. (Powers). Our
399 study suggests that riboflavin and FAD play critical roles in reproduction, specifically in germ cell
400 development, as loss of FAD biosynthesis or loss of *alh-6* specifically in the germline recapitulates the
401 sperm defects observed in whole animal knockdown or *alh-6* mutation. Importantly, these sperm-specific
402 defects can be corrected by dietary supplementation of vitamin B_2, which in light of the exceptional
403 conservation of mitochondrial homeostatic pathways, suggest the nutraceutical role vitamin B_2 could play
404 in sperm health across species.
405 Our study also demonstrates that spermatids lacking *alh-6* have increased mitochondrial fusion; a
406 perturbation at the mitochondrial organelle structure-level that contributes to the sperm-specific
407 phenotypes observed. In addition to prior work showing *fzo-1/MFN1/MFN2* and *drp-1/DRP-1* to be
408 important for mitochondrial elimination post-fertilization (Wang et al.), our work reveals that
409 mitochondrial fission and fusion machinery are present and active in spermatids and that perturbation of
410 these dynamics can affect sperm maturation and competitive fitness. Future work to define how *alh-6*
411 spermatids use mitophagy, which can clear damaged mitochondria, will be of interest. In conclusion, our
412 work identifies proline metabolism as a major metabolic pathway that can impact sperm maturation and
413 male reproductive success. Moreover, these studies identify specific interventions to reverse the redox
414 imbalance, cofactor depletion, and altered mitochondria dynamics, all of which play a part in sperm
415 dysfunction resulting from proline metabolism defects.
416

ACKNOWLEDGEMENTS

We thank N. Mih, K. Han, and L. Thomas for technical assistance and H. Dalton, A. Hammerquist, N. Stuhr, W, Escorcia, and J. Nhan for critical reading of the manuscript. Some strains were provided by the CGC, which is funded by the NIH Office of Research Infrastructure Programs (P40 OD010440). This work was funded by the NIH (R01GM109028, R01AG058610, to S.P.C., T32AG000037 to D.L.R., and T32GM118289 to C.D.T), and the American Federation of Aging Research (C-A.Y. and S.P.C).

AUTHOR CONTRIBUTIONS

S.P.C. designed the study; C-A.Y. performed the majority of all experiments with assistance from D.L.R., C.D.T., S.P., and S.P.C.; C-A.Y. and S.P.C. analyzed data. S.P.C. wrote the manuscript and C-A.Y. and S.P.C. revised the manuscript. All authors discussed the results and commented on the final version of the manuscript.

COMPETING INTERESTS

The authors declare no competing interests.

DATA AVAILABILITY

All relevant data are available from the authors. RNA-Seq data are deposited in GEO database (GSE121920).

METHODS

C. elegans strains and maintenance

C. elegans were cultured using standard techniques at 20°C. The following strains were used: wild type (WT) N2 Bristol, SPC321[*alh-6(lax105)*], SP326[*alh-6p::alh-6::gfp*], SPC447[laxEx025 - *pie-1p::alh-6*], SPC455[laxEx033 - *pie-1p::alh-6*], SPC473[laxEx051 - *pie-1p::alh-6*], CL2166[*gst4-p::gfp*], SPC223[*alh-6(lax105);gst-4p::gfp*], DCL569[mkcSi13(sun-1p::rde-1::sun-1 3'UTR + unc-119(+)) II; rde-1(mkc36) V], and CU6372[*drp-1(tm1108)*]. Double and triple mutants were generated by standard genetic techniques. *E. coli* strains used were as follows: B Strain OP50(Brenner) and HT115(DE3) [F^- mcrA mcrB IN(rrnD-rrnE)1 lambda$^-$ rnc14::Tn10 λ(DE3)](Timmons, Court, & Fire). For dietary supplement assays, riboflavin was added to the NGM plate mix to final concentration 2.5mM.

RNAi-based experiments

RNAi experiments were done using HT115-based RNAi (Timmons et al.), which yielded similar results as OP50 RNAi *E. coli* B strain as described in (Dalton & Curran). All strains were adapted to diets for at least three generations and strains were never allowed to starve. All RNAi clones were sequenced prior to use and RNAi knockdown efficiency measured. RNAi cultures were seeded on IPTG plates and allowed to induce overnight prior to dropping eggs on them for experiments.

Microscopy

Zeiss Axio Imager and ZEN software were used to acquire all images used in this study. For GFP reporter strains, worms were mounted in M9 with 10mM levamisole and imaged with DIC and GFP filters. For sperm number, assay samples were imaged with DIC and DAPI filters in z-stacks. For sperm size and activation assays, dissected sperm samples were imaged at 100x with DIC filter on two different focal planes for each field to ensure accuracy. For sperm mitochondria assays, dissected sperm samples were imaged at 100x with DIC, GFP, and RFP filters in z-stacks to assess overall mitochondria content within each spermatid.

Fertility assay

Worms were treated with alkaline hypochlorite and eggs were allowed to hatch overnight. The next day, synchronized L1 larvae were dropped on NGM plates seeded with either OP50 or HT115. 48 hours later, at least ten L4 hermaphrodites for each genotype were singled onto individual plates and moved every 12 hours until egg laying ceased. Progeny were counted 48 hours after the singled hermaphrodite was moved to a different plate. Plates were counted twice for accuracy.

Mated reproductive assay

Males were synchronized by egg laying, picked as L4 larvae for use as young adults for mating experiments. Singled L4 stage hermaphrodites were each put on a plate with 30ul of OP50 seeded in the center together with three virgin adult males. 24 hours post-mating, males were removed, and each hermaphrodite was moved to a new plate every 24 hours until egg laying ceased. Progeny were counted 48 hours after the hermaphrodite was moved from the plate. For sperm competition assay, progeny with GFP fluorescence were counted from the cohort. Plates were counted twice for accuracy.

Cofactor Measurements

Worms were treated with alkaline hypochlorite and eggs were allowed to hatch overnight. The next day, synchronized L1s were dropped on NGM plates with or without supplement seeded with 25X concentrated

488 OP50. FAD levels are measured following directions in FAD Colorimetric/Fluorometric Assay Kit (K357)
489 from BioVision. NAD/NADH levels are measured following directions in NAD/NADH Quantification
490 Colorimetric Kit (K337).
491
492 **Sperm Number Assay**
493 Worms were treated with alkaline hypochlorite and eggs were allowed to hatch overnight. The next day,
494 synchronized L1s were dropped on NGM plates with the indicated food source. At 48 hours (L4
495 developmental stage) males were isolated to new plates. 72 hours post-drop, day 1 adult virgin male
496 animals were washed 3x with 1xPBST, fixed with 40% 2-propanol, and stained with DAPI for 2 hrs.
497 Samples were washed for 30min with PBST, mounted with vectashield mounting medium, and covered
498 with coverslip to image. Spermatids in the seminal vesicle were counted through all planes in z-stack.
499
500 **Sperm Size Assay**
501 Males were isolated at L4 stage 24 hours before assay. For each strain, five day 1 adult males were
502 dissected in 35µL pH 7.8 SM buffer (50mM HEPES, 50mM NaCl, 25mM KCl, 5mM $CaCl_2$, 1mM
503 $MgSO_4$, 10mM dextrose) to release spermatids, which were immediately imaged.
504
505 **Sperm Activation with Pronase**
506 Males were isolated at L4 stage 24 hours before assay. For each strain, five day 1 adult males were
507 dissected in 35µL pH 7.8 SM buffer (50mM HEPES, 50mM NaCl, 25mM KCl, 5mM $CaCl_2$, 1mM
508 $MgSO_4$, 1mg/ml BSA) supplemented with 200µg/mL Pronase® (Millipore Sigma) to release spermatids.
509 Another 25ul of the same solution was added and the spermatids were incubated at RT for 30 min for
510 activation to occur.
511
512 **Sperm Mitochondria Staining**
513 Males were isolated at L4 stage 24 hours before assay. For each strain, five day 1 adult males were
514 dissected in 35µL pH 7.8 SM buffer (50mM HEPES, 50mM NaCl, 25mM KCl, 5mM $CaCl_2$, 1mM
515 $MgSO_4$, 1mg/ml BSA) with JC-1(Thermo Fisher Scientific T3168) added to 10µM final concentration.
516 Another 25ul of the same solution was added and the spermatids were incubated at RT for 10 min. The
517 slide was washed three times with 100ul SM buffer before imaging.
518
519 **RNA-sequencing**
520 Worms were egg prepped and eggs were allowed to hatch overnight. The next day, synchronized L1s were
521 dropped on NGM plates seeded with 25X concentrated OP50. 48 and 120 hours post drop, L4 animals
522 and day 3 adult animals, respectively, were washed 3 times with M9 and frozen in TRI Reagent at -80°C.
523 Animals were homogenized and RNA extraction was performed following the protocol in Zymo Direct-
524 zol RNA Isolation Kit. RNA samples were sequenced and analyzed by Novogene.
525
526 **Statistical analysis**
527 Data are presented as mean ± SEM. Comparisons and significance were analyzed in Graphpad Prism 7.
528 Comparisons between two groups were done using Student's Test. Comparisons between more than two
529 groups were done using ANOVA. For sperm activation assays, Fisher's Exact Test was used and p-values
530 are adjusted for multiple comparisons. *$p<0.05$ **$p<0.01$ *** $p<0.001$ ****<0.0001.

REFERENCES

Adams, E., & Frank, L. (1980). Metabolism of proline and the hydroxyprolines. *Annu Rev Biochem, 49*, 1005-1061. doi:10.1146/annurev.bi.49.070180.005041

Agarwal, A., Virk, G., Ong, C., & du Plessis, S. S. (2014). Effect of oxidative stress on male reproduction. *World J Mens Health, 32*(1), 1-17. doi:10.5534/wjmh.2014.32.1.1

Amaral, A., Lourenco, B., Marques, M., & Ramalho-Santos, J. (2013). Mitochondria functionality and sperm quality. *Reproduction, 146*(5), R163-174. doi:10.1530/REP-13-0178

An, J. H., & Blackwell, T. K. (2003). SKN-1 links C. elegans mesendodermal specification to a conserved oxidative stress response. *Genes & development, 17*(15), 1882-1893. doi:10.1101/gad.1107803

Argon, Y., & Ward, S. (1980). Caenorhabditis elegans fertilization-defective mutants with abnormal sperm. *Genetics, 96*(2), 413-433.

Baird, D. T., Collins, J., Egozcue, J., Evers, L. H., Gianaroli, L., Leridon, H., . . . Group, E. C. W. (2005). Fertility and ageing. *Hum Reprod Update, 11*(3), 261-276. doi:10.1093/humupd/dmi006

Barile, M., Giancaspero, T. A., Brizio, C., Panebianco, C., Indiveri, C., Galluccio, M., . . . Gianazza, E. (2013). Biosynthesis of flavin cofactors in man: implications in health and disease. *Current pharmaceutical design, 19*(14), 2649-2675. doi:10.2174/1381612811319140014

Berman, J., & Kenyon, C. (2006). Germ-cell loss extends C. elegans life span through regulation of DAF-16 by kri-1 and lipophilic-hormone signaling. *Cell, 124*(5), 1055-1068. doi:10.1016/j.cell.2006.01.039

Berman, J. R., & Kenyon, C. (2006). Germ-cell loss extends C. elegans life span through regulation of DAF-16 by kri-1 and lipophilic-hormone signaling. *Cell, 124*(5), 1055-1068.

Blackwell, T. K., Steinbaugh, M. J., Hourihan, J. M., Ewald, C. Y., & Isik, M. (2015). SKN-1/Nrf, stress responses, and aging in Caenorhabditis elegans. *Free radical biology & medicine, 88*(Pt B), 290-301. doi:10.1016/j.freeradbiomed.2015.06.008

Braeckman, B. P., Houthoofd, K., & Vanfleteren, J. R. (2009). Intermediary metabolism. *WormBook : the online review of C. elegans biology*, 1-24. doi:10.1895/wormbook.1.146.1

Brenner, S. (1974). The genetics of Caenorhabditis elegans. *Genetics, 77*(1), 71-94.

Burch, H. B., Combs, A. M., Lowry, O. H., & Padilla, A. M. (1956). Effects of riboflavin deficiency and realimentation on flavin enzymes of tissues. *The Journal of biological chemistry, 223*(1), 29-45.

Chavez, D. R., Snow, A. K., Smith, J. R., & Stanfield, G. M. (2018). Soma-germ line interactions and a role for muscle in the regulation of C. elegans sperm motility. *Development, 145*(24). doi:10.1242/dev.167734

Cocuzza, M., Athayde, K. S., Agarwal, A., Sharma, R., Pagani, R., Lucon, A. M., . . . Hallak, J. (2008). Age-related increase of reactive oxygen species in neat semen in healthy fertile men. *Urology, 71*(3), 490-494. doi:10.1016/j.urology.2007.11.041

Cocuzza, M., Sikka, S. C., Athayde, K. S., & Agarwal, A. (2007). Clinical relevance of oxidative stress and sperm chromatin damage in male infertility: an evidence based analysis. *Int Braz J Urol, 33*(5), 603-621.

Curtis, R., O'Connor, G., & DiStefano, P. S. (2006). Aging networks in Caenorhabditis elegans: AMP-activated protein kinase (aak-2) links multiple aging and metabolism pathways. *Aging cell, 5*(2), 119-126.

Dalton, H. M., & Curran, S. P. (2018). Hypodermal responses to protein synthesis inhibition induce systemic developmental arrest and AMPK-dependent survival in Caenorhabditis elegans. *PLoS genetics, 14*(7), e1007520. doi:10.1371/journal.pgen.1007520

de Lamirande, E., & Gagnon, C. (1993). A positive role for the superoxide anion in triggering hyperactivation and capacitation of human spermatozoa. *Int J Androl, 16*(1), 21-25.

Deuschle, K., Funck, D., Forlani, G., Stransky, H., Biehl, A., Leister, D., . . . Frommer, W. B. (2004). The role of [Delta]1-pyrroline-5-carboxylate dehydrogenase in proline degradation. *The Plant cell, 16*(12), 3413-3425. doi:10.1105/tpc.104.023622

581 Ghazi, A., Henis-Korenblit, S., & Kenyon, C. (2009). A transcription elongation factor that links signals
582 from the reproductive system to lifespan extension in Caenorhabditis elegans. *PLoS genetics,*
583 *5*(9), e1000639. doi:10.1371/journal.pgen.1000639
584 Glover-Cutter, K. M., Lin, S., & Blackwell, T. K. (2013). Integration of the unfolded protein and oxidative
585 stress responses through SKN-1/Nrf. *PLoS genetics, 9*(9), e1003701.
586 doi:10.1371/journal.pgen.1003701
587 Gomes, L. C., Di Benedetto, G., & Scorrano, L. (2011). Essential amino acids and glutamine regulate
588 induction of mitochondrial elongation during autophagy. *Cell cycle, 10*(16), 2635-2639.
589 doi:10.4161/cc.10.16.17002
590 Grad, L. I., & Lemire, B. D. (2004). Mitochondrial complex I mutations in Caenorhabditis elegans
591 produce cytochrome c oxidase deficiency, oxidative stress and vitamin-responsive lactic
592 acidosis. *Hum Mol Genet, 13*(3), 303-314.
593 Greenwald, I. (1989). Cell-cell interactions that specify certain cell fates in C. elegans development.
594 *Trends Genet, 5*(8), 237-241.
595 Guarente, L. (2016). CELL METABOLISM. The resurgence of NAD(+). *Science, 352*(6292), 1396-1397.
596 doi:10.1126/science.aag1718
597 Hansen, J. M., Chavez, D. R., & Stanfield, G. M. (2015). COMP-1 promotes competitive advantage of
598 nematode sperm. *Elife, 4*. doi:10.7554/eLife.05423
599 Hirsh, D., Oppenheim, D., & Klass, M. (1976). Development of the reproductive system of
600 Caenorhabditis elegans. *Developmental biology, 49*(1), 200-219.
601 Hsin, H., & Kenyon, C. (1999). Signals from the reproductive system regulate the lifespan of C.
602 elegans. *Nature, 399*(6734), 362-366.
603 Jonassen, T., Marbois, B. N., Faull, K. F., Clarke, C. F., & Larsen, P. L. (2002). Development and
604 fertility in Caenorhabditis elegans clk-1 mutants depend upon transport of dietary coenzyme Q8
605 to mitochondria. *The Journal of biological chemistry, 277*(47), 45020-45027.
606 doi:10.1074/jbc.M204758200
607 Kadandale, P., & Singson, A. (2004). Oocyte production and sperm utilization patterns in semi-fertile
608 strains of Caenorhabditis elegans. *BMC developmental biology, 4*, 3. doi:10.1186/1471-213X-4-
609 3
610 Kidd, S. A., Eskenazi, B., & Wyrobek, A. J. (2001). Effects of male age on semen quality and fertility: a
611 review of the literature. *Fertil Steril, 75*(2), 237-248.
612 Klass, M. R., & Hirsh, D. (1981). Sperm isolation and biochemical analysis of the major sperm protein
613 from Caenorhabditis elegans. *Developmental biology, 84*(2), 299-312. doi:10.1016/0012-
614 1606(81)90398-5
615 Kodama, H., Kuribayashi, Y., & Gagnon, C. (1996). Effect of sperm lipid peroxidation on fertilization. *J*
616 *Androl, 17*(2), 151-157.
617 LaMunyon, C. W., & Ward, S. (1995). Sperm precedence in a hermaphroditic nematode
618 (Caenorhabditis elegans) is due to competitive superiority of male sperm. *Experientia, 51*(8),
619 817-823.
620 LaMunyon, C. W., & Ward, S. (1998). Larger sperm outcompete smaller sperm in the nematode
621 Caenorhabditis elegans. *Proc Biol Sci, 265*(1409), 1997-2002. doi:10.1098/rspb.1998.0531
622 LaMunyon, C. W., & Ward, S. (2002). Evolution of larger sperm in response to experimentally
623 increased sperm competition in Caenorhabditis elegans. *Proc Biol Sci, 269*(1496), 1125-1128.
624 doi:10.1098/rspb.2002.1996
625 Leclerc, P., de Lamirande, E., & Gagnon, C. (1997). Regulation of protein-tyrosine phosphorylation and
626 human sperm capacitation by reactive oxygen derivatives. *Free radical biology & medicine,*
627 *22*(4), 643-656.
628 Lemaitre, J. F., & Gaillard, J. M. (2017). Reproductive senescence: new perspectives in the wild. *Biol*
629 *Rev Camb Philos Soc, 92*(4), 2182-2199. doi:10.1111/brv.12328

630 Liau, W. S., Gonzalez-Serricchio, A. S., Deshommes, C., Chin, K., & LaMunyon, C. W. (2007). A
631 persistent mitochondrial deletion reduces fitness and sperm performance in heteroplasmic
632 populations of C. elegans. *BMC Genet, 8*, 8. doi:10.1186/1471-2156-8-8
633 Libina, N., Berman, J. R., & Kenyon, C. (2003). Tissue-specific activities of C. elegans DAF-16 in the
634 regulation of lifespan. *Cell, 115*(4), 489-502.
635 Lima, A. R., Santos, L., Correia, M., Soares, P., Sobrinho-Simoes, M., Melo, M., & Maximo, V. (2018).
636 Dynamin-Related Protein 1 at the Crossroads of Cancer. *Genes (Basel), 9*(2).
637 doi:10.3390/genes9020115
638 Lin, K., Hsin, H., Libina, N., & Kenyon, C. (2001). Regulation of the Caenorhabditis elegans longevity
639 protein DAF-16 by insulin/IGF-1 and germline signaling. *Nature genetics, 28*(2), 139-145.
640 Luo, S., Kleemann, G. A., Ashraf, J. M., Shaw, W. M., & Murphy, C. T. (2010). TGF-beta and insulin
641 signaling regulate reproductive aging via oocyte and germline quality maintenance. *Cell, 143*(2),
642 299-312. doi:10.1016/j.cell.2010.09.013
643 Lynn, D. A., Dalton, H. M., Sowa, J. N., Wang, M. C., Soukas, A. A., & Curran, S. P. (2015). Omega-3
644 and -6 fatty acids allocate somatic and germline lipids to ensure fitness during nutrient and
645 oxidative stress in Caenorhabditis elegans. *Proc Natl Acad Sci U S A, 112*(50), 15378-15383.
646 doi:10.1073/pnas.1514012112
647 McCarter, J., Bartlett, B., Dang, T., & Schedl, T. (1999). On the control of oocyte meiotic maturation and
648 ovulation in Caenorhabditis elegans. *Developmental biology, 205*(1), 111-128.
649 doi:10.1006/dbio.1998.9109
650 Miller, G., Honig, A., Stein, H., Suzuki, N., Mittler, R., & Zilberstein, A. (2009). Unraveling delta1-
651 pyrroline-5-carboxylate-proline cycle in plants by uncoupled expression of proline oxidation
652 enzymes. *The Journal of biological chemistry, 284*(39), 26482-26492.
653 doi:10.1074/jbc.M109.009340
654 Mills, M., Rindfuss, R. R., McDonald, P., te Velde, E., Reproduction, E., & Society Task, F. (2011). Why
655 do people postpone parenthood? Reasons and social policy incentives. *Hum Reprod Update,*
656 *17*(6), 848-860. doi:10.1093/humupd/dmr026
657 Nakada, K., Sato, A., Yoshida, K., Morita, T., Tanaka, H., Inoue, S., . . . Hayashi, J. (2006).
658 Mitochondria-related male infertility. *Proc Natl Acad Sci U S A, 103*(41), 15148-15153.
659 doi:10.1073/pnas.0604641103
660 Nomura, M., & Takagi, H. (2004). Role of the yeast acetyltransferase Mpr1 in oxidative stress:
661 regulation of oxygen reactive species caused by a toxic proline catabolism intermediate. *Proc*
662 *Natl Acad Sci U S A, 101*(34), 12616-12621. doi:10.1073/pnas.0403349101
663 Ozkosem, B., Feinstein, S. I., Fisher, A. B., & O'Flaherty, C. (2015). Advancing age increases sperm
664 chromatin damage and impairs fertility in peroxiredoxin 6 null mice. *Redox Biol, 5*, 15-23.
665 doi:10.1016/j.redox.2015.02.004
666 Paek, J., Lo, J. Y., Narasimhan, S. D., Nguyen, T. N., Glover-Cutter, K., Robida-Stubbs, S., . . . Curran,
667 S. P. (2012). Mitochondrial SKN-1/Nrf mediates a conserved starvation response. *Cell*
668 *metabolism, 16*(4), 526-537. doi:10.1016/j.cmet.2012.09.007
669 Palikaras, K., Lionaki, E., & Tavernarakis, N. (2015a). Coordination of mitophagy and mitochondrial
670 biogenesis during ageing in C. elegans. *Nature, 521*(7553), 525-528. doi:10.1038/nature14300
671 Palikaras, K., Lionaki, E., & Tavernarakis, N. (2015b). Coupling mitogenesis and mitophagy for
672 longevity. *Autophagy, 11*(8), 1428-1430. doi:10.1080/15548627.2015.1061172
673 Pang, S., & Curran, S. P. (2014). Adaptive Capacity to Bacterial Diet Modulates Aging in C. elegans.
674 *Cell metabolism, 19*(2), 221-231. doi:10.1016/j.cmet.2013.12.005
675 Pang, S., Lynn, D. A., Lo, J. Y., Paek, J., & Curran, S. P. (2014). SKN-1 and Nrf2 couples proline
676 catabolism with lipid metabolism during nutrient deprivation. *Nat Commun, 5*, 5048.
677 doi:10.1038/ncomms6048
678 Powers, H. J. (2003). Riboflavin (vitamin B-2) and health. *Am J Clin Nutr, 77*(6), 1352-1360.
679 doi:10.1093/ajcn/77.6.1352

Ramalho-Santos, J., & Amaral, S. (2013). Mitochondria and mammalian reproduction. *Mol Cell Endocrinol, 379*(1-2), 74-84. doi:10.1016/j.mce.2013.06.005

Rambold, A. S., Kostelecky, B., Elia, N., & Lippincott-Schwartz, J. (2011). Tubular network formation protects mitochondria from autophagosomal degradation during nutrient starvation. *Proc Natl Acad Sci U S A, 108*(25), 10190-10195. doi:10.1073/pnas.1107402108

Redondo, A., Menasche, P., & Le Beau, J. (1975). [Operation for stenosis of the internal carotid artery secondary to irradiation (a propos of 1 observation)]. *Neurochirurgie, 21*(3), 239-245.

Shakes, D. C., & Ward, S. (1989). Initiation of spermiogenesis in C. elegans: a pharmacological and genetic analysis. *Developmental biology, 134*(1), 189-200.

Shaw, J. M., & Nunnari, J. (2002). Mitochondrial dynamics and division in budding yeast. *Trends in cell biology, 12*(4), 178-184.

Singson, A., Hill, K. L., & L'Hernault, S. W. (1999). Sperm competition in the absence of fertilization in Caenorhabditis elegans. *Genetics, 152*(1), 201-208.

Smirnova, E., Griparic, L., Shurland, D. L., & van der Bliek, A. M. (2001). Dynamin-related protein Drp1 is required for mitochondrial division in mammalian cells. *Molecular biology of the cell, 12*(8), 2245-2256. doi:10.1091/mbc.12.8.2245

Timmons, L., Court, D. L., & Fire, A. (2001). Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in Caenorhabditis elegans. *Gene, 263*(1-2), 103-112.

Tondera, D., Grandemange, S., Jourdain, A., Karbowski, M., Mattenberger, Y., Herzig, S., . . . Martinou, J. C. (2009). SLP-2 is required for stress-induced mitochondrial hyperfusion. *The EMBO journal, 28*(11), 1589-1600. doi:10.1038/emboj.2009.89

Treloar, A. E. (1981). Menstrual cyclicity and the pre-menopause. *Maturitas, 3*(3-4), 249-264.

van der Bliek, A. M., Sedensky, M. M., & Morgan, P. G. (2017). Cell Biology of the Mitochondrion. *Genetics, 207*(3), 843-871. doi:10.1534/genetics.117.300262

Wagner, H., Cheng, J. W., & Ko, E. Y. (2018). Role of reactive oxygen species in male infertility: An updated review of literature. *Arab J Urol, 16*(1), 35-43. doi:10.1016/j.aju.2017.11.001

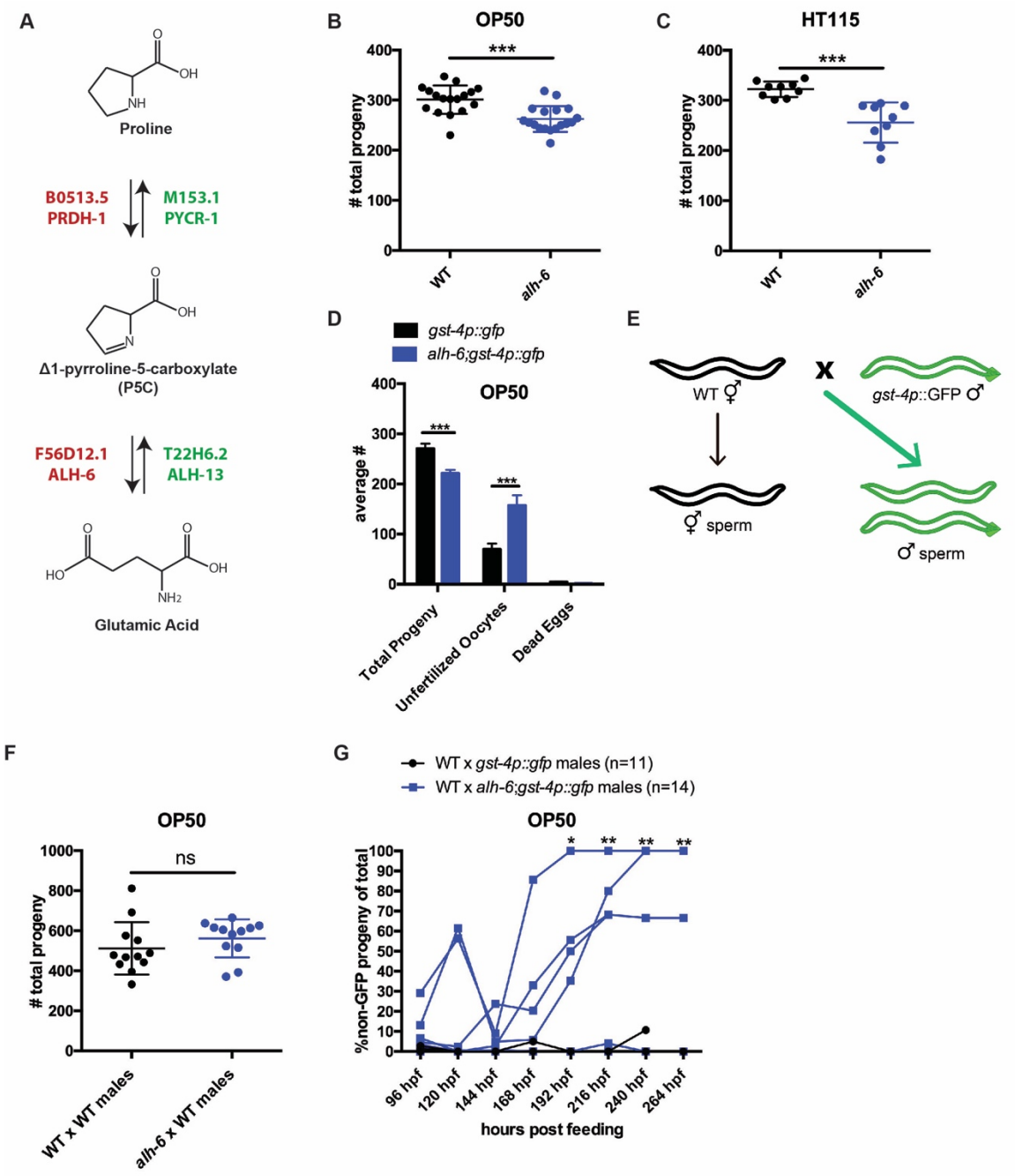
Wang, Y., Zhang, Y., Chen, L., Liang, Q., Yin, X. M., Miao, L., . . . Xue, D. (2016). Kinetics and specificity of paternal mitochondrial elimination in Caenorhabditis elegans. *Nat Commun, 7*, 12569. doi:10.1038/ncomms12569

Ward, S., & Carrel, J. S. (1979). Fertilization and sperm competition in the nematode Caenorhabditis elegans. *Developmental biology, 73*(2), 304-321.

Ward, S., Hogan, E., & Nelson, G. A. (1983). The initiation of spermiogenesis in the nematode Caenorhabditis elegans. *Developmental biology, 98*(1), 70-79.

Ward, S., & Miwa, J. (1978). Characterization of temperature-sensitive, fertilization-defective mutants of the nematode caenorhabditis elegans. *Genetics, 88*(2), 285-303.

716 **FIGURES**



717
718 **Figure 1. *alh-6* fertility defects are sperm-specific.** (A) Proline catabolism pathway. (B-C) *alh-6*
719 hermaphrodites have reduced brood size when fed OP50 (B) or HT115 (C) diets. (D) *alh-6*
720 hermaphrodites produce increased unfertilized oocytes, but few dead embryos. (E) Mated reproductive
721 assay scheme utilizes males to maximize reproductive output (as in F) and can exploit males harboring
722 GFP to differentiate progeny resulting from self- versus male-sperm (as in G). (F) Wild type (WT) and
723 *alh-6* hermaphrodites mated with WT males yield similar number of total progeny. (G) WT hermaphrodites
724 mated with *alh-6;gst-4p::gfp* yield more non-GFP progeny (indicating self-fertilization) than
725 hermaphrodites mated with wild type males harboring *gst-4p::gfp*. Statistical comparisons by unpaired t-
726 test. *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001. All studies performed in at least biological
727 triplicate; refer to Table S1 for n for each comparison.

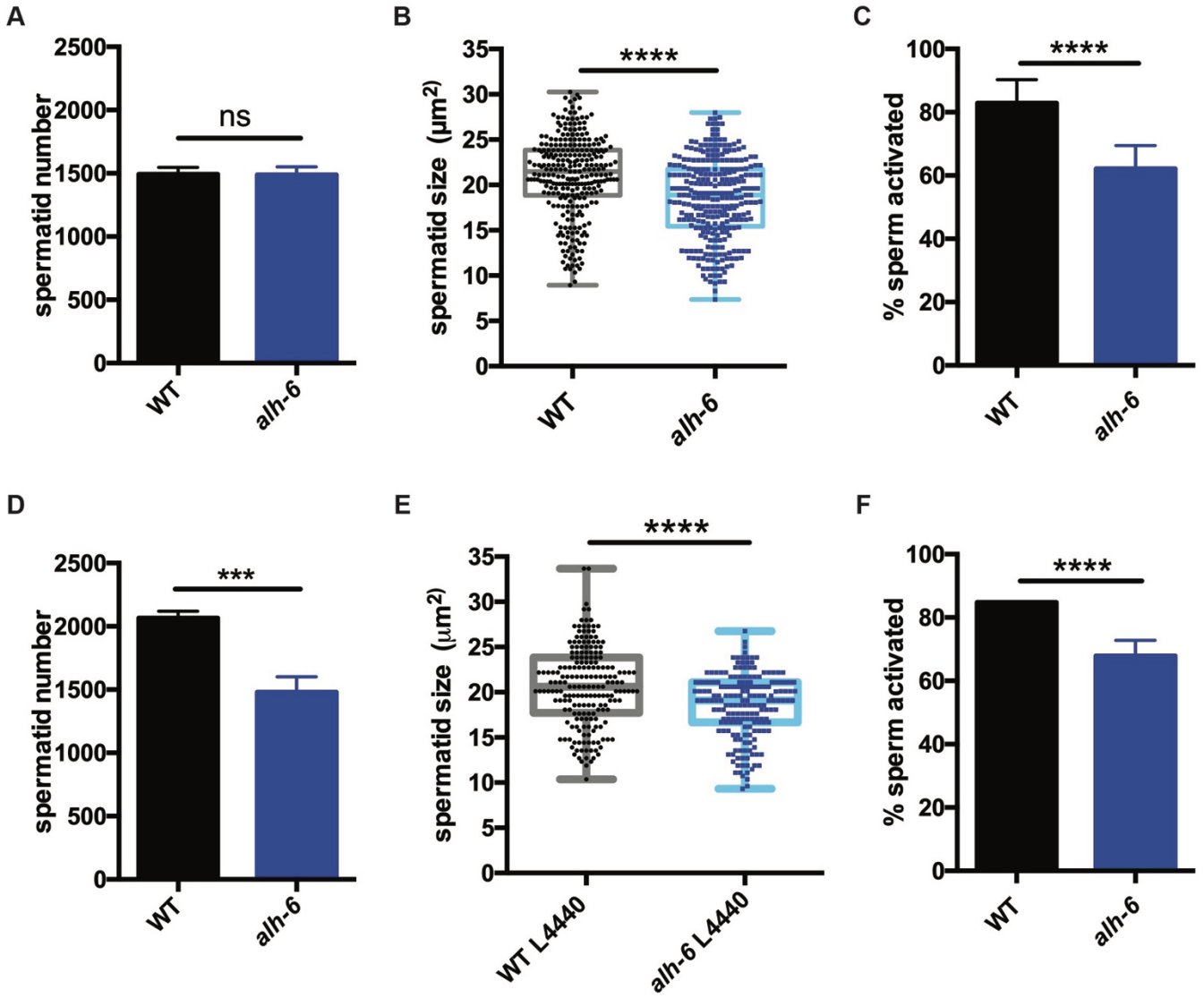


Figure 2. *alh-6* males have sperm defects on both OP50 and HT115 diets. (A-C) sperm phenotypes on OP50 diet. (A) Sperm quantity is similar between wild type (WT) and *alh-6* mutant day 1 adult males. (B) Spermatid size is reduced in *alh-6* mutant day 1 adult males as compared to age matched WT males. (C) Sperm activation is impaired in *alh-6* mutant day 1 adult males relative to age-matched WT males. (D-F) sperm phenotypes on HT115 diet. (D) Sperm quantity is reduced in *alh-6* mutant day 1 adult males compared to age-matched WT males. (E) Spermatid size is reduced in *alh-6* mutant day 1 adult males as compared to age matched WT males fed HT115. (F) Sperm activation is impaired in *alh-6* mutant day 1 adult males relative to age-matched WT males fed HT115. Statistical comparisons of sperm number and size by unpaired t-test and sperm activation by Fisher's exact test. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$. All studies performed in at least biological triplicate; refer to Table S1 for n for each comparison.

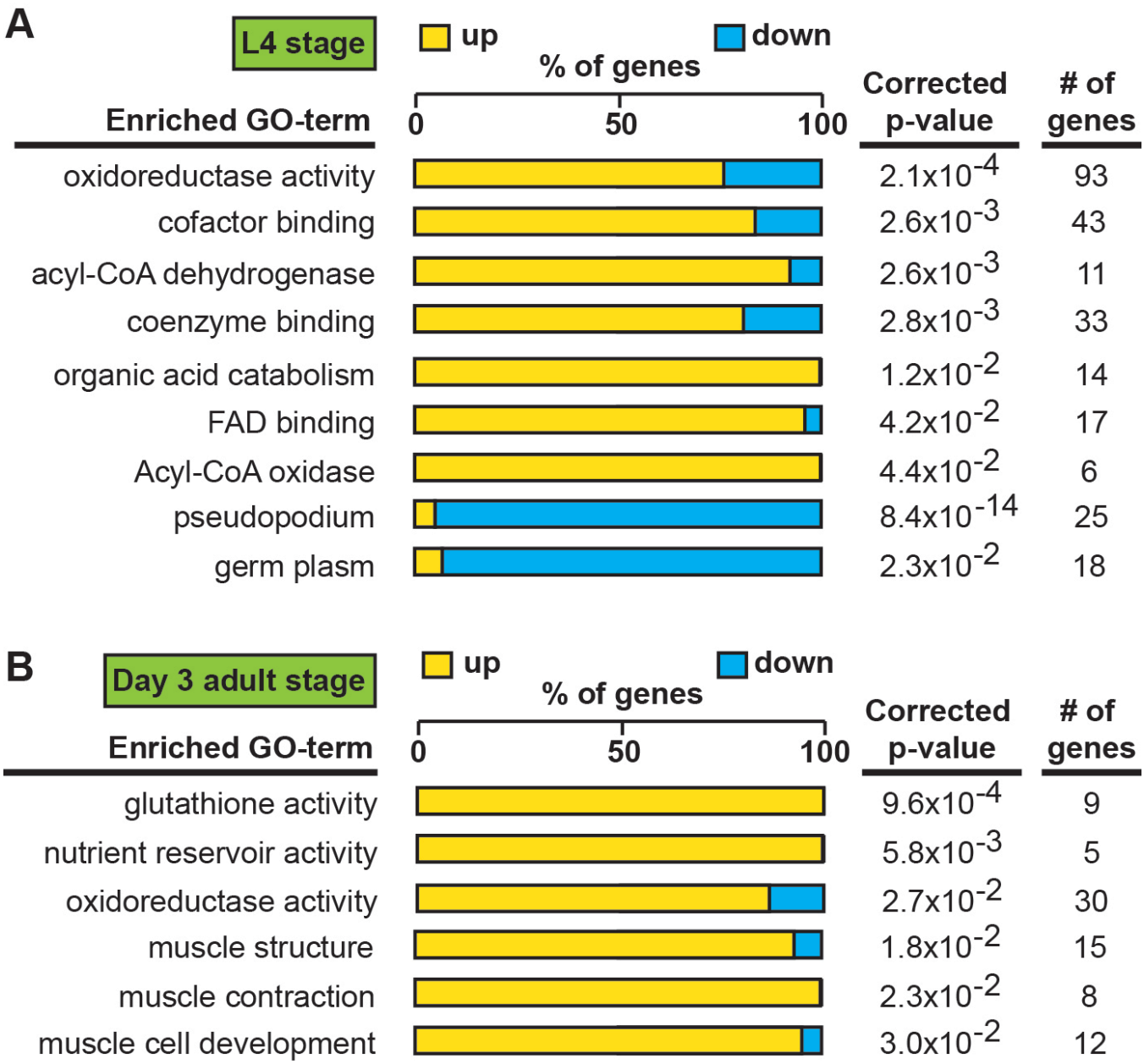


Figure 3. Transcriptional patterns define developmental- and adult-specific consequences to loss of *alh-6* activity. Gene Ontology (GO) term enrichment analysis of RNAseq data. (A) Transcriptional changes at L4 stage are enriched for metabolism and sperm-specific genes. (B) Transcriptional changes at day 3 adulthood are enriched for changes in glutathione activity, oxidoreductase activity, and muscle-specific genes. All studies performed in at least biological triplicate; refer to Table S1 for n for each comparison.

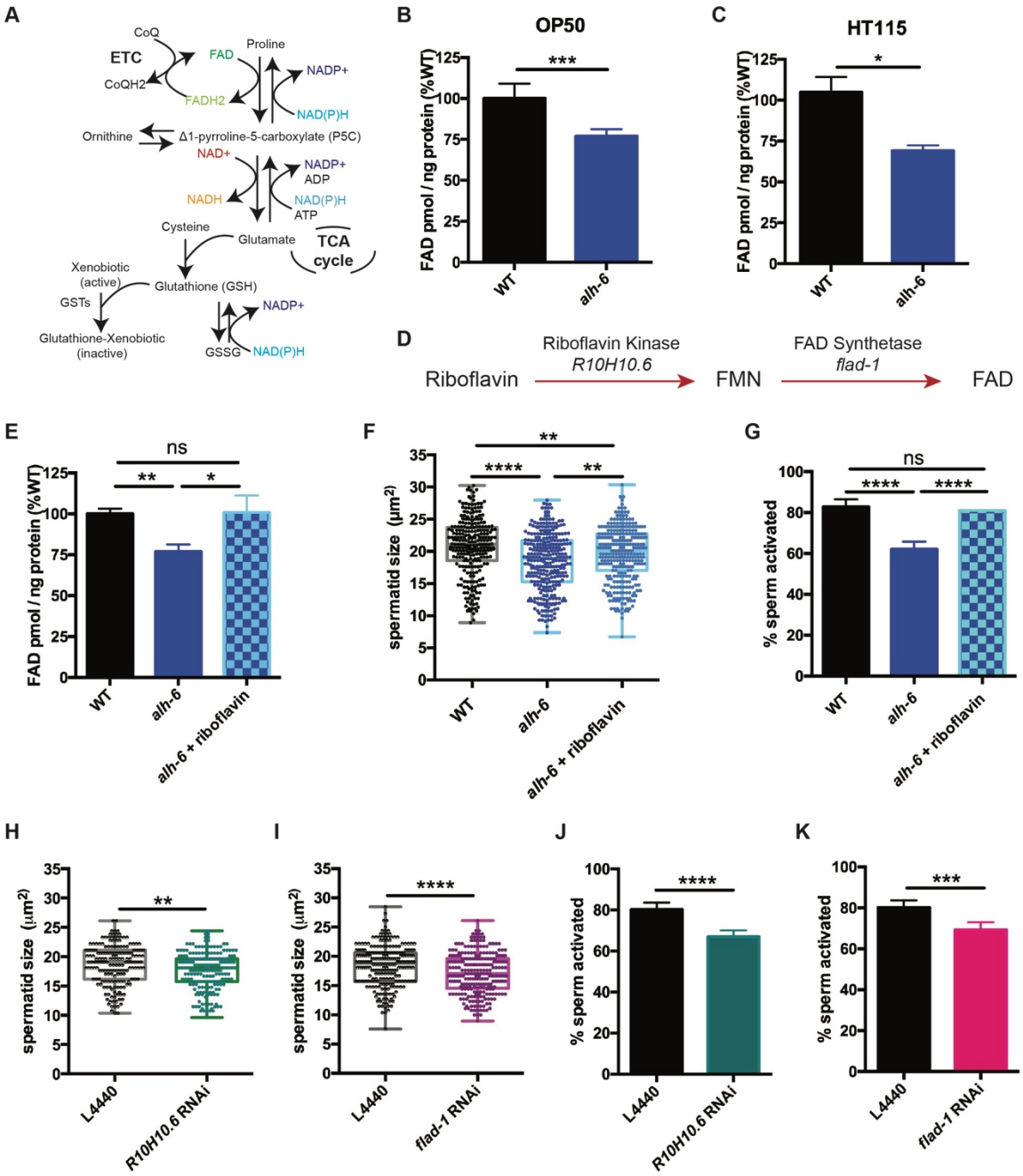
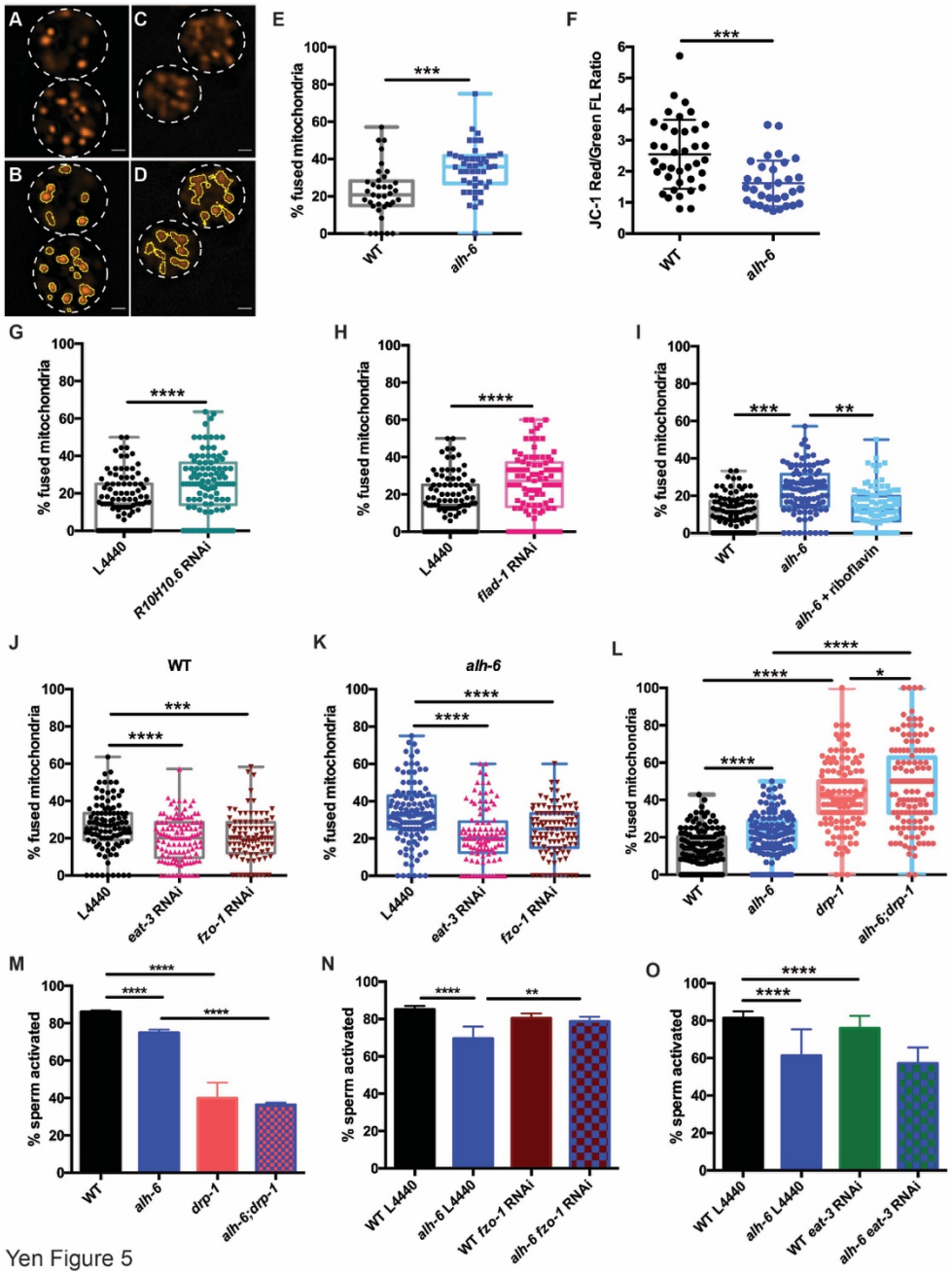


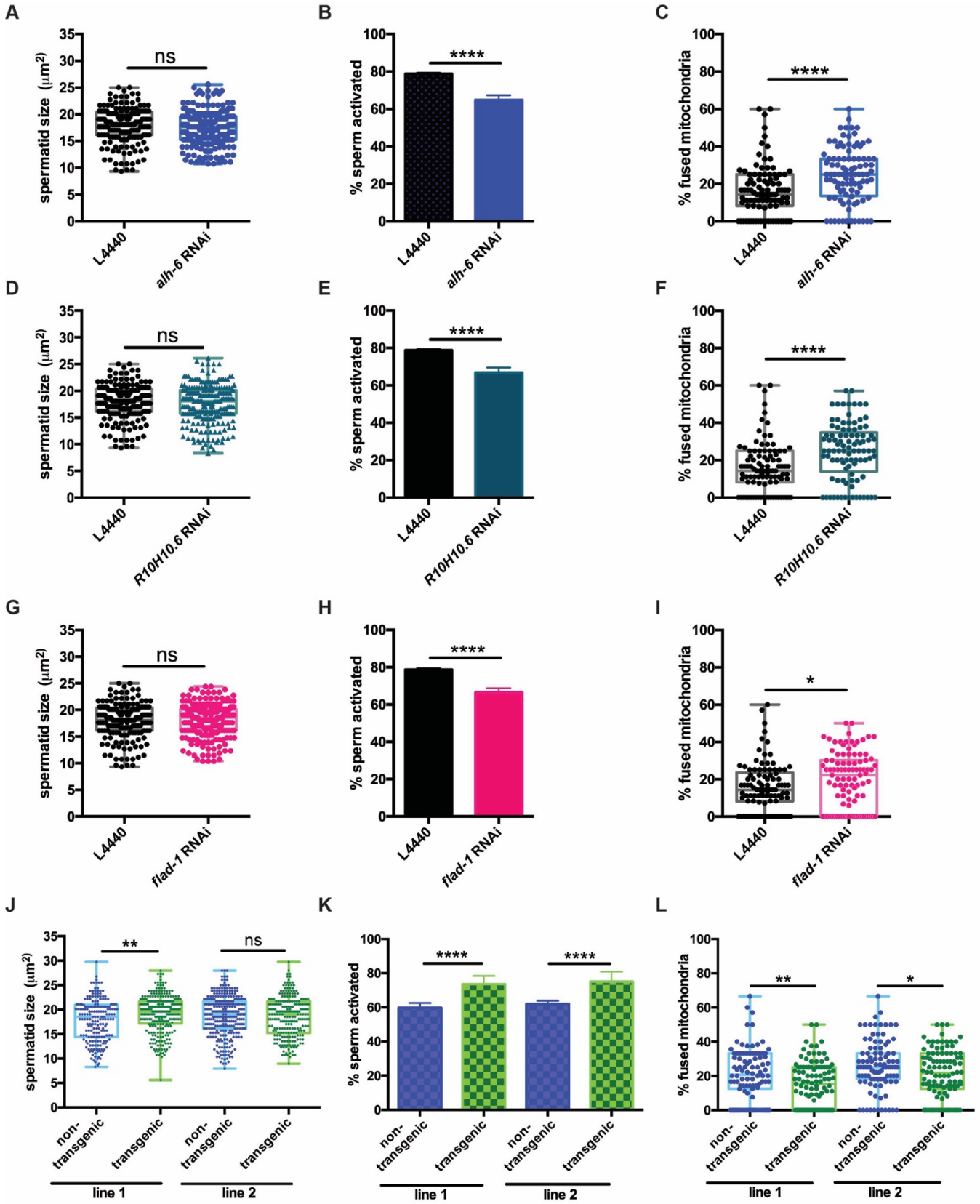
Figure 4. Loss of FAD homeostasis in *alh-6* mutants leads to sperm dysfunction. (A) Metabolic pathways utilize adenine dinucleotide cofactors to maintain redox balance in cells. (B-C) FAD+ levels are reduced in *alh-6* mutant animals fed OP50 (B) or HT115 (C) at the L4 developmental stage. (D) FAD biosynthetic pathway. (e-g) Dietary supplement of riboflavin restores FAD level (E), sperm size (F), and sperm activation (G) in *alh-6* mutants. (H-I) RNAi knockdown of *R10H10.*6 (H) or *flad-1* (I) in WT males reduces their sperm size compared to L4440 vector control. (J-K) RNAi knockdown of *R10H10.6* (J) or *flad-1* (K) in WT males impairs sperm activation upon Pronase treatment. Statistical comparisons of sperm size by ANOVA. Statistical comparisons of activation by fisher's exact test with p-value cut-off adjusted by number of comparisons. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$. All studies performed in biological triplicate; refer to Table S1 for n for each comparison.



Yen Figure 5

Figure 5. Mitochondrial dynamics drive sperm quality. (A-E) JC-1 dye stained mitochondria of WT (A-B), *alh-6* mutant (C-D); (B and D) are ImageJ detection of JC-1 stained sperm mitochondria area which are quantified in (E). (F) Mitochondria in *alh-6* mutant spermatids have reduced JC-1 red/green fluorescence ratio, indicating mitochondria depolarization. (G-H) RNAi knockdown of FAD biosynthetic pathway genes, *R10H10.6* (G) or *flad*-1 (H) increases mitochondrial fusion in WT spermatids. (I) Dietary supplement of FAD precursor riboflavin restores mitochondrial fusion in *alh-6* spermatids to WT level. (J-K) *eat-3* or *fzo-1* RNAi decreases mitochondrial fusion in both WT (J) and *alh-6* (K) mutant spermatids. (L) *drp-1* mutation increases mitochondrial fusion in spermatids. (M) *drp-1* mutation significantly impairs sperm activation in both WT and *alh-6* mutant spermatids. (N) *fzo-1* RNAi restores sperm activation in *alh-6* mutant. (O) *eat-3* RNAi reduces sperm activation in WT males but not *alh-6* males. Statistical comparisons of JC-1 Red/Green FL ratio by unpaired t-test. Statistical comparisons of mitochondria fusion by ANOVA. Statistical comparisons of sperm activation by Fisher's exact test with p-value cut-off

773 adjusted by number of comparisons. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$. All studies
774 performed in at least biological triplicate; refer to Table S1 for n for each comparison.
775
776


A
spermatid size (μm²)
ns
L4440
alh-6 RNAi
B
% sperm activated
****
L4440
alh-6 RNAi
C
% fused mitochondria
****
L4440
alh-6 RNAi
D
spermatid size (μm²)
ns
L4440
R10H10.6 RNAi
E
% sperm activated
****
L4440
R10H10.6 RNAi
F
% fused mitochondria
****
L4440
R10H10.6 RNAi
G
spermatid size (μm²)
ns
L4440
flad-1 RNAi
H
% sperm activated
****
L4440
flad-1 RNAi
I
% fused mitochondria
*
L4440
flad-1 RNAi
J
spermatid size (μm²)
**
ns
non-transgenic
transgenic
non-transgenic
transgenic
line 1
line 2
K
% sperm activated
****
****
non-transgenic
transgenic
non-transgenic
transgenic
line 1
line 2
L
% fused mitochondria
**
*
non-transgenic
transgenic
non-transgenic
transgenic
line 1
line 2

777

Figure 6. *alh-6* and FAD function cell autonomously in the germline to regulate sperm function. (A-C) Germline-specific RNAi of *alh-6* does not change sperm size (A), but does impair sperm activation (B) and increases mitochondrial fusion in sperm (C). Similarly, (D-I) germline-specific RNAi of *R10H10.6* and *flad-1* do not change sperm size (D,G), impair sperm activation (E,H), and increase mitochondrial fusion in sperm (F,I). (K-L) Germline-specific rescue of WT *alh-6* in *alh-6* mutant male animals increases sperm size (J) and restores activation (K) and mitochondrial dynamics (L). Statistical comparisons of sperm size and mitochondrial fusion in spermatids by unpaired t-test. Statistical comparisons of sperm activation by Fisher's exact test with p-value cut-off adjusted by number of comparisons. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$. All studies performed in biological triplicate; refer to Table S1 for n for each comparison.

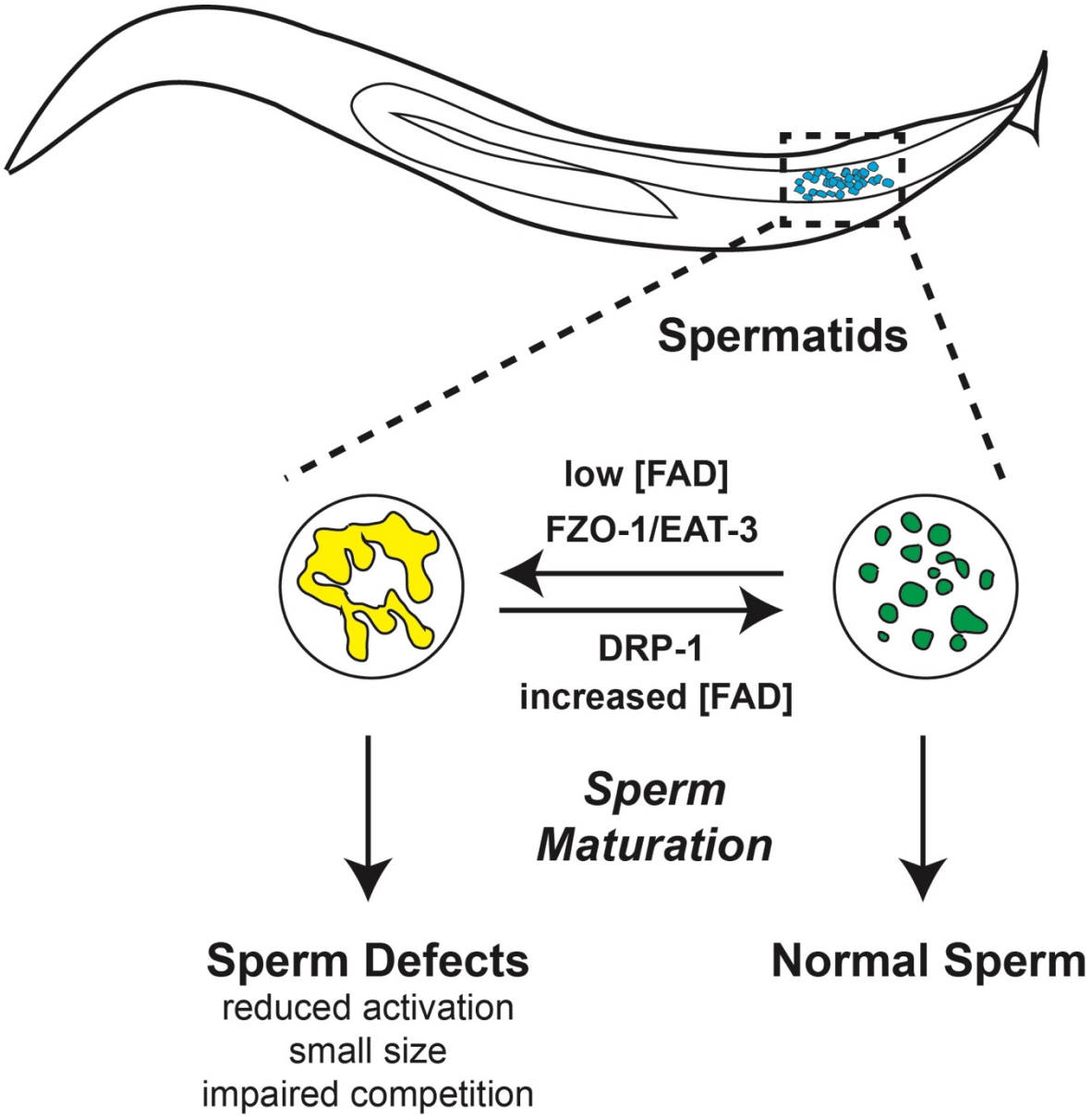
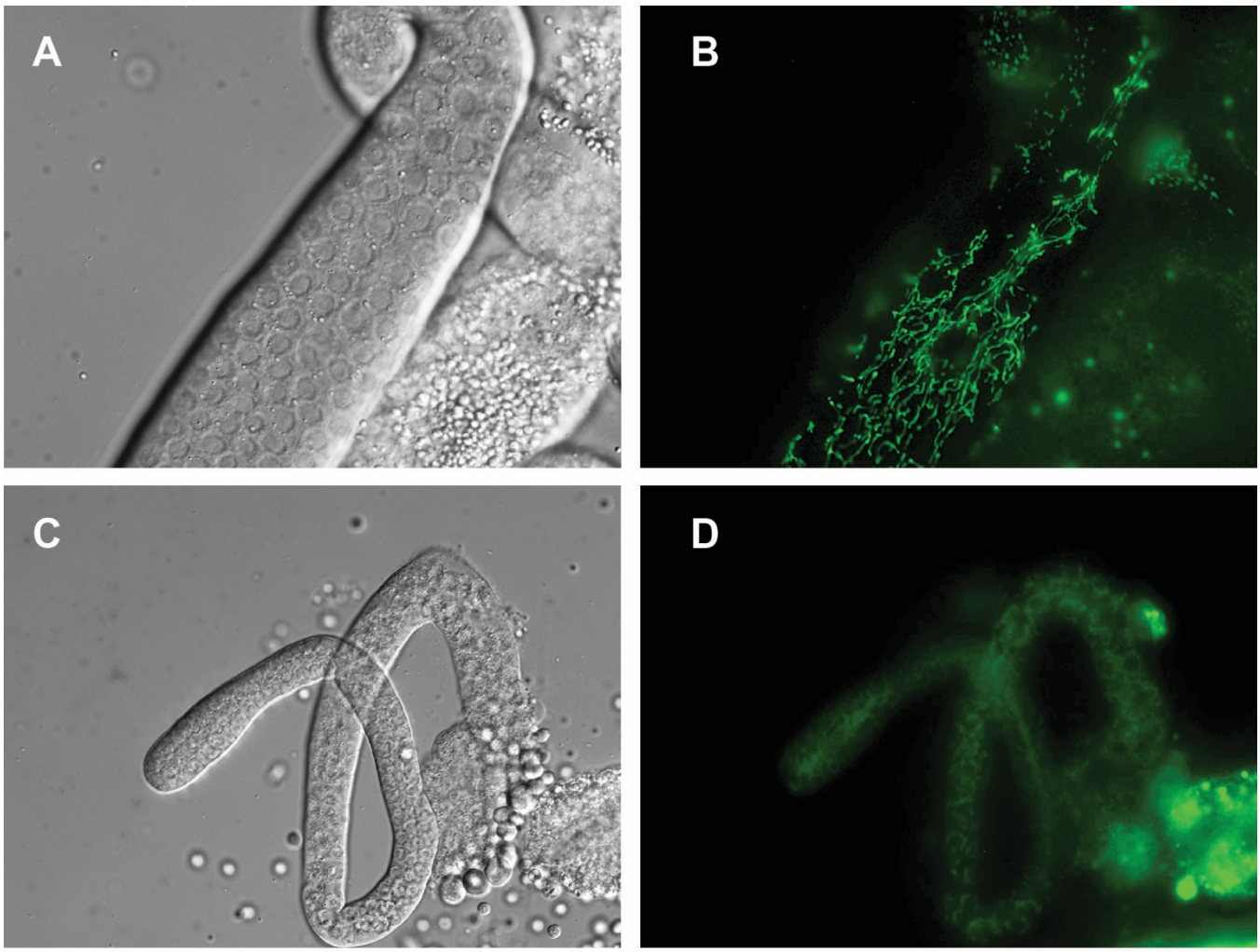


Figure 7. Model of *alh-6* and FAD mediated male reproductive senescence.

793 **SUPPLEMENTAL FIGURES**



794
795 **Figure S1. Mitochondrial localization of ALH-6 in the germline.** UV integrated *alh-6::gfp* strain under
796 its endogenous promoter reveal expression of ALH-6 in hermaphrodite (A-B) and male (C-D) germline.
797 a and c are DIC images while b and b are GFP images.
798

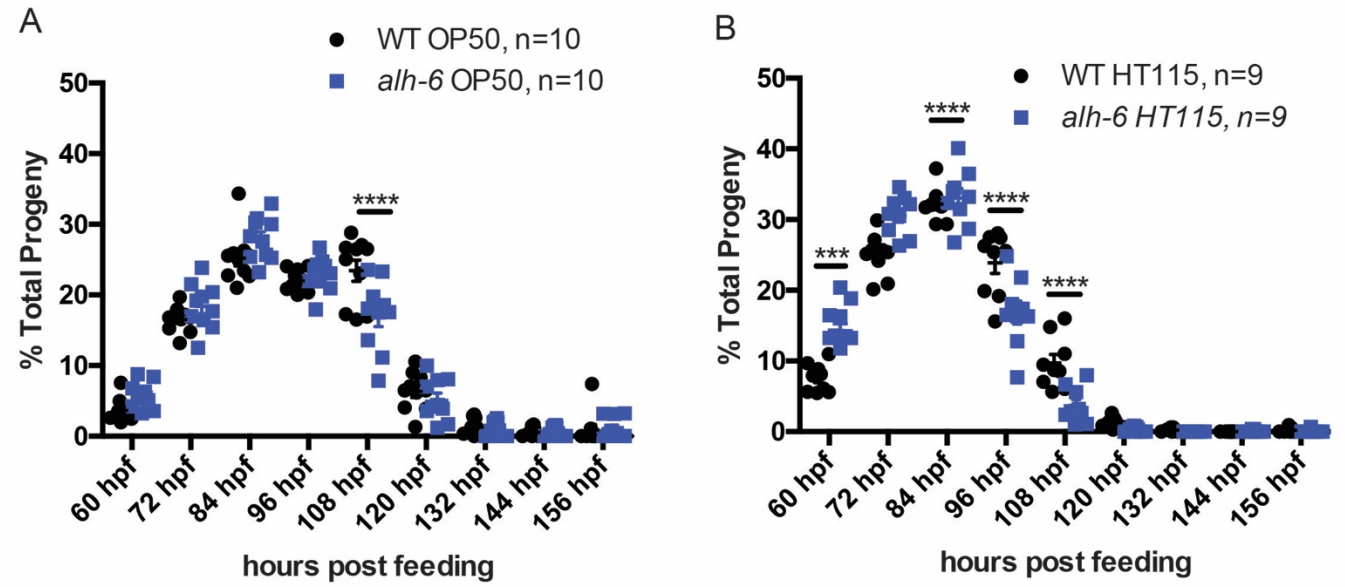
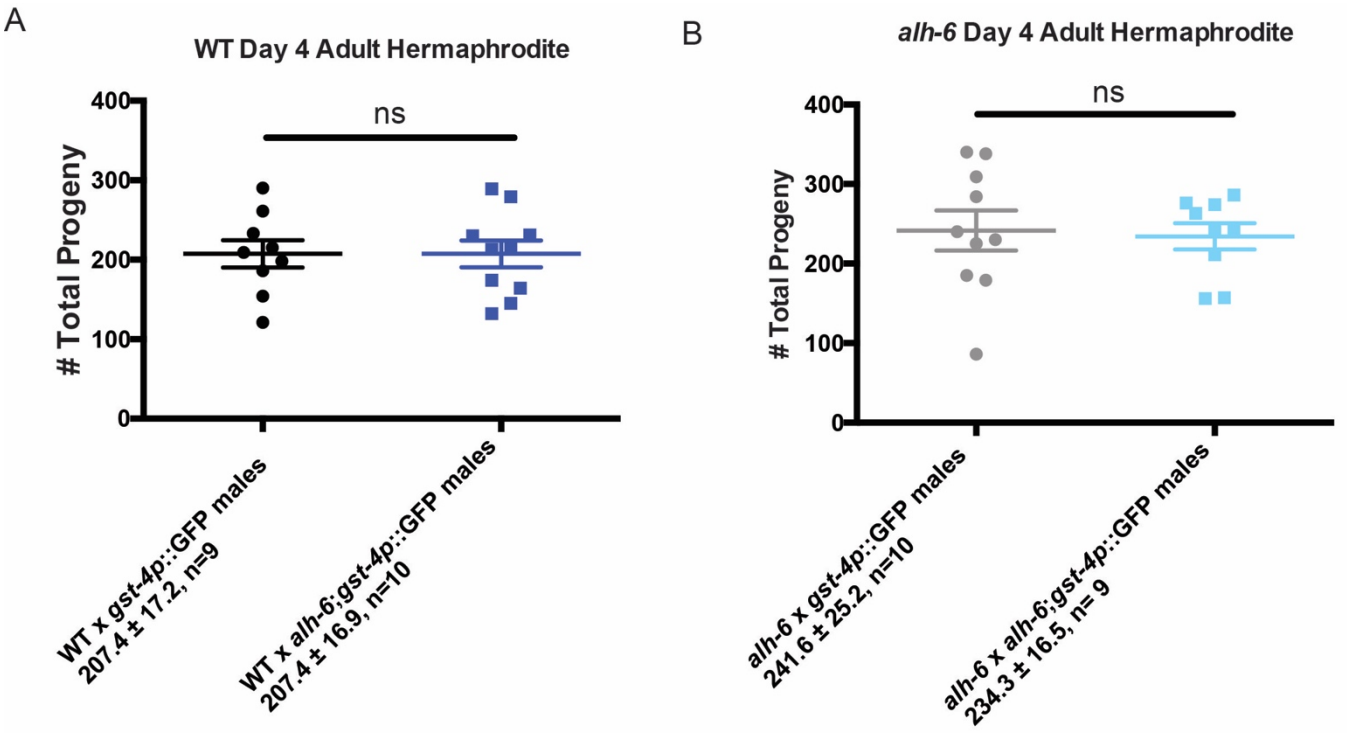


Figure S2. *alh-6* hermaphrodite reproductive span is similar to wild type (WT) on different diets. Progeny output time-courses are plotted as % total progeny for each time point. WT and *alh-6* mutant have similar output on OP50 (A) and HT115 (B). Significance indicate differences in progeny output at a particular time point done by multiple t-tests. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$. All studies performed in biological triplicate; refer to Table S1 for n for each comparison.



806
807 **Figure S3. *alh-6* fertility defects are sperm-specific.** (A) Day 4 adult WT hermaphrodites mated to
808 either *gst-4p::gfp* or *alh-6;gst-4p::gfp* males yield similar total brood size. (B) Day 4 adult *alh-6*
809 hermaphrodites mated to either *gst-4p::gfp* or *alh-6;gst-4p::gfp* males yield similar total brood size.
810 Comparisons made with unpaired t-test. *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$. All studies
811 performed in biological triplicate; refer to Table S1 for n for each comparison.
812

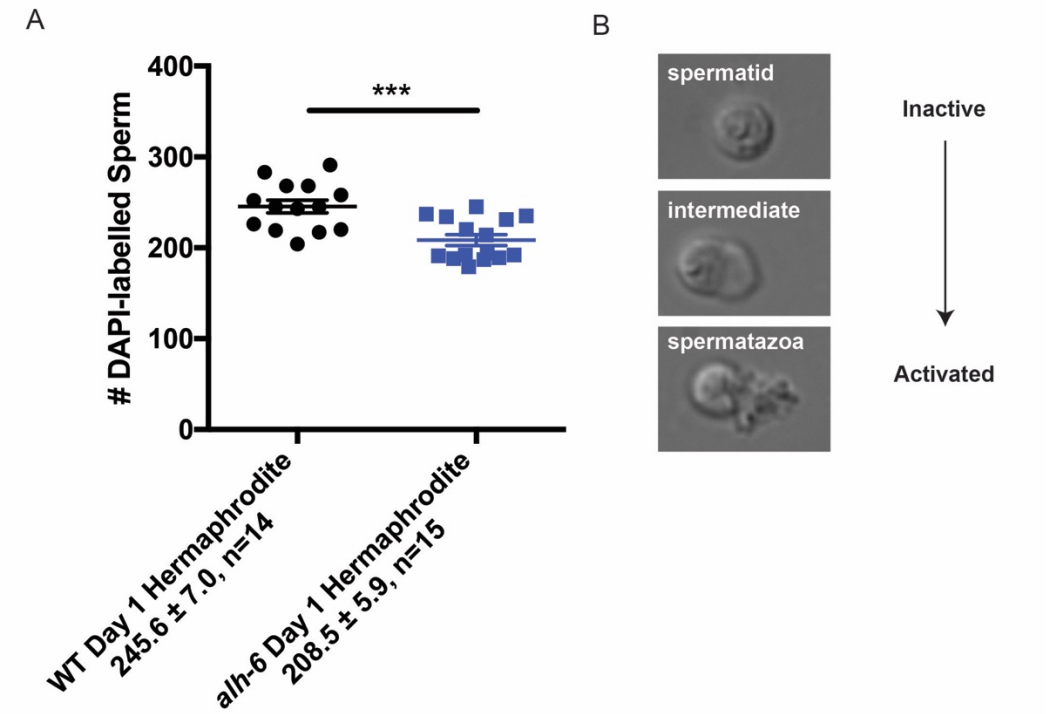
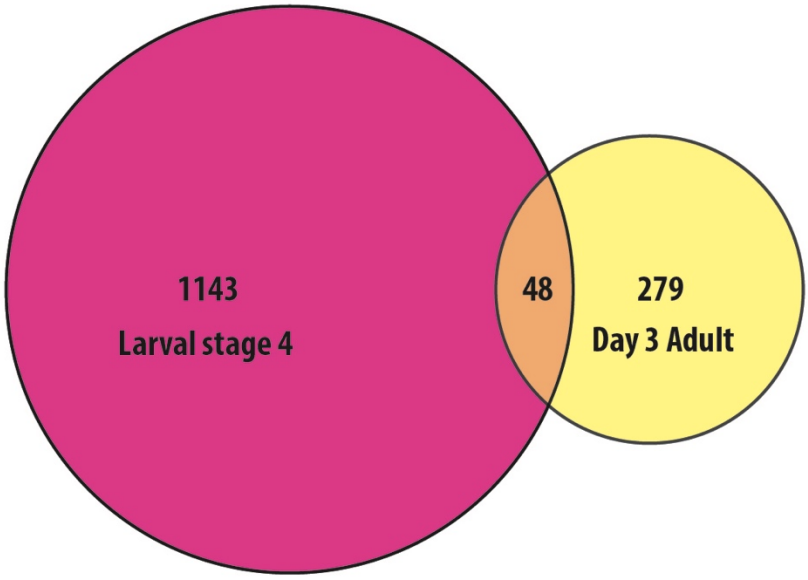


Figure S4. Sperm defects in *alh-6* mutants. (A) *alh-6* hermaphrodites have reduced sperm number as day 1 adults. (B) Spermiogenesis stages. Spermatozoa with fully formed pseudopods are considered activated.

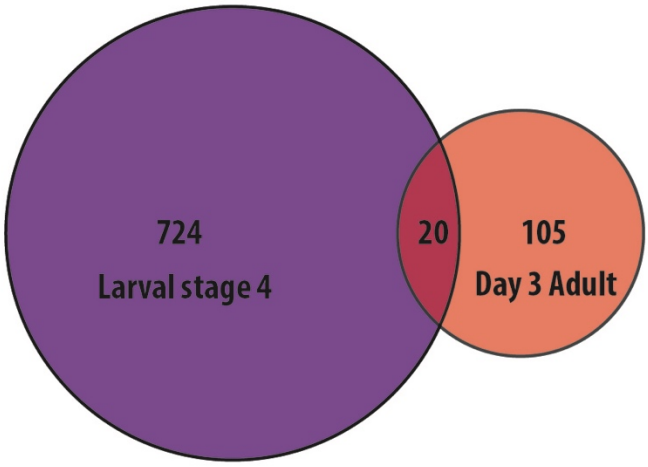
A

alh-6(lax105) vs wild type
2-fold increase in expression (0.05 FDR)

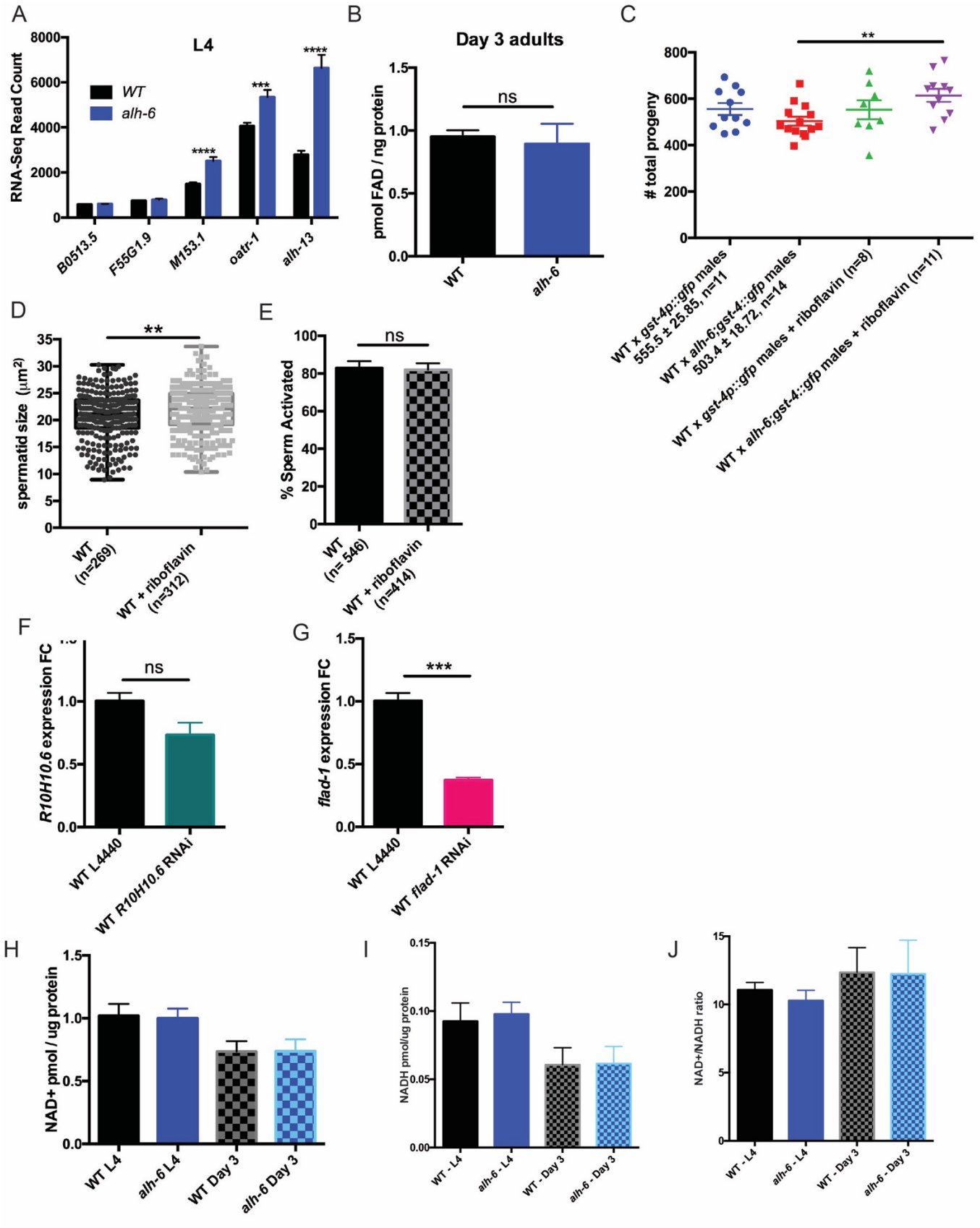


B

alh-6(lax105) vs wild type
2-fold decrease in expression (0.05 FDR)



818
819 **Figure S5. RNA-Sequencing data of WT and *alh-6* hermaphrodites at L4 and day 3 adulthood.** (A)
820 Number of genes that are significantly upregulated in *alh-6(lax105)* compared to WT at L4 and Day 3
821 adult stages. (B) Number of genes that are significantly downregulated in *alh-6(lax105)* compared to WT
822 at L4 and Day 3 adult stages. FDR = 0.05. All studies performed in biological triplicate; refer to Table S1
823 for n for each comparison.


A
L4
RNA-Seq Read Count
WT
alh-6
B0513.5
F55G1.9
M153.1
oatr-1
alh-13
B
Day 3 adults
pmol FAD / ng protein
ns
WT
alh-6
C
total progeny
WT x gst-4p::gfp males 555.5 ± 25.85, n=11
WT x alh-6;gst-4::gfp males 503.4 ± 18.72, n=14
WT x gst-4p::gfp males + riboflavin (n=8)
WT x alh-6;gst-4::gfp males + riboflavin (n=11)
D
spermatid size (μm²)
WT (n=269)
WT + riboflavin (n=312)
E
% Sperm Activated
ns
WT (n= 546)
WT + riboflavin (n=414)
F
R10H10.6 expression FC
ns
WT L4440
WT R10H10.6 RNAi
G
flad-1 expression FC
WT L4440
WT flad-1 RNAi
H
NAD+ pmol / ug protein
WT L4
alh-6 L4
WT Day 3
alh-6 Day 3
I
NADH pmol/ug protein
WT - L4
alh-6 - L4
WT - Day 3
alh-6 - Day 3
J
NAD+/NADH ratio
WT - L4
alh-6 - L4
WT - Day 3
alh-6 - Day 3

824

825 **Figure S6. Adenine nucleotide cofactor homeostasis is disrupted in *alh-6* mutants.** (A) *alh-6*
826 mutants display increased expression of metabolic enzymes to reduce P5C levels. (B) FAD levels are
827 unchanged between WT and *alh-6* mutant animals fed OP50 at day 3 adulthood. (C) WT hermaphrodites
828 mated to *alh-6;gst-4p::gfp* males fed OP50 supplemented with 2.5mM riboflavin results in increase in
829 total brood size compared to non-supplemented *alh-6;gst-4p::gfp* males. (D) Dietary riboflavin
830 supplement increased sperm size of WT males. (E) WT males fed OP50 diet supplemented with riboflavin
831 have similar % spermatid activated upon Pronase treatment as those without riboflavin supplement. (F)
832 *flad-1* expression is reduced by whole animal RNAi via RT-PCR verification. (G) *R10H10.6* expression
833 is modestly reduced by whole animal RNAi. (H) NAD levels are unchanged between WT and *alh-6*
834 animals at L4 and day 3 adulthood (I) NADH level is unchanged between aged matched WT and *alh-6*
835 hermaphrodites at both L4 and Day 3 adulthood. (J) NAD+/NADH level is unchanged between aged
836 matched WT and *alh-6* hermaphrodites at both L4 and Day 3 adulthood. *, $p<0.05$; **, $p<0.01$; ***,
837 $p<0.001$; ****, $p<0.0001$. All studies performed in biological triplicate; refer to Table S1 for n for each
838 comparison.
839
840

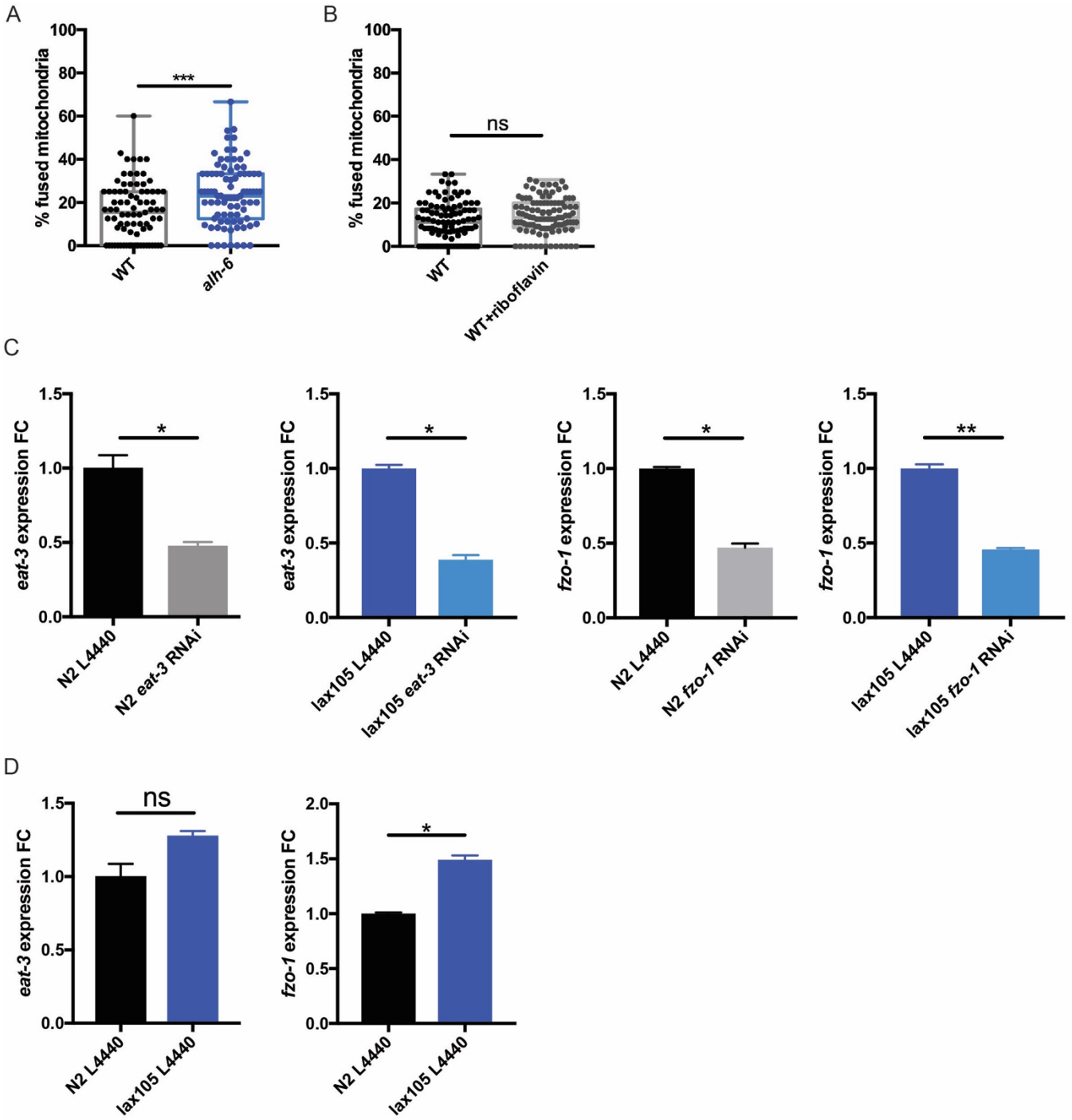
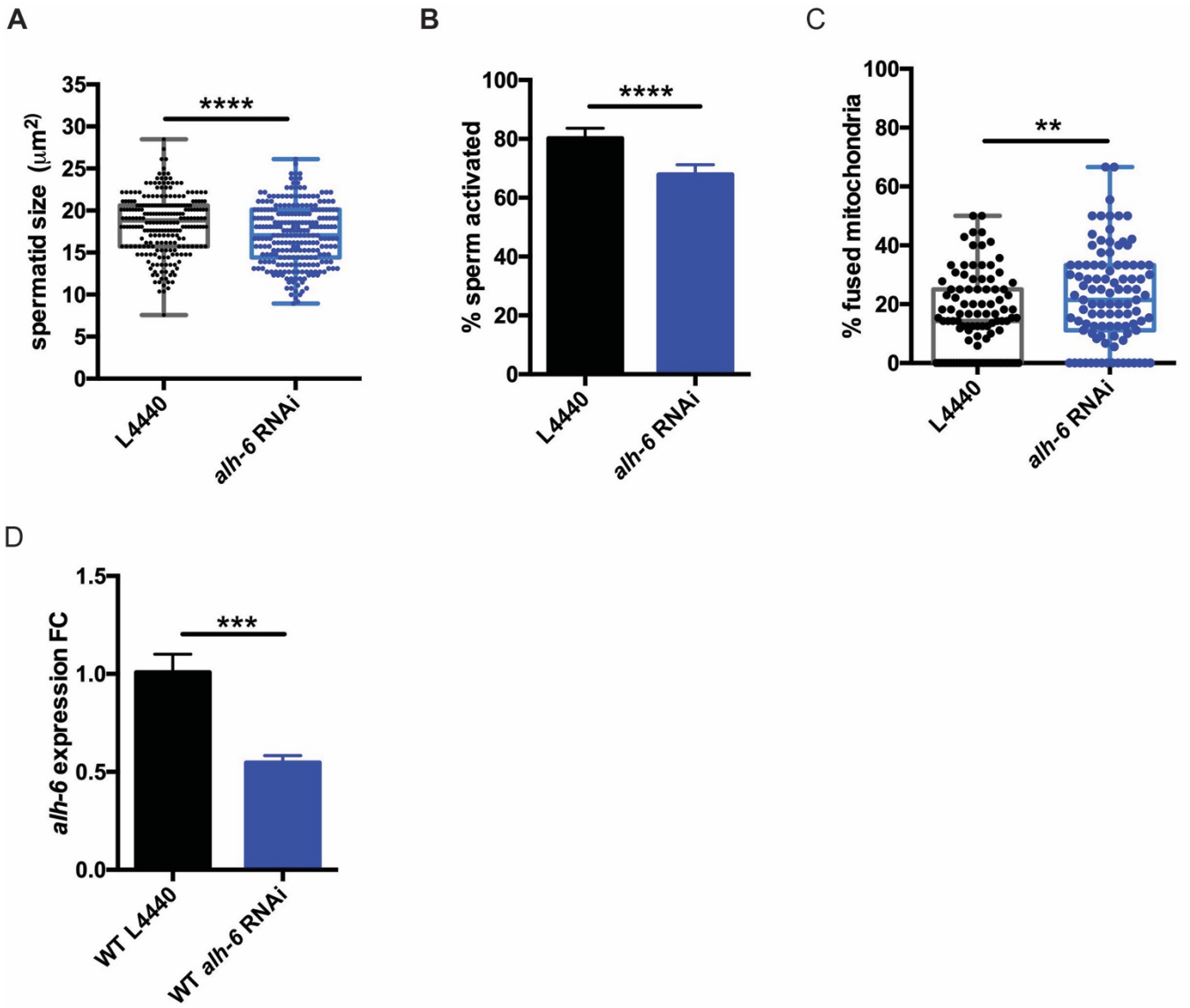


Figure S7. *fzo-1* is involved in mitochondrial dynamics aberration in *alh-6* spermatids. (A) *alh-6* males fed HT115 diet still have increased mitochondrial fusion in spermatids compared to age-matched WT males. (B) Riboflavin supplementation did not alter mitochondria in spermatids of WT males. (C) *eat-3* and *fzo-1* RNAi knockdown in WT and *alh-6* mutants are verified using RT-PCR. (D) *fzo-1* expression is increased in *alh-6* mutants, while *eat-3* expression is not significantly increased.



849
850 **Figure S8. Germline expression of WT *alh-6* is sufficient to rescue sperm defect.** (A-C) Whole
851 animal RNAi of *alh-6* reduces sperm size (A), impairs activation (B), and increases mitofusion in
852 spermatids (C). (D) *alh-6* expression is reduced by whole animal RNAi via RT-PCR verification.