The *C. elegans* Opa1 Homologue EAT-3 Is Essential for Resistance to Free Radicals

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Abstract

The *C. elegans eat-3* gene encodes a mitochondrial dynamin family member homologous to Opa1 in humans and Mgm1 in yeast. We find that mutations in the *C. elegans eat-3* locus cause mitochondria to fragment in agreement with the mutant phenotypes observed in yeast and mammalian cells. Electron microscopy shows that the matrices of fragmented mitochondria in *eat-3* mutants are divided by inner membrane septae, suggestive of a specific defect in fusion of the mitochondrial inner membrane. In addition, we find that *C. elegans eat-3* mutant animals are smaller, grow slower, and have smaller brood sizes than *C. elegans* mutants with defects in other mitochondrial fission and fusion proteins. Although mammalian Opa1 is antiapoptotic, mutations in the canonical *C. elegans* cell death genes *ced-3* and *ced-4* do not suppress the slow growth and small brood size phenotypes of *eat-3* mutants. Instead, the phenotypes of *eat-3* mutants are consistent with defects in oxidative phosphorylation. Moreover, *eat-3* mutants are hypersensitive to paraquat, which promotes damage by free radicals, and they are sensitive to loss of the mitochondrial superoxide dismutase *sod-2*. We conclude that free radicals contribute to the pathology of *C. elegans eat-3* mutants.

Introduction

Dominant optic atrophy (DOA) is one of the leading causes of inherited blindness. DOA is a progressive eye disease caused by degeneration of the retinal ganglion cell layer with ascending atrophy of the optic nerve [1]. The most prevalent form of DOA is caused by heterozygous mutations in the nuclear encoded, but mitochondrial targeted, Opa1 protein [2,3]. Opa1 is a member of the dynamin family of proteins. This family consists of several large GTP binding proteins with diverse cellular functions. The archetypal dynamin is required for endocytosis [4, 5], but two other dynamin-related proteins, Drp1 and Mitofusins in mammals, act along with Opa1 to control mitochondrial fission and fusion. Mitochondrial fission and fusion are dynamic processes required for the replenishment of mitochondria, for example in long neuronal projections and during cell growth and division. Mitochondrial fission facilitates the redistribution of mitochondria in response to local changes in the demand for ATP, while mitochondrial fusion is needed to exchange mtDNA and other components that may become damaged over time [6,7]. The rates of fission and fusion vary depending on cell type and environmental cues, but these rates are usually balanced. This balance is controlled by the opposing actions of the different dynamin family members on or in mitochondria.

The three dynamin-related proteins that affect mitochondria have different topologies and play different roles in fission and fusion. Mammalian Drp1 and the homologous proteins in *C. elegans* and yeast are cytosolic proteins that are required for mitochondrial division [8–10]. These proteins wrap around constricted parts of mitochondria where they control a late stage of mitochondrial outer membrane division [8,11]. Mutations in Drp1 homologues give rise to a highly interconnected mesh of mitochondria [8–10]. Fusion between mitochondrial outer membranes is mediated by a different set of dynamin family members [12]. These proteins are called Mitofusins in mammals and Fzo1 in yeast and Drosophila. They have two transmembrane segments that anchor the proteins in the mitochondrial outer membrane. There are two Mitofusins in mammals (Mfn1 and
Dominant Optic Atrophy is a progressive eye disease caused by degeneration of retinal ganglion cells. The most prevalent form of DOA is caused by mutations in the Opal protein. This protein is required for fusion between mitochondria, it has an anti-apoptotic function, and it is required for mitochondrial DNA segregation. It has, nevertheless, been difficult to understand why mutations in Opal specifically affect retinal ganglion cells. We used the nematode C. elegans as a model to study the underlying causes of Opal pathologies. C. elegans Opal is encoded by the eat-3 gene. Mutants are sluggish, grow slowly, remain small, and have small brood sizes. These phenotypes are not suppressed by mutations in cell death genes, suggesting that apoptosis does not contribute to eat-3 pathogenesis. Instead, eat-3 mutants are hypersensitive to paraquat, which promotes damage by free radicals, and they are sensitive to loss of the mitochondrial superoxide dismutase sod-2, which is needed to eliminate free radicals from the mitochondrial matrix. Moreover, eat-3 mutants overexpress SOD-2, most likely compensating for increased free radical production. These results show that C. elegans EAT-3 is important for resistance to free radicals and they raise the possibility that free radicals contribute to DOA in humans.

Mfn2), which are often coexpressed but are not redundant [13]. Mutations in Mfn2 cause peripheral neuropathy in Charcot Marie Tooth (CMT) disease [14]. Mutations in Fzo1 and Mitofusins give rise to fragmented mitochondria [12,15,16], but this fragmentation can be suppressed by mutations in Drp1 homologues in yeast and mammalian cells.

Evidence for the role of Opal in fusion between mitochondrial inner membranes initially came from studies of the yeast homologue of Opal, which is called Mgm1. The mitochondria of yeast Mgm1 mutants are fragmented, they form aggregates and they lose their mtDNA [17–19]. Conditional mutations show that the loss of mtDNA is preceded by the changes in mitochondrial morphology, indicating that loss of mtDNA is a secondary defect [18]. The mitochondrial fragments in Mgm1 mutants are converted into a closed network of mitochondria by additional mutations in Mfn1 homologues [20–23]. This role was substantiated by experiments in which two yeast cells with differently labeled mitochondria are allowed to fuse. The mitochondria of Mgm1 mutant cells do not mix the two labels showing that they are unable to fuse [24]. A direct role in mitochondrial fusion was then shown with in vitro reconstitution experiments using mitochondria isolated from yeast Mgm1 mutants [25].

Biochemical analysis shows that yeast Mgm1 and mammalian Opal1 are localized to the mitochondrial intermembrane space [19,24,26,27]. The mitochondrial leader sequences of Mgm1 and Opal1 are cleaved upon import into mitochondria. In yeast, roughly half of the protein is further processed by a rhomboid protease [28–32]. A homologue of this protease, called PARL, exists in mammals, but cleavage in higher eukaryotes may require other proteases [33]. Immuno-electron microscopy of mammalian cells shows the bulk of Opal protein distributed throughout cristae with only a small portion localized to the boundary space between mitochondrial inner and outer membranes [27].

The importance of Opal for housekeeping functions, such as mitochondrial fusion and redistribution of mtDNA, is apparent from these cell biological studies. It has, nevertheless, been difficult to establish the exact sequence of events leading to retinal ganglion cell death in DOA, even with the mouse models that have recently become available [34,35]. The effects on retinal ganglion cells are restricted both in time and place and they occur with the mild loss of Opal function that results from haploinsufficiency of the Opal gene [36]. In contrast, cultured mammalian cells transfected with Opal siRNA typically show the stronger effects that are associated with complete loss of Opal function. Late time points after transfection with Opal siRNA show mitochondria that are reduced to small dispersed fragments [27,37,38], while early time points show that this fragmentation is preceded by internal rearrangements of the mitochondrial inner membrane [27]. At these times the mitochondria swell and stretch forming localized constrictions, similar to the changes in mitochondrial morphology that are observed during early stages of apoptosis [39]. Transfection with Opal siRNA also increases susceptibility to apoptosis by promoting cytochrome c release [40]. Increased susceptibility to apoptosis, exacerbated by photo-damage, was therefore proposed as a possible cause of retinal ganglion cell death in patients with DOA [41]. However, alternatives, such as the effects of reduced levels of ATP, are also considered as possible causes of DOA [42].

Here we show that the previously described C. elegans eat-3(ad426) strain [43] has a mutation in the D2013.5 gene, which encodes the ortholog of yeast Mgm1 and mammalian Opal. The ad426 mutation leads to fragmented mitochondria similar to those cause by mutations in Opal1 and Mgm1. Electron microscopy shows that eat-3(ad426) mitochondria have disorganized inner membranes and a large number of inner membrane septae. We also find that eat-3(ad426) growth defects are attributable to impaired oxidative phosphorylation and increased damage from free radicals within mitochondria.

Results

C. elegans EAT-3 Is an Orthologue of Yeast Mgm1 and Mammalian Opal

BLAST homology searches show that C. elegans has a single homologue of yeast Mgm1 and mammalian Opal1. This protein is encoded by the D2013.5 gene. It has a predicted molecular weight of 106.8 kDa and 46% amino acid identity to human Opal1. Similar to yeast Mgm1 and mammalian Opal1, this C. elegans protein has a putative mitochondrial targeting sequence followed by domains that are typical of dynamin family members: a conserved GTPase domain, a middle domain and a GED or assembly domain [44] (Figure 1A). Pilot experiments with D2013.5 RNAi yielded worms that grew slowly, remained small and had small numbers of progeny. These phenotypes led us to investigate the eat-3 mutant, which was previously identified in a screen for mutations that cause abnormal or defective eating in C. elegans [43]. The D2013.5 gene is very close to the eat-3 locus (within 0.2 map units) and the overall appearance of D2013.5 RNAi animals is similar to that of eat-3 animals.

Upon sequencing the D2013.5 gene from eat-3(ad426) animals, we found a single point mutation, changing a valine at position 328 to an isoleucine (Figure 1B). Although this is a surprisingly conservative change, there are other examples where such a change has a dramatic effect on protein function [45]. The affected residue is just downstream of the G2 threonine in the effector binding loop of the dynamin-like GTPase, where it may disrupt the GTPase cycle. A C. elegans dynamin mutant, dyn-1(ky51), has a mutation that is also very close to the G2 threonine [46]. Surprisingly, this dynamin mutation can be suppressed by a second mutation at the same position as that mutated in eat-3(ad426), which further demonstrates the importance of this particular residue (Figure 1B).

Author Summary

Dominant Optic Atrophy is a progressive eye disease caused by degeneration of retinal ganglion cells. The most prevalent form of DOA is caused by mutations in the Opal protein. This protein is required for fusion between mitochondria, it has an anti-apoptotic function, and it is required for mitochondrial DNA segregation. It has, nevertheless, been difficult to understand why mutations in Opal specifically affect retinal ganglion cells. We used the nematode C. elegans as a model to study the underlying causes of Opal pathologies. C. elegans Opal is encoded by the eat-3 gene. Mutants are sluggish, grow slowly, remain small, and have small brood sizes. These phenotypes are not suppressed by mutations in cell death genes, suggesting that apoptosis does not contribute to eat-3 pathogenesis. Instead, eat-3 mutants are hypersensitive to paraquat, which promotes damage by free radicals, and they are sensitive to loss of the mitochondrial superoxide dismutase sod-2, which is needed to eliminate free radicals from the mitochondrial matrix. Moreover, eat-3 mutants overexpress SOD-2, most likely compensating for increased free radical production. These results show that C. elegans EAT-3 is important for resistance to free radicals and they raise the possibility that free radicals contribute to DOA in humans.
To verify that eat-3(ad426) is indeed a D2013.5 mutant, we injected this strain with a wildtype D2013.5 cDNA under control of the D2013.5 gene promoter. The number of progeny reaching the L4 larval stage increased from 10 per uninjected eat-3 animal (SD = 8, n = 28) to 30 per transgenic animal (SD = 22, n = 26), showing that a wildtype D2013.5 construct partially rescues the eat-3 mutant. Partial rescue is common for C. elegans genes with a maternal effect, since transgenes are often poorly expressed in the germline. We obtained further evidence that D2013.5 encodes the eat-3 locus with a second allele, named tm1107. The tm1107 allele is most likely a null, since it has a 419 bp deletion that causes a frameshift at position 329 and thus eliminates two thirds of the D2013.5 protein. The absence of EAT-3 protein in eat-3(tm1107) animals, but not in ad426 animals, was confirmed by Western blot analysis using an antibody raised against the C. elegans EAT-3 protein (Figure S1). Homozygous eat-3(tm1107) animals survive but they have fragmented mitochondria, a decrease in broodsize, sluggishness and slow growth phenotypes, similar to the phenotypes of eat-3(ad426) animals. More importantly, tm1107 fails to complement eat-3(ad426), indicating that ad426 and tm1107 are both alleles of eat-3 and that the phenotypes are due to mutations in the D2013.5 gene (data not shown). The C. elegans D2013.5 locus is henceforth called eat-3.

Additional alleles of eat-3 were isolated in an F2 screen for suppressors of eat-3(ad426) mutant phenotypes. The progeny of 36,000 F1 animals were screened for restored growth rate, size and fecundity. This screen yielded seven new mutants with restored growth rates. Five of these mutants have second site mutations in the eat-3 gene (cq6-cq10), while two mutants have mutations that lead to premature stops in the eat-3 gene (cq5 and cq11). The new mutations in the eat-3 locus all cause substitutions in the GTPase domain (Figure 1B). A similar screen with the dyn-1(ky51) also yielded a series of substitutions in the GTPase domain (Figure 1B). When the new mutations are mapped onto the crystal structure of the dynamin GTPase domain [47], they reveal a striking pattern of convergence on the G2 motif of the GTPase domain (Figure 1C). It seems likely that they restore the ability of the G2 threonine to interact properly with GTP or make the conformational changes that occur during GTP hydrolysis.

Figure 1. Mutations in C. elegans eat-3 and dyn-1 Mutants. (A) Dynamin family members in C. elegans. DYN-1 is required for scission of vesicles from the plasma membrane. DRP-1 is required for scission of mitochondrial outer membranes. FZO-1 is required for fusion of mitochondrial outer membranes. EAT-3 is required for fusion of mitochondrial inner membranes. The GTPase, Middle, and GTPase Effector (GED) domains are shared between dynamin family members. In addition, DYN-1 has a pleckstrin homology (PH) domain and a proline rich domain (PRD), FZO-1 has two transmembrane segments that anchor the protein in the mitochondrial outer membrane, and EAT-3 has a mitochondrial leader sequence (mls) that targets the protein to the mitochondrial intermembrane space. Some key alleles are shown on the right. (B) Sequence alignment of the GTPase domains of C. elegans EAT-3 (D2031.5), human Opal, human Dyn1, and C. elegans DYN-1. The GTP binding consensus sequences (G1-4) are indicated with white circles. The primary mutations in eat-3(ad426) and dyn-1(ky51) alleles are shown in the red circles. Secondary mutations in the intragenic revertants are shown in the blue circles. The dyn-1(ky51) revertants cq2, cq3, and cq4 are shown below the sequences and the eat-3(ad426) revertants cq6, cq7, cq8, and cq9 are shown above the sequences. (C) The positions of the GTP binding motifs (open circles labeled G1-4), the positions of primary mutations (red circles), and the positions of secondary mutations (blue circles) superimposed on the structure of the rat Dyn1 GTPase domain [47]. The arrows point to the primary mutation suppressed by each secondary mutation.

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Growth and Survival of C. elegans Opa1 Mutants

To investigate how eat-3 affects mitochondria, we focused on mitochondrial morphology in C. elegans body wall muscles. Mitochondria were detected with mitochondrial matrix markers, consisting of an N-terminal mitochondrial leader sequence fused to GFP, cyan fluorescent protein (CFP) or yellow fluorescent protein (YFP), and mitochondrial outer membrane markers, consisting of a resident outer membrane protein (TOM70) fused to GFP, CFP or YFP [8]. The functions of the EAT-3 protein were disrupted by expressing dominant negative mutant proteins or antisense cDNA, which effectively causes localized RNAi, under control of the muscle specific myo-3 promoter. The dominant negative mutations that we used here are T322A, which disrupts the G2 motif of the
GTPase domain, and K300A, which is analogous to the K44A mutation in the G1 motif of dynamin [5].

Both dominant negative mutations and the loss of function induced by antisense cDNA cause mitochondria to fragment into a large number of small pieces (Figure 2B and 2C; 83% of cells were affected, n = 200). Labeling with a mitochondrial outer membrane marker shows that the mitochondrial fragments are truly detached (not shown). The exact size and distribution of mitochondrial fragments varied between the different treatments, but it was not evident that these phenotypes represent different levels of severity (Figure 2B–2C). Mitochondrial fragmentation is also observed in eat-3(ad426) and in eat-3(tm1107) animals and this phenotype is reversed in transgenic animals expressing wildtype eat-3 cDNA under control of the myo-3 promoter (Figure 2D–2F).

Similar mitochondrial fragmentation is observed in muscle cells of fzo-1(tm1133) mutants (Figure 2G), which have a mutation in the C. elegans homologue of Drosophila and yeast Fzo1 and mammalian MitoFusins, proteins required for fusion of the mitochondrial outer membrane [12,13,15,16,48,49]. The mitochondria of eat-3 and fzo-1 mutants are similarly fragmented consistent with their roles in mitochondrial fusion. However, the gross anatomical defects (size, growth rate and broodsize) are less severe in fzo-1 (tm1133) mutants and fzo-1 RNAi animals than in eat-3 mutants (data not shown), even though fzo-1 (tm1133) is also a null allele (it has a chromosomal deletion that truncates the protein after 65 amino acids). These results suggest that there might be functional differences in the ways that EAT-3 and FZO-1 proteins work.
using electron tomography. In this technique, a thick section is viewed at different angles and the imaging data is used to reconstruct a three dimensional model. Images of an eat-3 mitochondrion are shown in Figure 4. This mitochondrion has several matrix “bubbles”, divided from the rest of the matrix by septae of inner membrane. These bubbles are sealed off, indicating that the septae observed in the thin sections reflect completed inner membrane divisions. The three-dimensional reconstructions of eat-3 mitochondria also show that some of the membrane inclusions that appear free floating in the matrix are indeed physically separated from the inner membrane (Figure 4). This separation suggests severing of membranes within the mitochondrial matrix by an as yet unknown mechanism. Similar free-floating structures were previously observed in a mitochondrial myopathy of unknown etiology in humans [50] and in mitochondria of apoptotic cells [51].

Inhibition of Growth by eat-3 RNAi

To investigate the role of eat-3 in whole worms we first determined the expression pattern of the eat-3 gene with transgenic animals that carry an extrachromosomal array with the eat-3 gene promoter fused to green fluorescent protein (GFP) and β-galactosidase coding sequences. This pattern was similar to that of C. elegans drp-1 [8] with high levels in intestinal cells, in muscle cells and in neurons and low levels in other cell types (data not shown). Cell types with high levels of expression may be metabolically more active than other cell types, but basal levels of this protein are most likely required in all cells. We then conducted experiments to assess the effects of eat-3 loss of function on the growth and brood size of worms. Worms

![Figure 3. Aberrant Internal Structures in eat-3(ad426) Mitochondria.](image)

![Figure 4. Tomographic Reconstruction of an eat-3(ad426) Mitochondrion.](image)
 injected with eat-3 dsRNA give viable progeny but their brood size is reduced (90 viable eggs per worm, SD = 14, n = 20, compared with 270 for wildtype, SD = 10, n = 20). The F1 worms remain small, are sluggish and develop slowly. Similar effects were observed with chromosomal mutations in the eat-3 gene. In an experiment with ad426 and tm1107 alleles, the averages were 302 for wildtype (SE = 8.2, n = 7), 51 for eat-3(ad426) (SE = 8.4, n = 7) and 50 for eat-3(tm1107) (SE = 10, n = 7). In an experiment with intragenic revertants of eat-3(ad426), the averages were 75 for eat-3(ad426g9) (SE = 4.8, n = 6), 190 for eat-3(ad426g7) (SE = 14, n = 6) and 65 for eat-3(ad426g8) (SE = 27, n = 6). These numbers are variable, as one might expect from different allele strengths, but they are all reduced when compared with wild type animals.

Growth was quantified by measuring the lengths of progeny from RNAi injected worms (Figure 5A). Progeny from worms injected with eat-3 dsRNA were on average only 0.15 mm in length at four days after hatching (SD = 0.02, n = 20), whereas wildtype animals were 1 mm in length (SD = 0.04, n = 5). Even after three weeks, the eat-3 RNAi worms rarely reach 0.5 mm, consistent with a previous study showing that the eat-3(ad426) mutant also remains small [52]. Similar effects on length were observed with chromosomal mutations in the eat-3 gene (Figure 5B). Worms with eat-3 deficiencies live longer than wildtype animals (33 days for RNAi worms, versus 20 days for untreated worms, and as shown previously with eat-3(ad426) animals [53]), but it also takes them longer to reach adulthood (10 days for eat-3 RNAi progeny whereas wildtype animals take 2 days). It would thus appear that developmental decisions are normal, but the rate of development is greatly reduced as one might expect from a general decrease in metabolic activity.

To see how mitochondria in the gonads of eat-3 RNAi animals are affected, we stained the gonads of injected worms with Rhodamine 123, as was previously done with C. elegans dpf-1 RNAi animals [8]. We find that the mitochondria are more dispersed, but do not appear to be less abundant than in untreated gonads (Figure 5C–5D). The effect of eat-3 RNAi on mitochondria is, however, much less dramatic than that of dpf-1 RNAi, which causes mitochondria to form large aggregates [8]. However, Hoechst staining shows that there is a paucity of nuclei when compared with wildtype (Figure 5C–5D). This paucity suggests reduced numbers of mitotic divisions at the tips of the gonads, which would lead to the production of fewer oocytes in agreement with the low brood sizes of eat-3 mutant and RNAi treated animals.

Genetic Interactions between C. elegans eat-3, drp-1, and fzo-1

Two of the mutants that were isolated in our screen for suppressors of eat-3(ad426) have premature stop codons in the drp-1 gene, showing that defects in mitochondrial fission suppress the defect in mitochondrial fusion caused by a mutation in eat-3. Similar genetic interactions were previously observed with mutations in the orthologous yeast genes [19,21]. Since mitochondrial fission and fusion proteins not only act antagonistically on mitochondrial morphology, but also affect the viability of worms, we conducted additional experiments to further determine the extent of eat-3 suppression by dpf-1 loss of function. First, we tested whether the fragmentation of mitochondria is reversed by the dominant negative mutant DRP-1[K40A], which blocks division of the mitochondrial outer membrane [8]. Constructs encoding Pmyo-3::DRP-1[K40A] and a mitochondrial outer membrane marker were injected into eat-3(ad426) worms or into wildtype worms along with the Pmyo-3::antisense-eat-3 construct. DRP-1[K40A] gives rise to interconnected mitochondria, regardless of whether it is expressed in a wildtype background, with antisense eat-3, or in an eat-3 mutant (100% of cells, n = 50, data not shown). The dpf-1(cq3) allele, which was isolated as a suppressor of eat-3(ad426), also causes hyperconnectivity of mitochondria in eat-3(ad426) animals, similar to the connectivity observed in the dpf-1(cq3) single mutant (Figure 6A–6D). We conclude that a functioning mitochondrial division apparatus is required for the mitochondrial fragmentation induced by mutant eat-3.

To find out whether other abnormalities of the eat-3(ad426) mutant are reversed by a defect in mitochondrial division, we determined the brood-size of eat-3(ad426) mutants grown with or without dpf-1 RNAi. Our results show that dpf-1 RNAi significantly restores the brood-size of eat-3(ad426) mutants (Figure 6E). A chromosomal mutation in dpf-1 also restores the brood size as shown with eat-3(ad426); dpf-1(cq5) animals (Figure 6E). We conclude that defects in C. elegans DRP-1 and EAT-3 proteins compensate each other’s physiological defects. Similar effects were observed in yeast, where the effects of mutations in the EAT-3 homologue Mgm1 are suppressed by mutations in the DRP-1 homologue Dnm1 [19,21]. To our surprise, however, the brood size defect of the eat-3(ad426) allele was also partially suppressed by fzo-1 RNAi, while the eat-3(tm1107) allele, which is most likely a null allele, was not suppressed by dpf-1 or fzo-1 RNAi (Figure 6E), even though mitochondrial fragmentation in eat-3(tm1107) animals is reversed by dpf-1 RNAi (Figure S2). These results suggest that the eat-3(ad426) allele has some residual protein function that is masked by wildtype DRP-1 and FZO-1 proteins. In support of this residual activity, we find that eat-3 RNAi reverses the restoration of brood size by the dpf-1 mutation in eat-3(ad426); dpf-1(cq5) animals (Figure 6E).

The suppressive effects of dpf-1 and fzo-1 loss of function can be explained by the fact that they both act upstream of inner membrane fusion. Loss of dpf-1 prevents the formation of inner membrane fusion intermediates by introducing a fission defect that is epistatic to fusion defects, while loss of fzo-1 does this by blocking outer membrane fusion, which also precedes inner membrane fusion. It seems likely that the inner membrane fusion intermediates, formed with wildtype dpf-1 and fzo-1, sequeser mutant EAT-3 protein, while loss of dpf-1 or fzo-1 function frees this
Failure of ced-3 and ced-4 Mutations To Suppress eat-3 Phenotypes

It is well-established that Opa1 has an anti-apoptotic function in mammalian cells [37,40,54,55]. We therefore tested whether apoptosis contributes to the various eat-3 phenotypes in C. elegans by making double mutants with eat-3(ad426) and ced-3(n717) or ced-4(n1894) mutations. The ced-3 gene encodes a caspase and the ced-4 gene encodes APAF-1. Mutations in either gene block programmed cell death in C. elegans. The effects on broodsize were determined by counting the numbers of progeny that survive to the L4 larval stage. The brood sizes were reduced to varying degrees in each of the single mutants, but the brood size defects of the eat-3(ad426) animals were not significantly affected by the additional mutations in ced-3 and ced-4 loci (Figure 7A). Although ced-3 encodes the caspase that is utilized for all programmed cell death in C. elegans and inducible cell death in C. elegans gonads [56], there are three other caspases (csp-1, csp-2 and csp-3) that might contribute to cell death under other circumstances. We tested these csp genes with feeding RNAs, but saw no effect on the brood size of eat-3(ad426) mutants. Some redundancy between the caspases remains possible, but redundancy does not apply to ced-4, since it encodes the single C. elegans homologue of APAF-1.

The ced-4 gene is central to all caspase dependent cell death in C. elegans. The absence of an effect of ced-4 mutations on the eat-3 broodsize defect, as shown here (Figure 7A), is therefore a reliable indication that caspase dependent cell death does not contribute to the reduced broodsize of eat-3 mutants. To verify that eat-3 mutants show no increase in cell death, we counted the numbers of dying cells by looking for light-refractory cells with DIC microscopy in eat-3(ad426) and eat-3(tm1107) embryos at the comma stage. Those numbers were not significantly different from the numbers for wildtype embryos (Figure 7B). To verify that the ced mutants used here were effective, the numbers of dying cells were also counted in ced-3(n717) and ced-4(n1894) mutant embryos. As expected, these mutants show strongly reduced numbers of dying cells. We conclude that ced-3 and ced-4 dependent cell death does not contribute to the reduced brood size of eat-3 animals. The eat-3; ced-3 and eat-3; ced-4 double mutants also grow slowly and remain small similar to the eat-3 single mutants (data not shown), suggesting that cell death does not contribute to these other maladies.

Parakquat Sensitivity of eat-3 Mutants

The growth and brood size defects of eat-3 mutants resemble those of gas-1 and mev-1 mutants, which have defects in Oxidative Phosphorylation complexes. Those mutants are also more susceptible to damage from free radicals, as shown by their sensitivity to parakquat, which produces superoxide radicals through a radical ion intermediate [57]. To test whether eat-3 mutants are also sensitive to free radicals, we grew eat-3(ad426) animals with increasing concentrations of parakquat. We find that eat-3(ad426) animals are significantly more sensitive to parakquat than wildtype animals (Figure 8A). Values for ICG50 were on
average 0.25 mM for eat-3(ad426) animals and 0.44 mM for wildtype (N2) animals (averages of four independent experiments). Increased sensitivity to paraquat is also observed with eat-3(tm1107) animals (Figure 8B), confirming that this effect is caused by loss of eat-3 function. The sensitivity of eat-3(ad426) animals to paraquat is suppressed by the ddp-1 mutations in eat-3(ad426); ddp-1(cq3) and in eat-3(ad426); ddp-1(cq11) animals (Figure 8A–8B). These two ddp-1 mutations were isolated independently, confirming that they are the cause of this reversal. Since these results suggest that mitochondrial outer membrane fission and fusion processes affect paraquat sensitivity, we tested whether the fzo-1(tm1133) mutant, which has a defect in mitochondrial outer membrane fusion, are also sensitive to paraquat. Our results show that this mutant is not more sensitive to paraquat than wildtype animals (Figure 8A), from which we conclude that mitochondrial fusion defects are not enough to promote free radical damage. The increased paraquat sensitivity of eat-3 mutants, but not of fzo-1 mutants, therefore indicates that the EAT-3 protein affects free radical formation or sequestration in ways that are unrelated to its role in mitochondrial fusion.

Enhancement of the eat-3 Mutant Phenotype by SOD-2 Loss of Function

To test whether the induction of superoxide dismutase genes aids survival of eat-3 mutants, we tested possible genetic interactions between eat-3 and superoxide dismutase genes in C. elegans. C. elegans has five superoxide dismutase genes. The sod-1, sod-4, and sod-5 genes encode Cu2+/Zn2+ superoxide dismutases. One splice variant of sod-1 and all variants of sod-4 have a signal peptide, suggesting that these proteins are sent through the secretory pathway to the extracellular matrix. Other sod-1 isoforms and all proteins encoded by sod-5 lack recognizable targeting sequences, suggesting that those are cytosolic. A fraction of Cu2+/Zn2+ superoxide dismutases might also be localized to the mitochondrial intermembrane even without recognizable targeting sequences, similar to Cu2+/Zn2+ superoxide dismutases in yeast and mammals [50]. The two remaining sod genes (sod-2 and sod-3) encode Fe/Mn superoxide dismutases. These proteins have mitochondrial leader sequences, which most likely target them to the mitochondrial matrix.

We first grew eat-3(ad426) animals on feeding RNAi bacteria with RNAi for the sod genes that are not secreted (sod-1, sod-2, sod-3 and sod-5), since those might affect the survival of eat-3 mutants. There were little or at best modest effects with sod-1, sod-3 and sod-5 RNAi treatments, but the effects of sod-2 RNAi on eat-3(ad426) animals were consistent and strong (Figure 9A). To verify these differences, we grew mutants of each of the sod genes on eat-3 RNAi bacteria. As with the converse experiment, sod-2(gk257) mutant animals grow much more poorly with eat-3 RNAi (Figure 9B). The effectiveness of eat-3(ad426) in one experiment and eat-3 RNAi in the second experiment confirms that the enhancement of sod-2 defects are indeed caused by eat-3 loss of function. We conclude sod-1, sod-3 and sod-5 are not necessary for survival of the eat-3 mutant, but a mutation in the sod-2 gene and sod-2 RNAi both strongly affect survival of animals with eat-3 deficiencies.

The weak or negligible enhancement of eat-3 by sod-3 RNAi and the sod-3(gk235) mutant is noteworthy since SOD-2 and SOD-3 have 88% amino acid identity and both proteins have mitochondrial leader sequences, indicating that they are both targeted to the mitochondrial matrix. The genetic interactions between sod-2 and eat-3 might, however, be different from those between sod-2 and eat-3, because sod-2 and sod-3 genes are differentially expressed [59] and their expression is regulated by different pathways [60]. We used Western blots probed with a cross reacting Fe/Mn-SOD antibody to determine whether differential expression of sod genes correlates with the different effects that we observe with sod-2 and sod-3 genes.

Our blots show that Fe/Mn-SOD expression is induced more than twofold in eat-3(ad426) animals (Figure 9C). This induction is almost entirely attributable to SOD-2 since sod-2 RNAi, but not sod-3 RNAi largely abolishes this expression (Figure 9D). The induction is reversed by a secondary mutation in ddp-1(cq3) and in the intragenic revertant of eat-3(ad426; cq8) (Figure 9C). Similar reductions were seen with other revertants (data not shown). Consistent with their lack of paraquat sensitivity, fzo-1(tm1133) animals show little or no induction of SOD expression. We conclude that SOD-2 protein levels are dramatically increased in eat-3(ad426) animals, but not in fzo-1(tm1133) animals. This increase is partially reversed in intragenic revertants and fully reversed by the ddp-1 mutation in the eat-3(ad426); ddp-1(cq5) double mutant. It seems likely that increased expression of SOD-2 helps prevent damage from free radicals, but this increase is still not enough to prevent the hypersensitivity of eat-3 mutants to paraquat.

Discussion

C. elegans eat-3 mutants have many of the same features that were previously observed with yeast Mgm1 mutants and mammalian cells transfected with Opal siRNA. The mitochondria in eat-3 mutants are fragmented, these fragmented mitochondria are further divided by inner membrane septae and fragmentation is reversed by loss of Drp1. C. elegans eat-3 mutants are also affected at
the organisinal level. The mutant animals grow slowly, are sluggish and have greatly reduced broodsize, consistent with severely compromised mitochondrial function. However, heterozygous eat-3 mutants have no overt defects in worms, unlike heterozygous Opa1 mutations in humans, which cause optic neuropathies through haploinsufficiency. The eat-3 mutants are nevertheless still useful for unraveling pathogenic mechanisms, since the phenotypes in C. elegans and in mammal are both due to loss of protein function and therefore their effects on other cellular pathways are also most likely similar.

It was conceivable that the broodsize defects of eat-3 mutants are due to increased apoptosis in the gonad. In wildtype worms, approximately 50% of germ cells die prior to oogenesis, but more death can be induced by DNA damage, by pathogens and by other forms of stress. These death-inducing conditions all converge through haploinsufficiency. The Opa1 mutations in humans, which cause optic neuropathies in mammal are both due to loss of protein function and therefore their effects on other cellular pathways are also most likely similar. The ability to suppress eat-3(ad426) defects with drp-1 RNAi and mutations in drp-1 is consistent with stochastic loss of mtDNA in eat-3 mutants. Mutations in the yeast DRP-1 homologue Dnm1 similarly suppress Mgm1 growth defects and they restore cristae morphology [20]. There are, however, several observations suggesting that the mitochondrial fusion defect and the resulting loss of mtDNA might not be the only causes of sickness in eat-3 mutants: First, drp-1 RNAi does not rescue the C. elegans eat-3(tm1107) deletion allele, while it does rescue the eat-3(ad426) allele. Second, C. elegans fzo-1(tm1133) mutant animals are not as severely affected as eat-3 mutants, nor are they rescued by drp-1 RNAi (data not shown), even though one might expect them to be

3 and the APAF1 homologue CED-4 [61]. We investigated the possibility that apoptosis contributes to the pathogenesis of eat-3 mutants by analyzing eat-3; ced-3 and eat-3; ced-4 double mutants and by counting the numbers of dying cells in eat-3 mutants. There was no increase in the numbers of dying cells in eat-3 embryos, nor was there suppression of the eat-3 broodsize defects in the double mutants. C. elegans does have several other caspases (csp-1, csp-2 and csp-5), but RNAi of these genes had no effect on eat-3 animals (data not shown) nor are they known to contribute to apoptotic cell death in C. elegans [56]. Redundancy is not an issue with ced-4, which encodes the only APAF1 homologue in C. elegans. In summary, none of the RNAi treatments or chromosomal mutations in cell death genes showed a suppressive effect on eat-3 mutants, from which we conclude that caspase-dependent cell death does not contribute to the pathology of eat-3 in worms.

Mammalian cells transfected with Opa1 siRNA are more sensitive to apoptosis inducing agents [62], but there is also evidence that patients with dominant optic atrophy have reduced levels of ATP, which could trigger retinal ganglion cell degeneration [42]. The gross anatomical phenotypes of C. elegans eat-3 mutants, such as small size, slow growth and reduced broodsize are consistent with caloric restriction as observed in feeding mutants with pharyngeal defects [43,52]. The small brood sizes of eat-3 mutants are not due to retention of eggs, nor are there increased numbers of dead eggs or larvae on plates (data not shown). There is, however, a paucity of nuclei in the gonads of eat-3 RNAi animals (Figure 5C), consistent with the production of fewer oocytes. Fewer oocytes could reflect reduced rates of mitotic division at the distal tip of the gonad, since it was previously shown that mutations in mitochondrial proteins can inhibit cell division through the actions of AMP kinase and cyclin E [63]. Oocyte production might also be compromised at later stages, since the availability of yolk protein and other major constituents of oocytes is affected by the metabolic state of the animal. Many of the eat-3 mutant phenotypes are therefore attributable to a general breakdown in mitochondrial function.

Earlier studies of yeast Mgm1 mutants show progressive loss of mtDNA [18,19]. Loss of mtDNA is also observed in patients with dominant optic atrophy where it will affect assembly of oxidative phosphorylation complexes [64]. Defects in oxidative phosphorylation proteins can result in fewer and shorter cristae [65], which would be confined to those matrix compartments that have lost their mtDNA. Selective loss of cristae due to stochastic loss of mtDNA agrees with our electron microscopy data, since that data shows a heterogeneous mixture of mitochondrial matrix compartments, some with severely disrupted cristae and others with seemingly wildtype cristae (pairs of arrows in Figure 3D). The observation of different types of matrices enclosed by a single mitochondrial outer membrane suggests that the outer membranes of eat-3 mutant mitochondria fuse irrespective of their mtDNA content, but the mitochondrial inner membranes fail to fuse, similar to the results obtained with Mgm1 in yeast [25].
equally susceptible to loss of mtDNA, since yeast Fzo1 mutants do lose their mtDNA [12,48] and are rescued by mutations in Dnm1 [19,21,22]. Extensive loss of mtDNA also occurs in mouse Mitofusin mutants (the mammalian homologues of Fzo1) [66]. We conclude that lack of ATP due to loss of mtDNA is not enough to explain why optic nerves are singled out for destruction in patients with dominant optic atrophy.

Our results suggest an alternative explanation for the sickness of C. elegans eat-3 mutants, which may also be relevant for the selective degeneration of retinal ganglion cells in patients with dominant optic atrophy. C. elegans eat-3 mutants are hypersensitive to paraquat and sod-2 RNAi, suggesting increased production of free radicals or an impaired disposal mechanism. A dip-1; eat-3 double mutant and an fzo-1 mutant are not more sensitive to paraquat, suggesting that there might be something specific about the effects of eat-3 on mitochondria, for example contributing to the maintenance of cristae, as was suggested for Opa1 in mammalian cells [27,37,40,53,67]. The enhancement of eat-3 phenotypes by sod-2 RNAi and a mutation in the sod-2 gene, but not by RNAi or mutations in other superoxide dismutase genes, suggests that damage from free radicals is confined to the mitochondrial matrix or the mitochondrial inner membrane. The effects are most likely not direct, since SOD-2 is a mitochondrial matrix protein while EAT-3 is primarily localized to the mitochondrial intermembrane space and other mutations that affect oxidative phosphorylation in C. elegans, such as the mev-1 and gas-1 mutants with mutations in complex I and II proteins, also show increased sensitivity to paraquat [57]. Disruption of the electron transport chain, for example through altered cristae morphology, can increase production of free radicals, while conversely free radicals in the mitochondrial matrix can further disrupt the electron transport chain. These two problems are therefore likely to reinforce each other, possibly leading to catastrophic breakdown of mitochondrial function.

If free radicals also contribute to dominant optic atrophy in humans, then the underlying cause of this disease might be more similar to that of other optic neuropathies than previously understood. Patients with Leber’s hereditary neuropathy (LHON) have mutations in submitis of Oxidative Phosphorylation complex I, which increases free radical production by disrupting the flow of electrons through complex I along with their more obvious effects on ATP production [68-70]. Optic neuropathies triggered by macular degeneration and optic neuropathies triggered by dietary deficiencies are also linked to damage from free radicals in mitochondria. Increased levels of free radicals in these diseases are compounded by the effects of light entering the eyes, since light triggers additional free radical production through absorption by cytochrome c oxidase and flavin containing oxidases in mitochondria [71]. Damage from free radicals will exacerbate the effects of ATP deficiency and increased susceptibility to apoptosis in patients with dominant optic atrophy. It is even possible that some of the increased susceptibility to apoptosis in Opa1 deficient cells is caused by damage from free radicals.

In conclusion, mutations in C. elegans eat-3 have many of the same effects on mitochondrial morphology that were previously observed with mutations in yeast Mgm1 and mammalian Opa1. Mutations in key components of the major cell death pathway show that this pathway does not affect the eat-3 phenotype. Instead, eat-3 mutants are sensitive to damage from free radicals and they show hallmarks of ATP deficiency. The effects of sod-2 loss of function and partial compensation by induced expression of SOD-2 suggest that damage from free radicals is localized to the mitochondrial matrix. These observations might help design more effective treatments for patients with DOA.

Materials and Methods

Molecular Cloning

The D2013.5 gene of eat-3(ad426) was sequenced using amplified genomic DNA from two independent PCR reactions. The C. elegans eat-3 cDNAs yk108h and yk21c2 were obtained from Y. Kohara (National Institute of Genetics, Mishima, Japan). The pPD expression vectors were kindly provided by A. Fire, J. Ahm, G. Seydoux, and S. Xu (Carnegie Institution of Washington, Baltimore, Maryland). The Pnt-3::NLS::GFP::β-galactosidase construct was made with an eat-3 gene promoter fragment (positions 23335 to 25288 of cosmID D2013), fused to the reporter sequences of pPD95.67. The rescue construct contained this same promoter fragment fused to the yk21c2 cDNA. This cDNA lacks the N-terminal 70 amino acids. The missing sequence was generated by PCR of genomic DNA. Mutations were introduced by PCR and verified by sequencing, EAT-3 was expressed in muscle cells using the myo-3 promoter of pPD96.52. The antisense construct has the insert of yk21c2 cloned in the antisense orientation in pPD96.52. Production of dsRNA, mitochondrial markers, microinjection, light microscopy and feeding RNAi procedures were described previously [8,72]. Feeding RNAi bacteria were kindly provided by Dr. J. Ahringer (University of Cambridge, UK).

Worm Strains

C. elegans strains were obtained from the C. elegans stock-center (CGC, University of Minnesota) and from Dr. S. Mitani (National Bioresource Project of Japan. Tokyo Women’s Medical University School of Medicine, Tokyo). Strains provided by Dr. Mitani were backcrossed with wildtype (N2) animals to remove adventitious mutations. Revertants of dyn-1(ly51) and eat-3(ad426) were generated with EMS mutagenesis. The dyn-1(ly51) is temperature sensitive for growth and motility [46]. L3 larvae of either strain were treated with 50 mM EMS as described [73]. F2 progeny of mutagenized animals were screened for revertants by looking for restored growth and motility. The dyn-1(ly51) animals were screened at the restrictive temperature (25°C) while eat-3(ad426) animals were screened at 20°C. Newly identified mutants were backcrossed with wildtype (N2) worms to determine whether the new mutations are intra- or extragenic and to rid them of adventitious mutations. The three revertants of dyn-1(ly51) were genetically inseparable from the original dyn-1 mutation and five of the seven eat-3(ad426) revertants were inseparable from eat-3, suggesting that these are intragenic revertants. New mutations in the intragenic revertants were identified by sequencing their respective dyn-1 and eat-3 genes. New mutations in the two extragenic revertants of eat-3(ad426) were identified by sequencing their dip-1 genes.

To determine paraquat sensitivity, increasing concentrations of paraquat (N,N'-Dimethyl-4,4'-bipyrindium dichloride from MP Biomedicals LLC, Solon Ohio) were added to 30 mm NGM agar plates. These plates were seeded with OP50 bacteria [73] and fifty L1 larvae were transferred to each plate. The plates with worms were incubated at 20°C and tracked for several days by counting the numbers of worms that reached adulthood.

Electron Microscopy

Young gravid worms were mixed with E. coli or dry baker’s yeast and 10% methanol [74]. This mixture was cryofixed in a Bal-Tec HPM 010 high pressure freezer (Technotrade, Manchester, New Hampshire), followed by freeze-substitution with 2% osmium tetroxide and 0.1% uranyl acetate in acetone. The temperature was slowly increased to ~20°C and then to room temperature. The samples were rinsed with acetone and infiltrated with Epon-Araldite (1 hr in 1 part resin and 3 parts acetone; 2 hr
in a 1:1 mixture; 4 hr in a 3:1 mixture; 1 hr and 16 hr in resin alone). The samples were then incubated in resin with accelerator for 4 hr, flat-embedded between Teflon-coated slides and cured in a 60°C oven for 40 hr. Longitudinal sections (60 nm thick) were post-stained with uranyl acetate and lead citrate. All specimens were examined using a Tecnai 12 transmission electron microscope at 100 kV. Membrane lengths and surface areas were measured with NIH Image software.

For tomography, 500 nm thick sections were cut and stained with uranyl-acetate and lead citrate. Colloidal gold particles (10 nm) were applied as alignment markers. A tilt series of 122 images was made on the Albany AEI EM MkJ HVEM at 1000 kV. The images were recorded around two orthogonal tilt axes, each over an angular range of 120° with a 2° tilt interval. The double-tilt images were aligned, further processed to make a tomographic reconstruction, followed by surface rendering as previously described [75].

### Western Blotting

Samples for Western blot analysis were prepared by freeze/thawing worms, followed by solubilization in SDS-PAGE sample buffer, boiling for 10 min and clearing of debris by centrifugation for 2 min at 3,000 rpm in an Eppendorf microfuge. Western blots were probed with superoxide dismutase antibody from Abcam (Cambridge, Massachusetts). Western blots were quantified with densitometry using a Personal Densitometer SI and ImageQuant software (Molecular Dynamics, Sunnyvale, California).

### Supporting Information

**Figure S1** Western blot showing EAT-3 expression levels in wild type and mutant *C. elegans*. An antibody raised against recombinant *C. elegans* EAT-3 protein detects a strong band of approximately 90 kDa in all strains except for *eat-3(tm1107)*, which has a deletion in the *eat-3* gene. This band is the size predicted for mature protein, assuming multi-step processing similar to that of yeast Mgm1. A faint upper band of approximately 100 kDa is also detected in all strains except for *eat-3(tm1107)*. This upper band most likely results from the initial cleavage of the mitochondrial leader sequence (computer algorithms predict a product of 99 kDa). The line between lanes with *eat-3(ad426); dp-1(cq5) and eat-3(tm1107)* samples shows that an empty lane between the two, which served as a buffer against spillover, was cut out. Tubulin and cytochrome served as loading controls. The EAT-3 antibody was raised in a rabbit against recombinant protein. The recombinant protein was made by expression in bacteria with a his-tag and purified with Ni-NTA column chromatography. The serum was blotted purified [76] and used for Western blotting as described in the Materials and Methods section. Tubulin antibody was from Sigma and cytochrome c antibody was from Pharmingen. Those were raised against mammalian proteins but show sufficient cross-reactivity with *C. elegans* proteins for Western blots.

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**Figure S2** Reversal of mitochondrial fragmentation in *eat-3(tm1107)* animals. (A) Mitochondria in muscle cells of an *eat-3(tm1107)* animal stained with the membrane potential dependent dye Rhodamine 6G [8]. (B) Mitochondria in muscle cells of an *eat-3(tm1107)* animal grown with *dp-1* feeding RNAi showing reversal of the fragmented phenotype. This indicates that *dp-1* loss of function is epistatic to an *eat-3* null allele. The scale bar is 5 μm.

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### Author Contributions

Conceived and designed the experiments: TK MZ AH KM CM. Performed the experiments: TK MZ AH AW EN KB KM CM. Wrote the paper: Av dB.
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