Research Article

Caenorhabditis elegans mitochondrial mutants as an investigative tool to study human neurodegenerative diseases associated with mitochondrial dysfunction

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In humans, well over one hundred diseases have been linked to mitochondrial dysfunction and many of these are associated with neurodegeneration. At the root of most of these diseases lay ineffectual energy production, caused either by direct or indirect disruption to components of the mitochondrial electron transport chain. It is surprising then to learn that, in the nematode *Caenorhabditis elegans*, a collection of mutants which share disruptions in some of the same genes that cause mitochondrial pathogenesis in humans are in fact long-lived. Recently, we resolved this paradox by showing that the *C. elegans* "Mit mutants" only exhibit life extension in a defined window of mitochondrial dysfunction. Similar to humans, when mitochondrial dysfunction becomes too severe these mutants also exhibit pathogenic life reduction. We have proposed that life extension in the Mit mutants occurs as a by-product of compensatory processes specifically activated to maintain mitochondrial function. We have also proposed that similar kinds of processes may act to delay the symptomatic appearance in many human mitochondrial-associated disorders. In the present report, we describe our progress in using the Mit mutants as an investigative tool to study some of the processes potentially employed by human cells to offset pathological mitochondrial dysfunction.

1 Introduction

1.1 Mitochondrial mutations associated with human neurodegenerative diseases

Over the last few decades, mitochondrial dysfunction, attributable to mutations in either mitochondrial DNA (mtDNA) or nuclear DNA (nDNA), has been established as a cause for numerous human diseases associated with neuronal degeneration [1]. Most of these mutations directly or indirectly affect mitochondrial electron transport chain (ETC) function. The subsequent alterations to the ETC have been shown to reduce ATP production, decrease mitochondrial membrane potential, impair calcium buffering, shift nucleotide pool ratios, increase reactive oxygen species (ROS) generation, and ultimately result in cell death [2, 3]. Most of the neurodegenerative disorders associated with mitochondrial dysfunction display a late onset and a chronic progressive course. Typically, symptoms associated with these diseases appear only after mitochondrial dysfunction and neuronal degeneration become very severe [4].

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Friedreich's Ataxia (FA) is the most commonly inherited ataxia and it is caused by pathologically low levels of frataxin, a nuclear-encoded protein involved in mitochondrial iron storage and Fe-S cluster biosynthesis [5, 6]. Several components of the mitochondrial ETC contain Fe-S clusters and hence require frataxin for their function. Chronic frataxin deficiency induces a vicious cycle that ultimately results in irreversible mitochondrial dysfunction and the complete dismantling of normal cell physiology. As for many mitochondrial-associated disorders, for FA, once a recognizable pathology presents, cell damage is usually so severe clinicians can only intervene with symptomatic treatment. It would be highly preferable, then, if treatments could be established duting the presymptomatic phase of FA. By doing so, it may be possible to delay the clinical appearance of this disorder, perhaps even preventing it permanently.

The Mitochondrial Threshold Effect Theory suggests that cells can cope with a certain degree of mitochondrial dysfunction until a critical threshold is reached. Experimental evidence suggests that several factors underlie the mitochondrial threshold effect including, mtDNA heteroplasmy, compensatory mitochondrial biochemical pathways, and age-related mitochondrial changes [7]. We recently proposed that several, mutually nonexclusive, stress response pathways might also act to help cells counter mitochondrial dysfunction. Such pathways could be responsible for the chronic nature of neurodegeneration and the delayed appearance of many mitochondrial-associated disorders [8]. In support of this idea, it was recently shown that mice with moderate levels of frataxin inactivation have a significant alteration in their pattern of expression of multiple genes; this was despite the fact that these animals manifested with no classical features of neurodegeneration at neither the phenotypic nor biochemical level [9]. It is thus highly conceivable that compensatory pathways can be induced in cells with compromised mitochondrial function well before phenotypic markers of an established pathology appear. The genetic nature of many mitochondrial diseases, coupled with the presence of an asymptomatic window preceding the onset of an established pathology, provides a great potential for presymptomatic diagnosis and preventive therapy.

1.2 Mitochondrial mutations associated with longevity in *Caenorhabditis elegans*

The Free Radical Theory of Aging asserts that oxidative stress underlies the causes of aging [10]. Since mitochondria are generally believed to account for the majority of endogenous ROS production, they too have been implicated as one of the main culprits responsible for normal cellular aging [11]. Aging is one of the greatest risk factors behind several neurodegenerative disorders that have been associated with defective mitochondrial function, including Parkinson's, Huntington's, and Alzheimer's dis-

eases [12]. Despite these considerations, recent advances in the nematode aging field have paradoxically revealed that mutations in many mitochondrial genes (collectively referred to as "Mit" mutations, and mainly affecting the ETC), can actually prolong the lifespan in this organism [13–15] (Table 1).

We recently resolved this human-worm lifespan paradox by showing that life extension in the *C. elegans* Mit mutants only occurs within a limited window of mitochondrial dysfunction (Shane L. Rea, Nastascia Ventura and Thomas E. Johnson, manuscript submitted). This life extension window was bounded by two thresholds: the first corresponded to moderate levels of mitochondrial dysfunction and was associated with slowed larval development, small adult size, decreased fertility, and increased lifespan. When mitochondrial disruption became too severe a second threshold became apparent – significant larval arrest occurred, or adult animals were invariably sterile, and lifespan was shortened. The *C. elegans* Mit mutants, with their paradoxical mitochondrial defects leading to lifespan extension, tell us that there can be an advantage in lowering the mitochondrial functionality, but only when it is kept within defined limits.

1.3 *C. elegans* mitochondrial mutants as a model for human neurodegenerative diseases associated with mitochondrial dysfunction

We recently proposed that the kinds of compensatory mechanisms activated to counteract the mitochondrial dysfunction in mammalian cells are potentially the same as those activated in the *C. elegans* Mit mutants that ultimately lead to life extension. Similar compensatory mechanisms might also act to delay the appearance of mitochondrial-related disorders in humans [8]. We have also proposed that the activation of such pathways represent a continuum of responses and that the ability of each to effectively mask overt mitochondrial dysfunction depends upon the severity of the lesion they are trying to counter. If mitochondrial dysfunction becomes too severe even these pathways are unable to compensate and we presume that this is the reason lifespan shortens in the Mit mutants and mitochondrial disorders suddenly appear in humans. As a first attempt to elucidate such compensatory pathways, we recently generated a *C. elegans* model of FA using a bacterial feeding RNAi against the worm frataxin ortholog, *frh-1*. Consistent with other Mit mutants, we found that mild reduction of frataxin expression initially increased the animal lifespan [15]. More severe reduction, as expected, shortened the lifespan (Shane L. Rea, Nastascia Ventura and Thomas E. Johnson, manuscript submitted).

We mentioned that several phenotypes coordinately track with lifespan outcome in the *C. elegans* Mit mutants including altered larval development rate, reduced adult size, and diminished fertility (Table 1). The severity in the

Table 1. The Mit mutants of *C. elegans* (genetic and RNAi-defined)

C. elegans gene	Age	Mit phenotypes[a]		Ste/Fer	Emb	Human ortholog	HMAD[b]
		Gro	Lva				
COMPLEX I (NADH:ubiquinone oxidoreductase)							
C09H10.3 (*nuo-1*, NDUFV1/51 kDa subunit)	✓ [54]	✓ [55]	✓ [56, 57]	✓ [55, 58]	✓ [56–58]	NDUFV1	✓ [59]
T10E9.7 (*nuo-2*, NDUFS3/30 kDa subunit)	✓ [14, 60]	✓ [61]	✓ [57]	–	✓ [57, 58, 61]	NDUFS3	✓ [23]
Y57G11C.12 (*nuo-3*, NDUFA6/B14 subunit)	✓ [14, 60]	✓ [56]	✓ [57]	–	✓ [56–58]	NDUFA6	–
K04G7.4 (*nuo-4*, NDUFA10/42 kDa subunit)	✓ [60, 62]	✓ [56]	✓ [57]	✓ [63]	✓ [56, 57]	NDUFA10	–
Y45G12B.1 (*nuo-5*, NDUFS1/75 kDa subunit)	✓ [60]	✓ [56]	✓ [57]	–	✓ [56, 58]	NDUFS1	✓ [64]
D2030.4 (NDUFB7/B18 subunit)	✓ [13, 62]	✓ [13, 61]	✓ [13, 57]	–	✓ [57, 58]	NDUFB7	✓ [65]
T20H4.5 (NDUFS8/23 kDa subunit)	✓ [62]	✓ [56]	✓ [57, 58]	–	✓ [56, 57, 58]	NDUFS8	✓ [65]
COMPLEX III (cytochrome *c* reductase)							
C54G4.8 (*cyc-1*, cytochrome *c*1)	✓ [14, 60]	✓ [61]	✓ [57]	✓ [58, 63]	✓ [57, 58, 61]	CYC1	–
F42G8.12 (*isp-1*, subunit RIP1, Rieske Fe-S protein)	✓ [27]	–	–	✓ [58, 66]	✓ [58, 66]	UQCRFS1	–
T02H6.11 (UQCRB)	✓ [13]	✓ [13]	✓ [13, 56, 57]	✓ [13, 58, 63]	✓ [5657, 58]	UQCRB	✓ [67]
COMPLEX IV (cytochrome *c* oxidase, COX)							
F26E4.9 (*cco-1*, subunit Vb/COX4)	✓ [13, 14, 60, 62]	✓ [13, 61]	✓ [57]	✓ [13, 58]	✓ [57, 58]	COX5B	–
Y37D8A.14 (*cco-2*, subunit Va/COX6)	✓ [60]	–	✓ [57]	✓ [56]	✓ [56–58, 63]	COX5A	–
F26E4.6 (subunit VIIc/COX8)	✓ [13, 14]	✓ [13, 56]	✓ [13, 56]	✓ [13, 56, 58]	✓ [56, 58]	COX7C	–
F54D8.2 (*tag-174*, subunit VIa/COX13)	✓ [62]	–	–	–	–	COX6A1	–
W09C5.8 (subunit IV/COX5b)	✓ [13, 14]	✓ [13, 61]	✓ [57]	✓ [58, 13]	✓ [57, 58]	COX4I2	–
COMPLEX V (F1F0-ATP synthase)							
C34E10.6 (*atp-2*, beta subunit)	✓ [54]	✓ [55]	✓ [54, 55, 57, 63]	✓ [55, 56]	✓ [55, 57, 58]	ATP5B	–
F27C1.7 (*atp-3*, subunit OSCP/ATP5)	✓ [14, 60]	–	✓ [57, 61, 63]	✓ [57, 58]	✓ [57, 61]	ATP5O	–
T05H4.12 (*atp-4*, subunit Cf6 (coupling factor 6))	✓ [60]	✓ [56]	✓ [56]	✓ [56, 57, 63]	✓ [56, 58]	ATP5J	–
C06H2.1 (*atp-5*, subunit d/ATP7)	✓ [60]	✓ [56, 63]	✓ [57]	✓ [63]	✓ [56–58]	ATP5H	–
C53B7.4 (*asg-2*, subunit g/ATP20)	✓ [62]	✓ [55–57]	–	✓ [55, 58]	✓ [55, 58]	ATP5L	–
F02E8.1 (*asb-2*, subunit b/ATP4)	✓ [60]	✓ [55]	✓ [57]	✓ [58, 63]	✓ [57]	ATP5F1	–
Other ETC related							
F59G1.7 (*frh-1*, defective Fe-S containing enzymes)	✓ [15]	✓ [56, 57]	✓ [15]	✓ [15]	–	FXN	✓ [68]
B0261.4 (mitochondrial S-ribosomal protein)	✓ [13]	✓ [13, 58, 61, 63]	✓ [57]	✓ [13, 57, 58]	✓ [58, 61]	MRL47	–
T06D8.6 (*cchl-1*, Holocytochrome *c* synthase/heme-lyase)	✓ [13]	✓ [13, 56, 57]	✓ [13]	✓ [56]	✓ [58, 69]	CCHL	✓ [70]
ZC395.2 (*clk-1*, DMQ mono-oxygenase/ ubiquinone biosynthesis protein COQ7/CLK-1/CAT5)	✓ [71, 72]	✓ [72]	–	✓ [72]	–	COQ7	–
ZC395.6 (*gro-1*,tRNA δ(2)-isopentenylpyrophosphate transferase)	✓ [73]	✓ [73]	–	✓ [56, 58]	–	TRIT1	–
ZK524.3 (*lrs-2*, leucyl-tRNA synthase)	✓ [13]	✓ [58, 63]	–	✓ [69]	✓ [69]	LARS2	✓ [74, 75]
T27E9.1 (*tag-61*, ANT2, ADP/ATP carrier)	✓ [62]	–	✓ [57]	✓ [63]	✓ [58]	ANT2	–
Other mitochondria-non-ETC							
F13G3.7 (mitochondrial carrier protein)	✓ [13]	–	–	–	–	carrier proteins	–
C33F10.12 (mitochondrial phosphate carrier protein)	✓ [62]	–	–	–	–	phosphate carriers	–
K01C8.7 (mitochondrial FAD carrier protein)	✓ [13]	✓ [56, 57]	–	✓ [58]	–	SLC25A32	–
F54H12.1 (*aco-2*, aconitase)	✓ [62]	–	✓ [57]	✓ [56]	✓ [56–58]	ACO2	–
ZK637.9 (*tpk-1*, thimaine pyrophosphokinase)	✓ [76]	–	–	–	–	TPK1	d)
F43G9.1 (isocitrate dehydrogenase, alpha subunit)	✓ [62]	✓ [57]	✓ [61]	✓ [58, 63]	✓ [57, 58, 61]	IDH3A	–
F59B8.2 (NADP-dependent isocitrate dehydrogenase)	✓ [62]	–	–	–	–	IDH2	–
C37H5.8 (*hsp-6*, mortalin, mot-2/mtHSP70)	✓ c)	✓ c)	✓ [57, 63], c)	✓ [56]	✓ [56–58]	mtHSP70	d)

a) Age: long-lived, Gro: slow larval growth, Lva: larval arrest, Ste/Fer: sterile or reduced fertility, Emb: embryonic lethal.
b) HMAD: Human mitochondrial-associated disorder.
c) This study.
d) Potentially.

presentation of these associated phenotypes appears to be dependent upon both the type and degree of target disruption. Several of these associated phenotypes are more easily scored than lifespan in worms. We believe that the coordinate appearance of these phenotypes is due to specific compensatory pathways activated upon mitochondrial dysfunction. Loss of cellular compensation inevitably leads to the coordinate disappearance of these phenotypes, thus providing us with surrogate markers for mitochondrial dysfunction.

In this study, we will describe our recent efforts showing how the *C. elegans* Mit mutants can be used as a novel and potentially powerful model to study neurodegenerative and other mitochondrial-related disorders in humans. By identifying signaling pathways regulating the various Mit phenotypes we hope to uncover similar signaling pathways that may be of relevance to offsetting human mitochondrial-related pathologies.

2 Materials and methods

2.1 Nematode maintenance

Standard nematode culturing techniques were employed [16]. The following strains were utilized: N2 (wild type), SJ4100 [*hsp-6::GFP*(*zcIs13*)], DA465 [*eat-2*(*ad465*)], LG100 [*sir-2.1*(*geIn3*)], VC389 [*frh-1*(*ok610*)/*mIn1*[*mIs14 dpy-10* (*e128*)].

2.2 Feeding RNAi

The following RNAi feeding constructs were derived from the Ahringer *C. elegans* RNAi library: *hsp-6* (C37H5.8), *atp-3* (F27C1.7), *nuo-2* (T10E9.7), *cco-1* (F26E4.9), *cyc-1* (C54G4.8), R53.4, D2030.4, *tag-55* (F42A8.2), F26E4.6, F13G3.7, B0261.4, and W09C5.8. Feeding RNAi constructs against *frh-1* and *isp-1* have been described previously ([15] and Shane L. Rea, Nastascia Ventura and Thomas E. Johnson, manuscript submitted, respectively). For RNAi dilutions, cultures of HT115 (DE3) containing empty vector (pL4440) or each gene of interest were prepared in LB broth containing 100 μg/mL ampicillin and 5 μg/mL tetracycline and grown overnight at 37°C. OD_{590} values were adjusted to 0.9 and bacteria mixed in the following target gene to pL4440 ratio – 0:1, 1:10, and 1:0. For each mix, 250 μL was added to a 6 cm RNAi plate (NGM agar + 1 mM IPTG, 100 μg/mL ampicillin and 5 μg/mL tetracycline) and grown overnight at 23°C. Eighty N2 eggs from a 4-h limited-lay were transferred from standard OP50 culture plates to each type of RNAi plate and allowed to develop at 20°C.

2.3 Consecutive generational RNAi

For studies involving *frh-1* and *nuo-2*, N2 animals were cultured on pL4440-containing, *nuo-2* RNAi-producing or *frh-1* RNAi-producing HT115 (DE3) bacteria for one to three consecutive generations as described previously [15].

2.4 Lifespan analysis

Lifespan was recorded at 20°C using synchronous populations of 60–80 animals per RNAi dilution or generation. Lifespan analyses began from the start of the egg laying. Data were analyzed using the Log Rank Test as previously described [17].

2.5 Western blot analysis

Whole-worm lysates were prepared from 200 animals in boiling 5% SDS, 0.02% β-mercaptoethanol, and 1 mM protease inhibitor cocktail (SIGMA P2714). Following protein quantitation (BCA, Pierce), protein levels were measured by Western analysis, quantified using densitometry, then normalized against actin. Antibodies employed were α-NUO-2 (MS112, MitoSciences, Oregon, 1:2000), α-actin (AC-15, Sigma, 1:5000), α-ANT (MSA02, MitoSciences, 1:1000), α -complex IV, subunit I (MS404, MitoSciences, 1:1000), α-complex V, alpha subunit (MS507, MitoSciences, 1:1000).

2.6 Microscopy

Individual worms were randomly selected from RNAi plates and then arranged for image analysis on 2% agarose pads. Nomarski and fluorescence images were collected using a PCO SensiCam charge-coupled diode camera connected to a Zeiss Axioskop running Slide-Maker 4.0 software. All other images were collected using a digital camera connected to a dissecting microscope.

3 Results and discussion

3.1 Mimicking human mitochondrial-associated diseases in *C. elegans*

Most of the mitochondrial-related disorders in humans are phenotypically complex; that is, different mitochondrial mutations can produce the same phenotype while the same mutation can lead to the appearance of different phenotypes. Generally, such diseases have a chronic progressive course and they present with symptoms of neurodegeneration, myopathy, and/or cardiomyopathy (encephalocardiomyopathies). The type and severity of a mitochondrial-related genetic lesion, its tissue-specific distribution as well as the activation of different compen-

satory pathways aimed at countering the lesion, dictate, in the end, the final presenting phenotype in patients. Similarly in *C. elegans*, disruption of different mitochondrial genes can produce the same phenotype, while disruptions in the same gene can result in the appearance of distinct phenotypic features depending on the degree of gene disruption (Table 1 and Shane L. Rea, Nastascia Ventura and Thomas E. Johnson, manuscript submitted).

In an effort to establish novel models to study human mitochondrial-related diseases, we have used bacterial feeding RNAi to systematically disrupt the function of multiple genes in *C. elegans* that are orthologous to known genes involved in human mitochondrial pathologies. To mimic the chronic course of these diseases we have developed a simple but powerful technique to modulate the degree of inactivation of *C. elegans* mitochondrial proteins (Materials and methods, Shane L. Rea, Nastascia Ventura and Thomas E. Johnson, manuscript submitted). By increasing the potency of each bacterial feeding RNAi (either by using bacterial dilution or by crossgenerational RNAi feeding), we have observed that disruption of many mitochondrial genes leads to a classic Mit phenotype – that is, at low levels of RNAi-mediated disruption larval development is slowed, adult size and fertility are reduced, and lifespan is extended. Following a more severe disruption (either by increasing RNAi potency or by gene knockout (KO)), deleterious phenotypes are often aroused – larval development becomes arrested or animals become sterile and/or lifespan shortens pathologically. Interestingly, we found that the disruption of several target genes displayed no phenotypic consequence, while others showed only mild effects. We suspect that differences in the efficacy of RNAi-mediated target knockdown, coupled with some unique properties of *C. elegans* physiology (such as the persistence of maternally contributed products), likely explain much of the gross variability in phenotypes that we observed. Nonetheless, several new models of human diseases have been established, three of which we will briefly mention.

HSP-6 is the *C. elegans* homolog of the mammalian mitochondrial protein HSP-70/mortalin/mthsp70 [18]. This protein, in cooperation with several other chaperonins, functions in promoting efficient mitochondrial protein import and folding. Previous work has shown that alterations in the mitochondrial import machinery can lead to human disease [19, 20]. Moreover, several reports have indicated that cells from patients affected by any one of a variety of mitochondrial diseases express altered levels of mRNAs and/or proteins encoding HSP-70 and other factors belonging to the import machinery [21, 22]. When we fed wild type *C. elegans* with a 1/10 dilution of RNAi directed against *hsp-6*, we observed that the larval development slowed and lifespan increased. When animals were fed undiluted *hsp-6* RNAi they instead arrested as early larvae and lifespan was brought back to untreated-control levels (Figs. 1A and B).
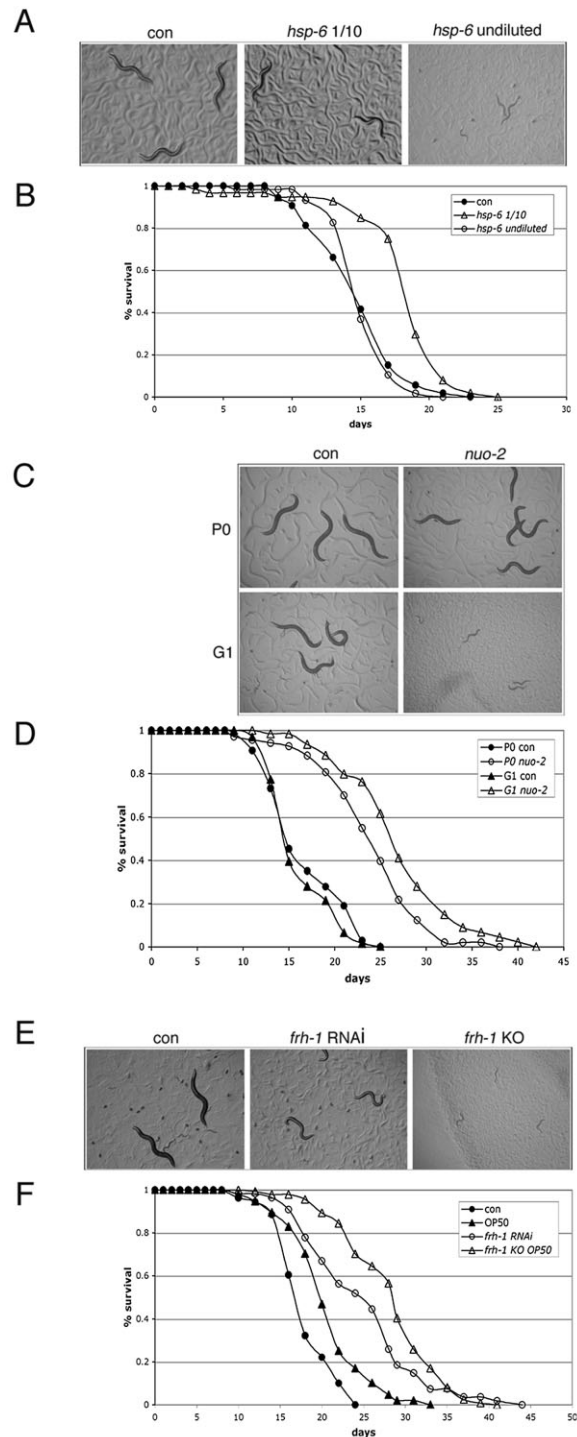

Figure 1. Phenotypes associated with *hsp-6*, *nuo-2*, and *frh-1*. Disruption in *C. elegans*. HT115 bacteria expressing dsRNA targeting *hsp-6* (A and B), *nuo-2* (C and D), or *frh-1* (E and F) were fed to N2 (wild-type) animals in undiluted form, or as a 1:10 mix with control bacteria (*hsp-6* only), from the time of hatching for one (*hsp-6*, *nuo-2*, P0), two (*nuo-2*, G1), or three (*frh-1*) consecutive generations. *frh-1* KO animals, and the parental wild-type behaving strain VC389, were cultured on OP50 bacteria. *C. elegans* lifespan is increased by ~3 days when grown on this bacteria, relative to growth on HT115. The effect on size (A, C, E) and lifespan (B, D, F) was subsequently recorded. Animals were photographed 2 days (A), 8 days (C, P0), 5 days (C, G1), or 4 days (E) after hatching.

Complex I (NADH:ubiquinone oxidoreductase), of the canonical ETC, is comprised of multiple protein subunits. In humans, disruptions to any of the 14 core complex I subunits results in the appearance of either Leigh's syndrome, LHON disease, or cardiomyopathy [23]. In worms, RNAi-mediated reduction in different complex I components leads to different phenotypes: Diminished expression of seven complex I genes (*nuo-1* to *nuo-5*, D2030.4, and T20H4.5) has been previously reported to result in lifespan extension (Table 1). Loss of *gas-1*, on the other hand, is pathologically life-shortening [24]. A single case report exists of a patient with a compound mutation in *nuo-2* leading to Leigh's syndrome [23]. When we reinvestigated the disruption of *nuo-2* in *C. elegans* using our feeding RNAi dilution methodology we found that the lifespan was greatly extended even using undiluted RNAi, as previously reported [14]. When *nuo-2* levels were more severely reduced following a crossgenerational feeding RNAi strategy, animals displayed an even greater life extension, yet larval development became arrested at the early L3 stage (Figs. 1C and D). Larval-arrest associated with life-extension may therefore reflect differences in the energy threshold for the progression of development *versus* cellular maintenance.

Complexes I, II, and III of the mitochondrial ETC as well as aconitase of the tricarboxylic acid cycle, each contain Fe-S clusters. Synthesis of these components requires the nuclear-encoded mitochondrial protein frataxin that, as mentioned above, leads to FA when disrupted in humans. In worms, mild reduction of *frh-1* by RNAi leads to life extension. When reduced more severely, using a crossgenerational feeding strategy on an RNAi enhancing strain, lifespan shortens (Shane L. Rea, Nastascia Ventura and Thomas E. Johnson, manuscript submitted and Figs. 1E and F). One interesting observation that we made involved animals genetically ablated for *frh-1*. These animals would seemingly represent the severest level of *frh-1* knockdown. However, we observed that despite arresting as early larvae, they are still long lived. This finding illustrates an important point about worm biology – that maternally-contributed product can sometimes mask a genetic phenotype. KO animals were derived from a heterozygous hermaphoditic parent and presumably either *frh-1* mRNA or protein was passed onto the homozygous *frh-1* animals, confounding the longevity data.

Our present results reinforce our earlier findings (Shane L. Rea, Nastascia Ventura and Thomas E. Johnson, manuscript submitted), showing that titrating RNAi against different mitochondrial components can result in seemingly vastly different phenotypic outcomes. Furthermore, the precise degree of target inactivation appears to play a major role in determining the phenotypic outcome. The core Mit phenotypes appear to be differentially sensitive to mitochondrial disruption. Different target tissues control each phenotype so we suspect that variations in the efficacy of target knockdown in each tissue ultimate-

ly dictate the final phenotypic mix seen at the organismal level. Alternatively, different compensatory pathways may be activated following different kinds of mitochondrial disruption and such pathways may be tissue specific. The Mit mutants provide an entrée into the elucidation of such processes.

3.2   Pathways potentially regulating Mit mutant phenotypes

3.2.1   A mitochondrial unfolded protein response

Understanding which mitochondrial parameters and which signaling pathways are affected following different levels of mitochondrial dysfunction in the Mit mutants could be extremely relevant to shedding light on different phases of disease progression in humans. Several features are generally altered in response to mitochondrial dysfunction including changes in protein folding, membrane import, free radical production, ATP levels, and nucleotide pools [2, 3]. Ron and coworkers [25] reported that disruption of normal mitochondrial function by RNAi directed against a variety of mitochondrial proteins (including, paradoxically, the mitochondrial heatshock protein HSP-6), or by chemical inhibition using ethidium bromide, induced a mitochondrial-specific, unfolded protein response (UPR_{mito}) [25]. This response was detectable using a *hsp-6_{promoter}::GFP* reporter construct.

Using the same *hsp-6_{promoter}::GFP* reporter system, we have observed that several Mit mutants constitutively activate a UPR_{mito} response (Fig. 2 and Table 2). This response was not universal, however, since not all the Mit mutants activated the reporter. Furthermore, we found that the level of *hsp-6_{promoter}::GFP* activation in those Mit mutants that did turn it on was dependent upon the degree of target gene inactivation. For most of the Mit mutants, higher levels of target disruption led to higher levels of reporter induction but this did not necessarily correlate with greater lifespan increases (Fig. 2, Table 2 and data not shown). These findings suggest that there may well be an optimal window of *hsp-6* expression that correlates with maximal lifespan increase and that very strong reporter induction presumably instead reflects major mitochondrial problems. Consistent with the notion of an optimal window of *hsp-6* expression leading to life extension, Yokoyama *et al.* [26] have reported that overexpression of *hsp-6* alone can increase *C. elegans* lifespan. We conclude therefore that *hsp-6_{promoter}::GFP* induction in those Mit mutants that turn it on likely reflects a general mitochondrial stress response and, although probably necessary for their survival, it may not be sufficient to account entirely for their life extension.

Isp-1(*qm150*) is one of the few genetically defined *C. elegans* Mit mutants. *Isp-1* encodes the Rieske iron–sulfur protein subunit of complex III and the *qm150* allele contains a missense mutation, most likely affecting the redox potential of the protein. This mutant is characterized by increased longevity, reduced fertility, and delayed
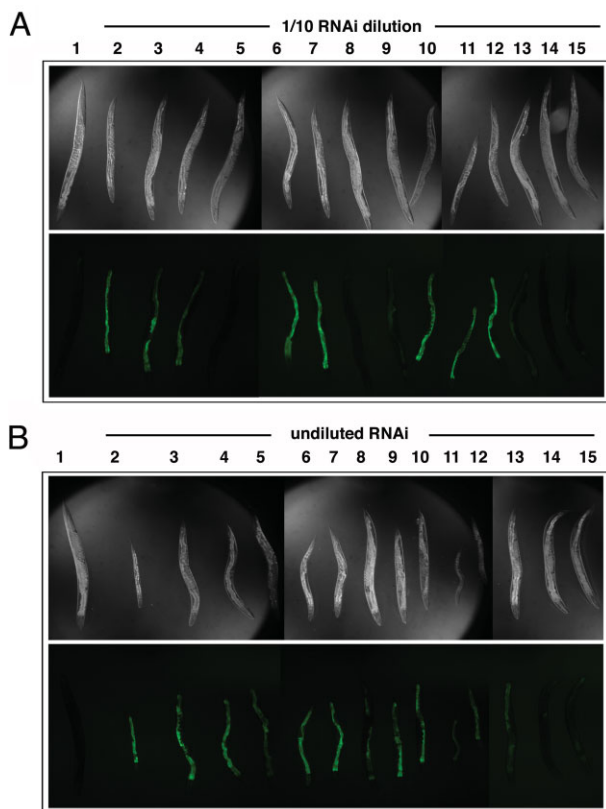
Figure 2. Unfolded protein response in the Mit mutants. Development is delayed, and a mitochondrial-specific UPR$_{mito}$ is activated, in multiple RNAi-induced Mit mutants. N2 animals were exposed to RNAi against various mitochondrial components: left to right: **1.** control (vector), **2.** *hsp-6*, **3.** *nuo-2* (complex I), **4.** D2030.4 (complex I), **5.** *tag-55* (complex II), **6.** *isp-1* (complex III), **7.** *cco-1* (complex IV), **8.** W09C5.8 (complex IV), **9.** *cyc-1* (complex IV), **10.** F26E4.6 (complex IV), **11.** *atp-3* (complex V), **12.** R53.4 (complex V), **13.** *frh-1*, **14.** F13G3.7 (carrier protein), **15.** B0261.4 (rRNA) from the time of hatching (except *frh-1* which had been cultured on RNAi for two prior generations), then imaged as one day-old adults using normaski (A, B top panels), and fluorescence (A, B bottom panels) optics. All the RNAi targets except *tag-55* have been shown previously to result in a *bona fide* Mit phenotype. For many of the targeted genes, the effect of RNAi on reporter induction is concentration dependent (compare 1:10 RNAi dilution [A], with undiluted RNAi [B]). UPR$_{mito}$ was detected using a *hsp-6$_{promoter}$::GFP* reporter strain [25].

embryonic and postembryonic development [27]. When we crossed the *hsp-6$_{promoter}$::GFP* reporter strain with *isp-1(qm150)* we found that the GFP reporter was robustly activated. Identical results were also found using *isp-1* RNAi in the *hsp-6$_{promoter}$::GFP* reporter background (Fig. 2B). We are currently using our *isp-1(qm150)*; *hsp-6$_{promoter}$::GFP* strain to look for both genes and drugs that can modulate expression of the Isp-1 phenotype as well as the *hsp-6$_{promoter}$::GFP* reporter. Similarly, we are also employing other GFP reporter strains that are indicative of a variety of additional stress responses (*gst-4::GFP, etc.*), to elucidate genes and pathways affected in the *isp-1* mutant background. Our preliminary observations support the notion that different stress response pathways are differentially activated following mitochondrial dysfunction and that they are dependent upon both the kind and severity of gene inactivation.

3.2.2 Calorie restriction

As in other species, in *C. elegans* caloric restriction delays aging [28, 29]. How exactly organisms benefit from this intervention is unclear, but it has been proposed that a mild metabolic stress could cause cells to respond by modulating their energy metabolism, redox status, protein biosynthesis, mitochondrial function, and/or genomic integrity. Ultimately, it is thought that there is an enhanced turnover of damaged molecules and/or the prevention of their formation, leading to improved cellular and organismal survival [30–32]. We believe that a related phenomenon may, in part, also be functioning in the Mit mutants where an initial mild mitochondrial stress could similarly promote the induction of a whole organism's protective response. Support for this idea comes from the observations of Tsang and Lemire [33] who noticed that mitochondrial mutants that arrested as early larvae have empty guts and appear starved.

Two interventions which increase *C. elegans* lifespan with a caloric restriction-like mechanism are genetic mutations in the *eat* genes (which produce a defect in pharyngeal pumping) [34], and overexpression of *sir-2.1(+)* (which encodes an NAD-dependent deacetylase that is activated following conditions of food deprivation) [35–37]. A powerful approach afforded by the *C. elegans* system is the ability to map genes to known or novel signaling pathways using epistatic analysis. Using such an approach, we have found that RNAi directed against frataxin further prolongs the longevity of both *eat-2* loss of function (Fig. 3A) and *sir-2.1(+)* overexpressing strains (Fig. 3B). This synergistic effect suggests two opposite interpretations which will need a further investigation: Frataxin might define a completely different longevity pathway than either that specified by *sir-2.1(+)* and *eat-2*; or in fact it might work on the same pathway, reinforcing the function of both alterations.

3.2.3 Exploring mitochondrial biogenesis in the Mit mutants

The beneficial effects of caloric restriction have been ascribed to the induction of mitochondrial biogenesis and to the improvement of bioenergetic efficiency [38]. Peroxisome proliferative-activated receptor coactivator 1 (PGC1) has emerged as a central regulator in mammalian cells of both the adaptive response to caloric deprivation [39] and mitochondrial biogenesis [40]. Many of the mitochondrial and energy metabolism genes that are under the control of PGC1 in mammals are also repressed with aging. This repression can be partially reversed by caloric restriction [41–43]. Several metabolites are altered in cells following the mitochondrial dysfunction including ROS, Ca^{2+}, NADH, and AMP. Changes in these compounds can

Table 2. Hsp-6_promoter_::GFP expression level in different Mit mutant backgrounds

RNAi target	Control	hsp-6	nuo-2	D2030.4	tag-55	isp-1	cco-1	W09C5.8	cyc-1	F26E4.6	atp-3	R53.4	frh-1	F13G3.7	B0261.4
Mitochondrial Function [a]	–	Chaperone	I	I	II	III	IV	IV	IV	IV	V	V	Fe-S cluster synthesis	Carrier protein	rRNA
Fig. 2 worm [b]	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
48 h															
Developmental stage [c]															
Undiluted RNAi	L4/YA	L3	L4/YA	L4/YA	L4	L3	late L3	L4/YA	L4	L4/YA	late L3	late L3	L4	L4	L3-YA
1:10 RNAi	L4/YA	L3/L4	YA	YA	L4	L4	L4/YA	L4	L4	YA	L3-YA	L4/YA	L4/YA	L4/YA	YA
hsp-6_promoter_::GFP expression level [d]															
Undiluted RNAi	2	8	7	7	3	8	7	2	6	7	8	8	3	3	3
1:10 RNAi	2	8	6	4	2	7	6	2	2	5	7	7	3	2	3
72 h															
Developmental stage [c]															
Undiluted RNAi	GA	L3 arrest	GA	GA	GA	L4/YA	YA/GA	GA	GA	GA	L3 arrest	L4	GA	GA	GA
1:10 RNAi	GA	GA	GA	GA	GA	GA	GA	GA	GA	GA	GA	GA	GA	GA	GA
hsp-6_promoter_::GFP expression level [d]															
Undiluted RNAi	2	8	8	8	5	8	8	5 [e]	8	8	6	6	6	2	5 [e]
1:10 RNAi	2	8	7	5	3	8	8	2	5 [e]	7	8	8	4	2	3

a) Roman numerals refer to complexes I, II, III, IV, and V of the ETC. Genes with these annotations encode components of the respective ETC complex.
b) Representative fluorescent images of 72 h worms are shown in Fig. 2.
c) L1, L2, L3, and L4 represent consecutive C. elegans Larval stages; YA: Young Adult; GA: Gravid Adult.
d) N2 animals were exposed to RNAi from the time of hatching ($n = 40$ worms/RNAi; 20°C) and then GFP expression was scored at +48 h and then again at +72 h by two different investigators. The average level of GFP expression in each population was subjectively assigned a value from 1 to 8 (with 1 being the lowest level of expression and 8 the highest). Reporter expression in the vector control treatment (background) was defined as 2.
e) Heterogeneous GFP expression across population. Some worms show this level of expression, others show only background GFP level.
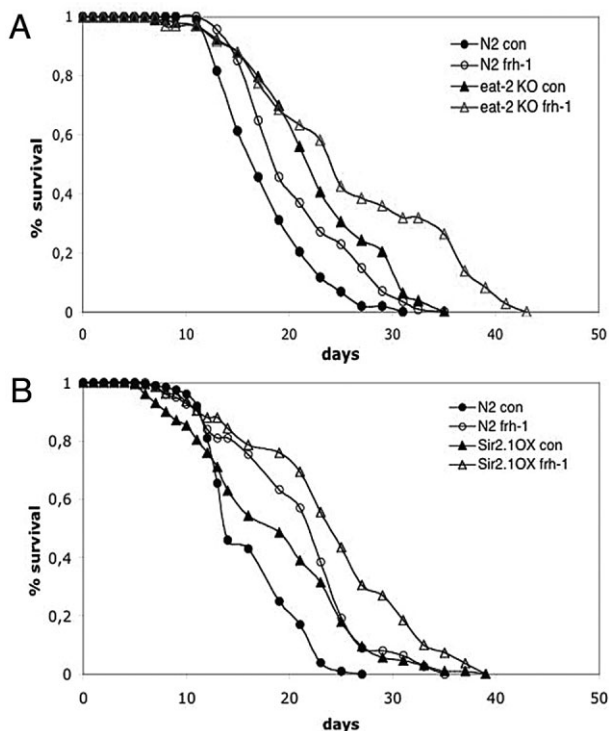
Figure 3. Epistatic analysis of life extension pathways in the *frh-1* Mit mutant. N2 wild type (A and B, circles), *eat-2*(*ad465*) (A, triangles), and *sir-2.1* over-expressing animals (B, triangles) were subjected to vector control (closed symbols) or frataxin RNAi (open symbols) for three consecutive generations and lifespan recorded in the third generation. *Eat-2*(*ad465*) and *sir-2.1* over-expressing strains are both long-lived. Exposure to *frh-1* RNAi extends the lifespan of both strains even further.

be sensed by several proteins, such as AMP kinase, p38/JNK stress/mitogen-activated kinase, mammalian target of rapamicin (mTOR), the deacetylase sirtuin 1 (SIRT1), and calcium/calmodulin-dependent protein kinase IV (CaMKIV) [39], all of which finely tune PGC1 activation. Stimulation of PGC1 induces the doubling of mtDNA [44] as well as a plethora of transcription factors (PPARγ, NRF1, NRF2, and mtTFA) that in turn regulate a suite of genes (such as COXIV, COXI, b-ATP synthetase, cyt-c) necessary for the proper assembly and integration of new mitochondria into the existing mitochondrial reticulum [45]. The *C. elegans* genome apparently lacks orthologs of the PGC1 proteins [46]. However, a partial functional overlap between mammalian PGC1 and *C. elegans* MDT-15 has been strongly suggested by the data of Taubert *et al.* [47]. Interestingly, we recently identified a target of the MDT-15 coactivator in a screen we undertook for factors affecting *isp-1* viability (unpublished observations).

When a metabolic stress (such as cold or exercise) places chronic demands upon ATP level, many cells respond by altering their metabolism and increasing mitochondrial biogenesis. Ragged-red fibers, with their ex-

panded subsarcolemmal mitochondria, are common to myopathies associated with several HMADs and presumably represent an attempt by cells to counter their reduced ETC activity [45]. Mitochondrial biogenesis may also then be one of the strategies employed by the Mit mutants to counter their mitochondrial defects. Interestingly, in our first attempts to characterize the mitochondrial morphology at the ultrastructural level using electron microscopy (EM) in a *C. elegans* Mit mutant, we have observed that compared to control animals, *frh-1* RNAi-treated animals contain an increased number of mitochondria and of thin membranous bridges connecting them which may be indicative of mitochondrial biogenesis (Gin Fonte, N.V., unpublished observation). Other studies also support the notion that mitochondrial dynamics are dysregulated in the Mit mutants [13].

As an alternative approach to studying mitochondrial biogenesis in the Mit mutants we have focused our attention on changes in mitochondrial protein expression. mtDNA copy number increases during *C. elegans* development, mostly in coincidence with germline maturation [48]. *C. elegans* embryos have about 2.5×10^4 copies of mtDNA and this number remains unchanged until the L3 larval stage. By the beginning of the L4 larval stage this value has increased fivefold, 50% of this increase is attributable to somatic cells duplication while 50% to the germline expansion (by both oocytes and sperm). mDNA copy number then increases a further sixfold by the time young adulthood is reached, and this latter expansion is due entirely to hermaphroditic oocyte production. These increases in mtDNA copy number during nematode development suggest a parallel increase in the number of mitochondria, or at least of mitochondrial protein expression, although so far this has not been formally shown. With the few antibodies available against *C. elegans* mitochondrial proteins, we have found that at least one nuclear-encoded subunit of complex I (NUO-2), and a mitochondrial-encoded subunit of complex IV (COX1), both clearly increase during the development, in accord with mitochondrial expansion. Surprisingly, the α-subunit of complex V seemed to remain mostly unchanged (Fig. 4A). Since we analyzed whole worm extracts, we presume that these differential changes reflect tissue-specific alterations. Expansion of the gonad presumably underlies many of these differences. Alternatively, such changes could reflect larval-specific subunit requirements during animal development.

When we explored the expression of our small selection of mitochondrial proteins in three RNAi-induced Mit mutants (*frh-1*, *cco-1*, and *nuo-2*), we invariably found that multiple complexes were affected by the loss of a single target protein (Fig. 4B). One interpretation is that reduction of multiple complexes merely reflects the loss of gonadal cells, since each RNAi also affects fertility. If this were true, one would have expected to have seen a consistent decrease in all the ETC subunits, but, as shown in
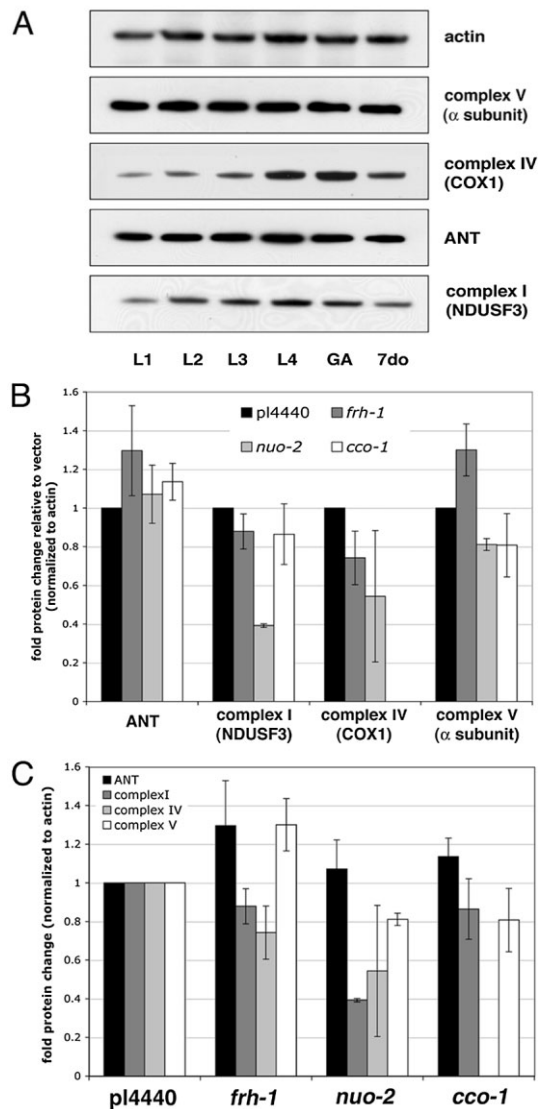
Figure 4. Mitochondrial protein composition is differentially altered in several Mit mutants. (A) Western analysis showing the expression profile of four mitochondrial proteins (complex I (NDUSF3 subunit), complex IV (COX1 subunit), complex V (α subunit), ANT (adenine nucleotide transporter)) during the postembryonic development of *C. elegans*. COX1 is mitochondrially-encoded. Actin is shown for normalization purposes and all the data are representative of three independent experiments. (L1–L4, larval stages one through four; GA, gravid adult; 7do, 7 day-old). (B and C) N2 animals were exposed to a 1:10 dilution of RNAi against *cco-1* or *nuo-2* from the time of hatching, or to undiluted *frh-1* RNAi for three consecutive generations, and then all processed as 1 day-old gravid adults for Western analysis against the same four proteins described in (A). Protein levels were densitometrically quantitated using Image J (NIH). Sample loading was normalized on the basis of actin. Subunit alterations are expressed in fold change relative to vector-treatment alone (pl4440). Error bars represent SEM of the fold change from experiments carried out on two different biological samples. (B) and (C) are alternative representations of the same data.

Fig. 4B, this did not appear to be the case. While loss of mitochondrial-encoded COX1 did consistently decrease following each RNAi treatment, and probably reflecting, in part, reduced gonad expansion, the other ETC subunits we analyzed did not follow the same trend. Indeed, in some instances the other ETC subunits actually increased in concentration. This effect becomes more apparent when the data of Fig. 4B are reorganized into the format of Fig. 4C. The unique pattern of subunit disruption following each RNAi treatment is obvious.

Our current findings on subunit alterations are consistent with earlier studies in mammalian cells showing that different mitochondrial mutations lead to distinct changes in mitochondrial ETC protein expression profiles [49, 50]. Such findings support the relevance of the Mit mutant model in the study of human mitochondrial-related diseases. Presently, we are establishing global profiles of mitochondrial protein changes, across different levels of gene inactivation, for a variety of Mit mutants. It is anticipated that such data will help shed light on different phases of the corresponding human diseases.

4　Concluding remarks

Many human neurodegenerative disorders are associated with mitochondrial dysfunction and severely compromised energy generation, and arise because of genetic defects in the mitochondrial or nuclear genome [51]. Several of the affected genes have orthologs in *C. elegans*. We have successfully disrupted a number of these genes in worms using RNAi in an effort to establish new models to study the human disorders. In this paper, we have outlined our progress on this front.

Aging is the major risk factor for the appearance of neurodegenerative disorders and alteration in mitochondrial function proceeds, or is associated with, several of these diseases. The kinds of mitochondrial alterations that are associated with neurodegeneration resemble those that occur normally during the aging process and caloric restriction has been shown to delay both aging and age-associated diseases. It is probable, then, that there is a significant overlap in the pathogenic mechanisms leading to both processes.

Worms appear to be able to compensate for the partial mitochondrial dysfunction by paradoxically increasing their lifespan. We are currently using this phenotype as well as others, to gain insight into the ways mutant worms offset the severity of their mitochondrial disruptions with the ultimate hope of applying this knowledge to the prevention of the corresponding human diseases. Recent advances have permitted *C. elegans* to be used as a test bed for drug screening [52, 53]. The pharmacologic alteration of the Mit phenotype represents a promising strategy to discover new targets potentially exploitable for

early preventive therapy in human neurodegenerative diseases and other mitochondrial-related disorders.

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