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Compartment-specific perturbation of protein handling activates genes encoding mitochondrial chaperones

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Summary

Protein folding in the mitochondria is assisted by nuclear-encoded compartment-specific chaperones but regulation of the expression of their encoding genes is poorly understood. We found that the mitochondrial matrix HSP70 and HSP60 chaperones, encoded by the Caenorhabditis elegans hsp-6 and hsp-60 genes, were selectively activated by perturbations that impair assembly of multi-subunit mitochondrial complexes or by RNAi of genes encoding mitochondrial chaperones or proteases, which lead to defective protein folding and processing in the organelle. hsp-6 and hsp-60 induction was specific to perturbed mitochondrial protein handling, as neither heat-shock nor endoplasmic reticulum stress nor manipulations that impair mitochondrial steps in intermediary metabolism or ATP synthesis activated the mitochondrial chaperone genes. These observations support the existence of a mitochondrial unfolded protein response that couples mitochondrial chaperone gene expression to changes in the protein handling environment in the organelle.

Supplemental data available online

Key words: Protein folding, Chaperones, Organelle, Signaling, Genetics

Introduction

Chaperones are a diverse group of proteins with important roles in nascent polypeptide folding, assembly of multimeric protein complexes, protein translocation across membrane barriers, integration into membranes and protein degradation (Bukau and Horwich, 1998; Horwich et al., 1999; Hartl and Hayer-Hartl, 2002). In eukaryotes, chaperones are segregated to specific organelles. For example, the cytoplasm, endoplasmic reticulum (ER) lumen and mitochondrial matrix each contain unique DnaK/Hsp70-type and DnaJ/Hsp40-type chaperones encoded by specific nuclear genes (Martin, 1997; Fewell et al., 2001; Hartl and Hayer-Hartl, 2002), whereas distinct GroE/Hsp10/60-type chaperonins are found in the cytoplasm and mitochondrial matrix but not in the ER lumen (Horwich and Willison, 1993).

Chaperone engagement depends on the balance between chaperone levels and client protein load. The latter is set by the rate of protein synthesis and translocation into specific organelles and is influenced by the intrinsic ability of client proteins to fold, enter into productive complexes with other proteins and retain their properly folded state. Increased chaperone engagement by perturbations that impede proper protein folding, encourage protein complex disassembly or promote malfolding, activates chaperone-encoding genes. For this reason, many chaperones were first identified as heat-shock proteins, induced at elevated temperature (Lindquist and Craig, 1988).

Studies of chaperone gene expression have revealed a conserved paradigm in which signaling pathways activating chaperone-encoding genes are repressed by free chaperones that are not engaged by client proteins. In E. coli the transcription factor activating the heat shock operon, σ32, is repressed by free DnaK (Bukau, 1993; Biaszczak et al., 1999). In eukaryotes signal transduction pathways that regulate expression of ER chaperones are repressed by binding of the ER chaperone BiP to the lumenal domains of the proximal signal transducers IRE1, PERK and ATF6 (Bertolotti et al., 2000; Okamura et al., 2000; Liu et al., 2002; Shen et al., 2002). By contrast, cytosolic chaperones specifically repress the heat-shock sensitive transcription factors (HSFs) that regulate their expression (Morimoto, 1998). These observations suggest that cells monitor the folding environment in specific compartments by determining the sufficiency of their chaperone reserves and defend this reserve selectively in each compartment. Thus, segregation of chaperones to specific organelles implies that chaperone-encoding genes respond selectively to the needs of specific organelles.

Compartment specificity in signaling to chaperone-encoding genes has been demonstrated for the ER and cytoplasm. This was established by studies that relied on biochemical tools to
perturb protein folding in a compartment-selective manner. For example, treatment of cells with arsenite, which preferentially perturbs protein folding in the cytoplasm, selectively activates cytoplasmic chaperone gene expression (Mosser et al., 1993; Liu et al., 1994; Harding et al., 1999; Calfon et al., 2002), whereas tunicamycin, an agent that blocks the ER-specific modification of N-linked glycosylation preferentially activates pathways promoting the expression of ER-localized chaperones (Gething and Sambrook, 1992). The molecular basis for this specificity is partially understood, as some of the proximal transducers that selectively activate genes encoding cytoplasmic or ER chaperones have been identified (Morimoto, 1998; Kaufman, 1999; Patil and Walter, 2001; Harding et al., 2002).

Less well understood are the mechanisms that regulate genes encoding chaperones localized to mitochondria. It has recently been shown that over-expression of a folding-incompetent mitochondrial matrix protein, mutant ornithine transcarbamylase, activates mitochondrial chaperone-encoding genes in mammalian cells. The stress-induced transcription factor CHOP was shown to play an important role in this signaling pathway (Zhao et al., 2002). However, the pathways linking CHOP activation to mutant OTC expression are not understood. Furthermore, given that CHOP is a vertebrate-specific transcription factor, whereas mitochondria are present in all eukaryotes, it seemed likely that other pathways for activating mitochondrial chaperones existed. We report on the establishment of genetic and pharmacological tools for perturbing protein handling in the mitochondria of the nematode *C. elegans* and used these to reveal a mitochondrial unfolded protein response that depends on transmitting information from the organelle to the nucleus.

### Materials and Methods

**Transgenic *C. elegans***

The strain containing the ER stress reporter *hsp-4::gfp(zcIs4)* V has been previously described (Calfon et al., 2002). The mtHSP70 reporter, *hsp-6::gfp* was constructed by ligating a 1.7 kb HindIII-BamHI PCR fragment derived by amplification of *C. elegans* genomic DNA with the primers: C37H5.5.2AS (TCGAGTCCATA-GC60::gfp were produced for each reporter, with identical GFP expression extrachromosomal arrays were integrated into the chromosome with been previously described (Calfon et al., 2002). The mtHSP70 (*C. elegans*) was a gift from Jim Morley and Richard Morimoto from Northwestern University (Chicago, IL, USA).

**Pharmacological treatment**

Gravid animals were bleached to obtain eggs that hatched to produce a synchronous population of young adults that were used in the treatments described below.

Ethidium bromide (Sigma) was dissolved in water at 10 mg/ml and added to agar plates to a final concentration of 5-125 µg/ml. 2,4 dinitrophenol (Sigma) was dissolved in ethanol at 1 M and added to agar plates to a final concentration of 0.1-2.5 mM. Bacteria were seeded on these plates and the animals were allowed to feed and produce a brood. The brood was harvested for study on day 3 after egg laying. Animals were heat shocked at 30°C or treated with 1 µg/ml tunicamycin, for 6 hours.

Tetramethylrhodamine ethyl ester (TMRE; Molecular Probes), a lipophilic cation whose mitochondrial uptake depends on the ΔΨ potential (Farkas et al., 1989; Loew et al., 1993) was dissolved in DMSO at 50 µM and applied to the agar plate at final concentration of 0.1 µM. Untreated and DNP-treated animals were placed on the TMRE plates for 12 hours, removed, washed with M9 medium, embedded in agarose and examined using rhodamine filters in a Zeiss Axioptph microscope. Fluorescence and transmission photomicrographs were obtained using a digital CCD camera and processed in Adobe Photoshop.

**RNAi**

Interference with gene function by RNAi followed an established protocol (Timmons et al., 2001). Briefly, two double stranded RNA were produced in the HT115 strain of *E. coli* transformed with pPD129 plasmids containing cDNA fragments of genes being studied: a 675 bp *EcoRI*-*XhoI* of the *hsp-60* EST yk515d7 (a gift from Yoji Kohara), a 735 bp *KpnI*-BamHI fragment of the Paraplegin homologue (*Y47G6A.10*, *spg-7*)-derived EST *yk282e3*, a 595 base-pair *SacI*-BamHI fragment of *hsp-6* (*C37H5.8*)-derived EST *yk313b1*, a 423 nucleotide *Chl-XhoI* fragment from *eno-1* (*Y105E5B.8*) a 794 bp PCR fragment of the PDI homologue (H06O01.1, *pdi-3*) derived by the primers TTCCAGCCCAAGACTACGA and *aco-2::gfp(zcEX13) V* were used in this study. The cytoplasmic HSP70 (*C128.1*) reporter, *hsp-70::gfp(rml8)* was a gift from Jim Morley and Richard Morimoto from Northwestern University (Chicago, IL, USA).

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individually and produced their brood. RNAi phenotypes were evaluated in their F1 progeny 3 days later.

Northern blots, Southern blots and immunoblots

Total RNA was prepared using the guanidine thiocyanate–acid–phenol extraction method and 15 μg were applied to each lane of a 1.5% agarose gel. The hybridization probes used to detect hsp-6, spe-7, cts-1, aco-2, F1FO ATP synthase α-chain (H28O16.1) and cytochrome c oxidase subunit IV (W09C5.8) were derived from the fragments used in RNAi (see above). Mitochondrially encoded cox-1 mRNA was detected using a 1075 bp PCR fragment derived by the primers TTACATGCGAGGGTTAAG and TCAGACTGATACTGTG-ACC and hsp-4 was detected as previously described (Calfon et al., 2002).

Fig. 1. Attenuated function of the mitochondrial genome induces mtHSP70 (hsp-6) mRNA. (A) Autoradiogram of a Southern blot of EcoRI-digested total DNA from animals raised in the indicated concentration of ethidium bromide (EtBr; μg/ml), or tunicamycin (Tun; 1 μg/ml). The blot was hybridized sequentially to a labeled DNA fragment of the mitochondrially encoded cox-1 gene and the nuclear encoded K08H10.2a gene. (B) Autoradiogram of the northern blot of total RNA from animals cultured on agar plates containing the indicated concentration of ethidium bromide or tunicamycin. (Upper panel) The blot was hybridized to a labeled cox-1 DNA fragment. (Lower panel) Ethidium bromide staining of the ribosomal bands of the RNA. (C) Autoradiogram of a northern blot of total RNA from animals treated with ethidium bromide, tunicamycin or heat-shock (HS). The blot was hybridized sequentially to radiolabeled fragment from the mtHSP70 gene (hsp-6) and the C. elegans BiP homologue, hsp-4. Bar charts on the right of each panel show the relative intensity of the hybridization signal.

Results

Proteins encoded by nuclear genes are imported into mitochondria. During import, they associate with mitochondrial chaperones and are released upon proper folding, often only after incorporation into multi-subunit complexes (Neupert and Pfanner, 1993; Neupert, 1997). Many such complexes also contain mitochondrialy encoded proteins, which are required for complex stability (Stuart and Neupert, 1996). Thus, the fixed stoichiometry of the nuclear and mitochondrialy encoded components of these multimeric complexes predicts that disrupting the supply of mitochondrialy encoded proteins will interfere with complex assembly and increase the association of the mitochondrialy chaperones with unassembled components. Ethidium bromide preferentially impairs replication and transcription of the mitochondrial genome in cultured cells, reducing the synthesis of mitochondrialy encoded polypeptides (King and Attardi, 1989; Hayashi et al., 1990). We tested whether C. elegans raised on medium containing ethidium bromide would also have reduced mitochondrial DNA and reduced expression of mitochondrialy encoded genes.

Hybridization analysis revealed a dose-dependent decrease in mitochondrial cox-1 DNA and mRNA in animals raised on ethidium bromide (Fig. 1A,B). Remarkably, animals survived ethidium bromide treatment, but generation time was prolonged and brood size reduced. We studied the effect of ethidium bromide on activation of the mitochondrial matrix HSP70 (mtHSP70) gene, hsp-6. Northern blots showed a dose-dependent increase in hsp-6 mRNA in animals raised on ethidium bromide (Fig. 1C). By contrast, ethidium bromide did
not activate hsp-4, which encodes a *C. elegans* homologue of the ER-localized chaperone, BiP. As previously noted, hsp-4 was activated by treatment with tunicamycin or by culture at elevated temperature (Fig. 1C). However, these stressful treatments did not increase hsp-6 mRNA levels.

We generated an *hsp-6::gfp* transcriptional reporter and introduced this fusion transgene into animals. GFP was expressed broadly in multiple transgenic lines and expression was noted from L1 larva to adults. ethidium bromide caused time- and dose-dependent increase in *hsp-6::gfp* expression

![Image](image.png)

**Fig. 2.** Reporter genes activated by mitochondrial perturbation.
(A) Fluorescence photomicrographs of untreated (UT), ethidium bromide (EtBr; µg/ml) or tunicamycin-treated (Tun; 1 µg/ml) or heat-shocked (HS) animals with reporter transgenes for mitochondrial chaperones (*hsp-6::gfp*, *hsp-60::gfp*), an endoplasmic reticulum chaperone (*hsp-4::gfp*), a cytoplasmic chaperone (*hsp-70::gfp*), and the mitochondrial tri-carboxylic acid cycle enzymes citrate synthetase (*cts-1::gfp*) and aconitase (*aco-2::gfp*). (B) Immunoblot of soluble proteins extracted from the *hsp-6::gfp* animals described in A. The blot was reacted with anti-GFP serum (upper panel) or antiserum to the broadly expressed UNC-32 protein (lower panel). (C) Northern blot of untreated and ethidium bromide-treated *hsp-6::gfp* animals. The blot was hybridized sequentially with a *hsp-6*-coding region probe that detects the endogenous gene and a GFP probe that detects the transgene.
A similar pattern of ethidium bromide inducibility was observed in animals transgenic for a mitochondrial chaperonin-encoding gene reporter, hsp-60::gfp (Fig. 2A, Fig. 5B). The transgenic reporters were much more sensitive to ethidium bromide than the endogenous genes (compare the levels of endogenous hsp-6 mRNA to that of hsp-6::gfp mRNA in ethidium bromide-treated hsp-6::gfp transgenic animals, Fig. 2C). However, the reporter’s specificity for mitochondrial perturbations was retained, as reflected in their indiffERENCE to tunicamycin or heat shock. By contrast, the hsp-4::gfp reporter, like the endogenous gene, was unaltered by ethidium bromide but was markedly induced by either tunicamycin or heat shock and the cytoplasmic hsp-70::GFP reporter was inducible only by heat shock (Fig. 1C, Fig. 2A). Furthermore, the induction of hsp-6 and hsp-60 was not shared by nuclear genes that encode enzymes active in the mitochondria, as neither the cts-1::gfp, which reports on the activity of C. elegans citrate synthase, nor the aco-2::gfp, which reports on the activity of mitochondrial aconitase, were activated by ethidium bromide (Fig. 2A). These last observations suggest that activation of the mitochondrial chaperones can occur independently of a general upregulation of nuclear genes encoding mitochondrial proteins.

We exploited the heightened sensitivity of the transgenic reporters to explore other perturbations predicted to selectively impede protein folding in the mitochondria. C. elegans is highly susceptible to disruption of gene expression by RNA-mediated interference (RNAi). Therefore we examined the impact of hsp-6(RNAi) and hsp-60(RNAi) on the activity of the hsp-6::gfp and hsp-60::gfp reporters. hsp-6(RNAi) and hsp-60(RNAi) animals had severe growth delay and many were arrested before hatching or at early larval stages. Nonetheless, these growth-arrested animals expressed high levels of the GFP reporter. By contrast, hsp-6(RNAi) and hsp-60(RNAi) did not induce hsp-4::gfp, cytoplasmic hsp-70::gfp, cts-1::gfp or aco-2::gfp. RNAi of the C. elegans homologues of the ER oxidase, ero-1 or the ER protein disulfide isomerase, pdi-3, markedly activated hsp-4::gfp but did not affect hsp-6::gfp and hsp-60::gfp (Fig. 3).

We sought to perturb mitochondrial protein handling by inactivating a gene that might not exert as profound an impact on animal health and development as hsp-60(RNAi) or hsp-6(RNAi). Paraplegin (or SPG7) is a nuclear encoded, mitochondrial localized, membrane-associated protease, mutations in which cause human spinal cord dysfunction (spastic paraplegia) (Casari et al., 1998). As the clinical syndrome associated with loss of function mutations in SPG7 affects individuals that have otherwise developed normally, we hoped that inactivating a C. elegans homologue of SPG7, spg-7, might likewise have only a modest impact on animal development. Yeast homologues of SPG7 are involved in proteolytic degradation of mitochondrial membrane proteins and play an important role in assembly of mitochondrial multi-protein complexes (Langer et al., 2001). Therefore, inactivation of the gene might promote accumulation of abnormal mitochondrial proteins, as interfering with protein degradation in other compartments increases the load of malfolded proteins in a compartment-specific manner (Bence et al., 2001; Nishitoh et al., 2002).

To determine if spg-7(RNAi) affected protein processing in the mitochondria, we used a transgenic strain expressing GFP linked to a mitochondrial import signal in the body wall muscle (myo-3::mtGFP) (Labrousse et al., 1999). Following import into the mitochondria mtGFP folds to a soluble protein that is identical in size to conventional GFP. In E. coli GFP folding is chaperone dependent (Wang et al., 2002), we therefore expected that the ability of the processed mtGFP to fold to its soluble form might also be chaperone dependent and report on the capacity of the mitochondria to chaperone matrix proteins. Immunoblot analysis of soluble and Triton insoluble fractions from untreated mtGFP and GFP transgenic animals showed that most of the mtGFP signal is in the soluble fraction and that this signal co-migrates on SDS-PAGE with conventional (cytoplasmic) GFP, which is mostly soluble too (Fig. 4A). The modest amount of insoluble mtGFP in extracts from untreated animals has slightly reduced mobility on the SDS-PAGE (lane 9) suggesting that it represents a precursor form. spg-7(RNAi) did not alter the solubility of conventional cytoplasmic GFP, however, it markedly increased the mtGFP signal in the insoluble fraction. These observations are consistent with an adverse affect of spg-7(RNAi) on the folding environment in mitochondria, which impairs the ability of the organelle to handle a model nuclear-encoded matrix protein. A similar increase in insoluble processed mtGFP was also observed in animals raised on medium containing ethidium bromide (Fig. 4A, lower panel). This last observation is consistent with the idea that impaired mitochondrial gene expression and an unbalanced load of unassembled components of multi-subunit complexes had taxed the organelle’s capacity to process an imported model polypeptide such as GFP.

Ethidium bromide and spg-7(RNAi) specifically activated hsp-6::gfp and hsp-60::gfp but not hsp-4::gfp or cytoplasmic hsp-70::gfp (Fig. 3). Northern blot analysis showed that spg-7(RNAi) also activated the endogenous hsp-6 gene but had no effect on the endogenous aco-2 and cit-1 genes that encode enzymes of the tri-carboxylic acid cycle or on the α-chain of the F1F0 ATP synthase or cytochrome c oxidase subunit IV-encoding genes, involved in oxidative phosphorylation (Fig. 4B). These observations suggest that the mitochondrial chaperones are not activated as part of a general increase in organelle biogenesis attendant upon the perturbation in protein handling, as biogenesis would be expected to increase the expression of genes involved in key mitochondrial metabolic activities and oxidative phosphorylation. As the spg-7(RNAi) animals were readily recovered in large quantities, it was also possible to confirm by northern blotting the expected decreased expression of spg-7 mRNA (Fig. 4B).

The experiments described above revealed a link between perturbed mitochondrial protein handling and activation of genes encoding mitochondrial chaperones. However, it remained to be determined if any perturbation of mitochondrial function would activate the chaperone-encoding genes or if there were specificity in their response to perturbations affecting protein handling in the organelle. 2,4 dinitrophenol (DNP) uncouples oxidative phosphorylation in mammalian and C. elegans mitochondria (Murfitt et al., 1976). It does so by promoting a proton leak across the inner mitochondrial membrane, thus dissipating the electromotive chemical gradient (ΔΨ) required for ATP synthesis and aspects of mitochondrial import. Therefore, to determine if DNP treatment impacts on mitochondrial function in C. elegans, we exposed live untreated and DNP-treated animals to tetramethylrhodamine ethyl ester (TMRE), a lipophilic
fluorescent cation whose mitochondrial uptake depends on $\Delta \Psi^-$ (Farkas et al., 1989; Loew et al., 1993).

As shown in Fig. 5A, *C. elegans* takes up TMRE from the culture medium and incorporates it broadly into its tissues. Animals exposed to DNP exhibited a dose-dependent decrease in TMRE fluorescence, consistent with dissipation of their mitochondrial proton gradient, $\Delta \Psi^-$. DNP-mediated decrease in TMRE fluorescence accrued slowly, over several days, indicating that direct interference with TMRE fluorescence by DNP is unlikely. Furthermore, DNP treatment also delayed development of the animals and reduced their fertility, indicating that exposure to this mitochondrial uncoupler has significant effects in vivo. As TMRE can also be taken up by other organelles, a more general ATP shortage affecting other electrochemical gradients may have contributed to reduced TMRE fluorescence in DNP-treated animals. Despite evidence that DNP strongly impairs mitochondrial function in vivo, it did not activate mitochondrial chaperone-encoding genes (Fig. 5B,C).

Mitochondria are a major source of reactive oxygen species and perturbed mitochondrial function might further promote their accumulation by interfering with the normal flow of electrons to molecular oxygen (Shigenaga et al., 1994). To examine the potential role of reactive oxygen species in signaling the activation of the mitochondrial chaperones we compared the activation of the *hsp-60::gfp* reporter to that of *sod-3::gfp*, reporting on the manganese superoxide dismutase gene that is known to respond to accumulation of reactive oxygen species (Honda and Honda, 1999; Libina et al., 2003).

RNAi of the mitochondrial chaperone *hsp-60*, the
A mitochondrial unfolded protein response

mitochondrial protease spg-7 or phb-2 (the latter encodes a component of the prohibitin complex that is implicated in assembly of components of the respiratory chain (Artal-Sanz et al., 2003) all strongly activated the hsp-60::gfp reporter, as predicted. However, no activation of sod-3::gfp was noted (Fig. 5D). Exposure of animals to Paraquat, an agent that promotes accumulation of reactive oxygen species, potently activated sod-3::gfp, serving as a positive control. Interestingly, Paraquat exposure also activated hsp-60::gfp, consistent with the damaging effect of reactive oxygen species on mitochondrial proteins. Conversely, exposure to ethidium bromide activated both hsp-6::gfp and sod-3::gfp (not shown). These experiments show that while conditions that promote expression of mitochondrial chaperones may lead to accumulation of reactive oxygen species, and accumulation of reactive oxygen species may promote the putative mitochondrial unfolded protein response, the two can be clearly uncoupled.

The observation cited above suggested that perturbations affecting the metabolic activity of mitochondria do not necessarily activate mitochondrial chaperone gene expression. To further examine the link between mitochondrial chaperone expression and perturbations affecting the folding environment in the organelle, we performed an unbiased screen for genes whose loss of function activated mitochondrial chaperones in C. elegans. We sequentially exposed hsp-6::gfp and hsp-60::gfp animals to RNAi directed to all the genes on C. elegans chromosome I that were predicted to encode mitochondrial proteins and to all the 1160 predicted genes in a randomly selected interval on C. elegans chromosome I, between Y58G1C and unc-13. The latter component of the collection was included in an effort to uncover a potential class of genes whose RNAi might affect the expression of hsp-6::gfp and hsp-60::gfp but who are not annotated as encoding mitochondrial proteins.

RNAi of 32 predicted genes in this collection reproducibly activated the mitochondrial chaperones in the untreated point was set as 1.

![Image](48x325 to 342x719)

**Fig. 4.** Interference with spg-7 (predicted to encode a mitochondrial protease) or ethidium bromide treatment perturbs protein processing in the mitochondria and activate the endogenous hsp-6 gene. (A) Immunoblot of GFP in detergent extracts from transgenic animals expressing conventional GFP (a cytoplasmic protein) or GFP with an N-terminal mitochondrial import sequence (mtGFP), both in the body wall muscle. The animals were exposed to mock RNAi or spg-1(RNAi) or cultured on 125 µg/ml ethidium bromide (EtBr) as indicated. Equal fractions of the total extract (Total), 100,000 g soluble (Soluble) and 100,000 g pellet (Pellet) of the whole animal detergent extract were loaded on the gel as indicated.

(B) Autoradiogram of a northern blot of total RNA from mock-RNAi, spg-7(RNAi) or ethidium bromide (EtBr) treated animals. The blot was hybridized sequentially to radiolabeled fragments from the hsp-6, spg-7, aco-2, cts-1, F1F0 ATP synthase α-chain (F1F0α) and cytochrome c oxidase subunit IV (Cyt C Ox IV) genes. (C) Quantification of the radiolabeled signal on the blots shown in B. The hybridization signal for each gene at the untreated point was set as 1.
expression (Fig. 2). Two genes in this pool of activators are predicted to be involved in processing of mitochondrial proteins, one, a protease, SPG-7, has been described above and the second, a *C. elegans* homologue of yeast Oxa1p, is predicted to play a role in membrane insertion of mitochonrdially encoded components of the respiratory apparatus. Sixteen activators encode mitochondrial proteins with other roles (oxidative phosphorylation, intermediary metabolism, protein import). Remarkably, 15 of these are subunits of multi-protein complexes, whose assembly requires precise stoichiometry of nuclear and/or mitochondrially encoded components. Only one of these 16 genes, that encoding cytochrome C1, is not part of a heteromeric complex.

Therefore, 27 of the 32 genes whose loss of function activated the mitochondrial chaperones are predicted to function directly in protein metabolism or to have the potential to tax chaperone reserves by causing accumulation of unassembled subunits of mitochondrial complexes. Of the remaining five genes whose RNAi activated *hsp-6::gfp* and *hsp-60::gfp*, one, *rts-1*, encodes a protein related to transcriptional repressors of the polycomb group and whose RNAi non-specifically up-regulated other reporter transgenes (data not shown). A second gene encodes a DnaJ-like chaperone whose subcellular localization is not known. Two genes encode a pair of related predicted cytoplasmic ribosomal proteins whose RNAi may cross-react with mitochondrial ribosomal protein genes, and the fifth gene encodes the aforementioned cytochrome C1.

The genes in this collection whose RNAi did not activate the chaperones were also informative as 30 of them encode predicted mitochondrial proteins (Table 1). Remarkably, 24 of these 30 encode monomeric proteins or proteins that form homo-oligomeric complexes. Their inactivation is therefore not predicted to result in accumulation of unassembled subunits of mitochondrial complexes. Four of the remaining six genes do encode components of multimeric complexes, however it is not known if the RNAi procedure sufficiently diminished their expression. Similar considerations apply to a fifth gene, which encodes a predicted homologue of a mitochondrial Lon-like protease, whereas the oligomeric status of the sixth gene, encoding a homologue of Metataxin, is not known.

To further explore the role of impaired mitochondrial metabolism in activation of the chaperones, we inactivated, by RNAi, four genes that encode key monomeric or homo-oligomeric enzymes of the tri-carboxylic acid cycle. RNAi of all four genes resulted in significant embryonic lethality and arrested growth (as previously described) (Kamath et al., 2003). However the *hsp-6::gfp* or *hsp-60::gfp* reporter genes were either inactive [in the case of *aco-2*(RNAi) and *mdh-1*(RNAi)] or were activated very late in development of the RNAi animals [in the case of *fum-1*(RNAi) and *cts-1*(RNAi)] (Table 1). The associated RNAi phenotypes are unlikely to
<table>
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<th>No.</th>
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<td>2</td>
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Activators: Genes on chromosome I in the interval between Y48G1C and unc-13 whose RNAi activated hsp-6::gfp and hsp-60::gfp.

Non-activators: Genes encoding predicted mitochondrial protein, localized to the above interval that did not activate hsp-6::gfp and hsp-60::gfp. Also listed under non-activators are four genes from chromosome III who encode enzymes of the tri-carboxylic acid cycle and whose RNAi presumably results in a severe disruption of mitochondrial metabolism reflected in a high incidence of larval arrest and embryonic lethality (Kamath et al., 2003) but whose effect on hsp-6::gfp and hsp-60::gfp is confined to late induction in adult RNAi animals (in the case of aco-2 and cit-1).

Description: Predicted protein function.

Class: Hypothesized mechanism by which the RNAi procedure perturbs (or fails to perturb) protein processing in the mitochondria (see text for further discussion).
have obscured reporter activation, as many of the activators listed in Table 1 had similar severe RNAi phenotypes and these did not interfere with detection of reporter activity in early embryonic or larval stages (also see Fig. 2A). Collectively these data indicate that mitochondrial chaperones are activated by manipulations predicted to tax the organelle’s protein handling machinery, but not by manipulations that impede its metabolic activity.

Discussion
The lack of tools to specifically manipulate the folding environment in the mitochondria had restricted the ability to study mitochondrial chaperone gene expression. In this study we took advantage of the ease with which gene function can be manipulated in C. elegans to show that reduced function of genes encoding chaperones and proteases specifically involved in protein processing in the mitochondria selectively activates genes encoding two major mitochondrial chaperones. Using sensitive reporter genes we found that mitochondrial chaperones are upregulated transcriptionally in response to perturbations that affect protein processing in the mitochondria. The robustness of the responses we observed and their specificity strongly suggests the existence of a mitochondrial unfolded protein response. It is important, however, to emphasize that we have not proved that signaling in the pathway culminating in hsp-6 and hsp-60 expression initiates with direct recognition of unfolded proteins or recognition of reduced capacity to handle proteins in the organelle; this remains a working hypothesis to be tested as the components of the pathway are revealed by future work.

In this study, we have cast the problem of regulating mitochondrial chaperone-encoding genes in the context of the need to respond to compartment-specific changes in protein folding capacity and chaperone occupancy; in other words in the context of an unfolded protein response. However, the problem also needs to be considered in the broader context of the communication that must take place between the mitochondrial and the nuclear genome. Inactivation of genes involved directly in protein processing by the mitochondria gives rise to a very early and strong activation of the chaperone reporter genes. However, impaired protein folding in the mitochondria will also affect organelle function. Therefore, it is informative that manipulations primarily predicted to degrade mitochondrial energy production, such as dissipation of the mitochondrial inner membrane proton gradient with DNP or RNAs of genes encoding enzymes of the tri-carboxylic acid cycle (which interrupts the supply of electrons for oxidative phosphorylation), induced the chaperone reporters only minimally, or resulted in markedly delayed activation. Furthermore, manipulations that impair protein handling in the organelle selectively activate chaperone genes as these do not result in measurable upregulation of genes encoding mitochondrial enzymes (Fig. 2A and Fig. 4B). Together these observations suggest that genes encoding mitochondrial chaperones are responsive to a signaling pathway(s) that is selectively activated by perturbed protein folding and processing in the organelle rather than being activated by pathways primarily responsive to the metabolic consequences of mitochondrial dysfunction.

Previous studies have addressed the role of transcriptional control in cellular adaptation to mitochondrial dysfunction (Liao and Butow, 1993; Amuthan et al., 2001) and have begun to shed light on mechanisms that coordinate upregulation of nuclear genes encoding mitochondrial proteins (Wu et al., 1999; Lin et al., 2002). As mitochondrial biogenesis entails expression of many nuclear-encoded genes whose products must be processed and folded by mitochondrial chaperones, one would expect activation of nuclear-encoded mitochondrial chaperones to accompany mitochondrial biogenesis, as has been described in mammalian adipocytes (Wilson-Fritch et al., 2003). It is tempting to speculate that the delayed induction of hsp-6::gfp and hsp-60::gfp observed in the cts-1(RNAi) and aco-2(RNAi) (data not shown) reflects such an indirect response to the metabolic consequences of mitochondrial dysfunction. Furthermore, Lemire and colleagues have emphasized the physiological increase in mitochondrial mass during C. elegans germline development (Tsang and Lemire, 2002). It is interesting in this regard to note that activation of the hsp-6::gfp and hsp-60::gfp reporter by spg-7(RNAi) or ethidium bromide treatment was most conspicuous in L4 larvae and in young adults, stages of development associated with expansion of the germline. This observation is consistent with crosstalk between the pathways that control mitochondrial biogenesis and the hypothesized mitochondrial UPR. Our study did not further address this issue as we have not identified conditions that selectively manipulate mitochondrial biogenesis in C. elegans.

It is also important to emphasize the limitations inherent in the use of pharmacological and genetic criteria to distinguish between signals initiated by altered metabolism and altered protein handling by the mitochondria. The potential for diminished chaperone reserve to secondarily affect metabolic function has been discussed above. But altered metabolism may also affect protein handling by the organelle. For example, dissipation of the inner membrane proton gradient (by DNP) impairs mitochondrial import (Fig. 5A), which is likely to have pleiotropic effects on mitochondrial function. Perhaps the reduced expression of mitochondrial genes in animals exposed to high dose DNP (Fig. 5C) offsets the predicted imbalance between imported and mitochondrially encoded components, explaining why chaperones are not induced in DNP-treated animals.

Another variable addressed by our study is the role of reactive oxygen species in activating mitochondrial chaperone-encoding genes. Interactions between these two processes are revealed by the observations that both Paraquat, which leads to accumulation of massive amount of reactive oxygen species and ethidium bromide, which primarily affects the integrity of the mitochondrial genome, activate hsp-60::gfp and the oxidative stress inducible reporter sod-3::gfp (Fig. 5D and data not shown). However, the two responses are uncoupled by milder manipulations that primarily affect protein processing in the mitochondria. Thus, our data do not support an essential role for reactive oxygen species in signaling the putative mitochondrial unfolded protein response.

The severity of the hsp-6(RNAi) and hsp-60(RNAi) phenotype is consistent with the essential role of HSP60 and mtHSP70 in yeast (Cheng et al., 1989; Craig et al., 1989). More surprising is the relative resistance of C. elegans to degradation of mitochondrial function by culture in ethidium bromide or DNP. Though brood size was markedly diminished, consistent
with the established effect of mitochondrial dysfunction on germline development (Tsang et al., 2001; Tsang and Lemire, 2002), the treated animals remained viable and surprisingly mobile under conditions in which much of their mitochondrial DNA had been lost (Fig. 1) or in face of evidence for loss of mitochondrial membrane potential (Fig. 5). This tolerance of nematodes for impaired mitochondrial function suggests that it may be possible to recover animals with mutations affecting the hypothesized mitochondrial UPR.

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References


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A mitochondrial unfolded protein response


