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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 chaperonin-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 *Hin*dIII-*Bam*HI PCR fragment derived by amplification of *C. elegans* genomic DNA with the primers: C37H5.5.2AS (TCGAGTCCATACAAGCACTC) and C37H5.8.2AS (GGGGGGATCCGAAGACAAGAATGATCGTGC) into the GFP reporter plasmid pPD95.75 (a gift of Andy Fire, Baltimore MD, USA). *hsp-6::gfp* contains the 5′ flanking region and encodes the predicted first 10 amino acids of HSP-6 fused to GFP. The *hsp-60::gfp* reporter was constructed by ligating a 2.3 kb *Sal*I-*Bam*HI PCR fragment derived by amplification of *C. elegans* genomic DNA with the primers: CeHSP10.6AS (AAGA*GTC*GACTCGCGGAAGATTGAGTATTCC) and CeHSP60.2AS (CTGA*GGAT*CCTTTCTGGCGAGGGGAAGCATC) into pPD95.75. *hsp-60::gfp* contains the 5′ flanking region and encodes the predicted first 7 amino acids of HSP-60 fused to GFP. The *myo-3::*$_{mt}$*gfp* expressing a mitochondrially localized GFP with a cleavable mitochondrial import signal peptide (Labrousse et al., 1999) was gift from Alexander van der Bliek (University of California Los Angeles, USA) and the strain expressing *sod-3::gfp* (muIs84, CF1553) (Libina et al., 2003) was a gift from Malene Hansen and Cynthia Kenyon (University of California San Francisco, USA). The *hsp-6::gfp* plasmid (25 μg/ml) and the *hsp-60::gfp* plasmid (5 μg/ml) were co-injected with a *lin-15* rescuing plasmid, pSK1 (25 μg/ml) into *lin-15(n765ts)* strain. The extrachromosomal arrays were integrated into the chromosome with ultraviolet/trimethylpsoralen treatment. Several independent lines were produced for each reporter, with identical GFP expression patterns and one of each, *hsp-6::gfp(zcIs13) V*, and *hsp-60::gfp(zcIs9) V* were backcrossed four times into wild-type N2, eliminating the *lin-15* mutation. The *cts-1::gfp* reporter was constructed by ligating a 0.8 kb *Sal*I-*Bam*HI PCR fragment derived by amplification of *C. elegans* genomic DNA with the primers: T20G5Sal.1S (TTTGG*TC*GACATGGTTCATCGGCGGAAGAG) and T20G5Bam.2AS (GCGA*GGATC*CATTCCAGAGAGCGACATTTTCTG) into pPD95.75 and the *aco-2::gfp* was constructed by ligating a 0.9 kb *Sal*I-*Bgl*II PCR fragment derived by amplification of *C. elegans* genomic DNA with the primers: F54H12Sal.1S (GCGG*GTC*GACGCTCCTCAACGATCTTTGC) and F54H12Bgl.2AS (AGGT*AGATCT*AAGTGGCTCAACCGAAGAAGAG) into pPD95.75. Three independently produced extrachromosomal arrays of each line were maintained and two, *cts-1::gfp(zcEX12)* and *aco-2::gfp(zcEX13)* were used in this study. The cytoplasmic HSP70 (C12C8.1) reporter, *hsp-70::gfp(rmIs8)* 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 $\Delta\Psi^{-}$ 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 Axiophot 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, double stranded RNA was produced in the HT115 strain of *E. coli* transformed with pPD129 plasmids containing cDNA fragments of genes being studied: a 675 bp *Eco*RI-*Xho*I of the *hsp-60* EST yk515d7 (a gift from Yoji Kohara), a 735 bp *Kpn*I-*Bam*HI fragment of the Paraplegin homologue (Y47G6A.10, *spg-7*)-derived EST yk282e3, a 595 base-pair *Sac*I-*Bam*HI fragment of *hsp-6* (C37H5.8)-derived EST yk313b1, a 423 nucleotide *Cla*I-*Xho*I fragment from *ero-1* (Y105E8B.8) a 794 bp PCR fragment of the PDI homologue (H06O01.1, *pdi-3*) derived by the primers TTCCAGCCCAAGACTACGA and TTTCCAACGGCAACCTTAAC. Inactivation of *aco-2* (F54h12.1a), *mdh-1* (F20H11.3), *fum-1* (H14A12.2a) and *cts-1* (T20G5.2) was carried out by the primer pairs as described in Wormbase (http://www.wormbase.org/). RNAi of genes on chromosome I was carried out by sequential feeding of an arrayed library of validated genomic fragments obtained from the United Kingdom Human Genome Mapping Project Resource Centre (Cambridge, UK) as described previously (Fraser et al., 2000). Isopropyl-β-D-thiogalactopyranoside (IPTG, 1 mM) was added to the bacterial growth medium to induce transcription of the double stranded RNA and L4 staged animals of defined genotype were added to plates

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 hybrization probes used to detect *hsp-6*, *spg-7*, *cts-1*, *aco-2*, F1F0 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 TTACATGCAGCAGGGTTAAG and TCAGACTGATAACTGTG-ACC and *hsp-4* was detected as previously described (Calfon et al., 2002).

Total cellular DNA (mitochondrial and nuclear), 10 μg, was digested with *Eco*RI resolved on a 1% agarose gel and hybridized sequentially with the radiolabeled *cox-1* probe described above or with a 1065 bp fragment of the nuclear gene K08H10.2, derived by the PCR primers TGCTAAAGACAAGACTTCGG and ACGA-TACGGAGTCAGCAACG.

Immunoblot analysis of protein levels were performed on whole animal extracts prepared by washing animals in M9 medium to remove adherent bacteria, crushing on ice with a mechanical Teflon homogenizer in 3 volumes of lysis buffer (20 mM Hepes, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EDTA 10 mM tetrasodium pyrophosphate, 100 mM NaF, 17.5 mM β-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, 4 μg/ml aprotonin, and 2 μg/ml pepstatin A). The lysate was clarified by centrifugation for 15 minutes at 13,000 ***g*** at 4°C. Aliquots containing 50 μg of protein were loaded in each lane of a 15% SDS-PAGE for electrophoresis and the immunoblot developed with a rabbit polyclonal serum to GFP at 1:10,000 dilution (gift from Pam Silver, Boston MA, USA) or goat polyclonal antiserum to UNC-32 (Santa Cruz Biotechnology sc-15649, lot #A042). To measure the solubility of GFP extracted by Triton X-100, the crude lysates were clarified by centrifuging at 14,000 ***g*** for 10 minutes in a table-top centrifuge and the supernatant was then centrifuged at 100,000 ***g*** for 1 hour to obtain an insoluble pellet and soluble supernatant which was then solubilized in SDS. Equal fractions of the total lysates, 100,000 ***g*** supernatant and pellet were resolved by SDS-PAGE and subjected to immunoblot as described above.

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 mitochondrially encoded proteins, which are required for complex stability (Stuart and Neupert, 1996). Thus, the fixed stochiometry of the nuclear and mitochondrially encoded components of these multimeric complexes predicts that disrupting the supply of mitochondrially encoded proteins will interfere with complex assembly and increase the association of the mitochondrial chaperones with unassembled components. Ethidium bromide preferentially impairs replication and transcription of the mitochondrial genome in cultured cells, reducing the synthesis of mitochondrially 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 mitochondrially 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

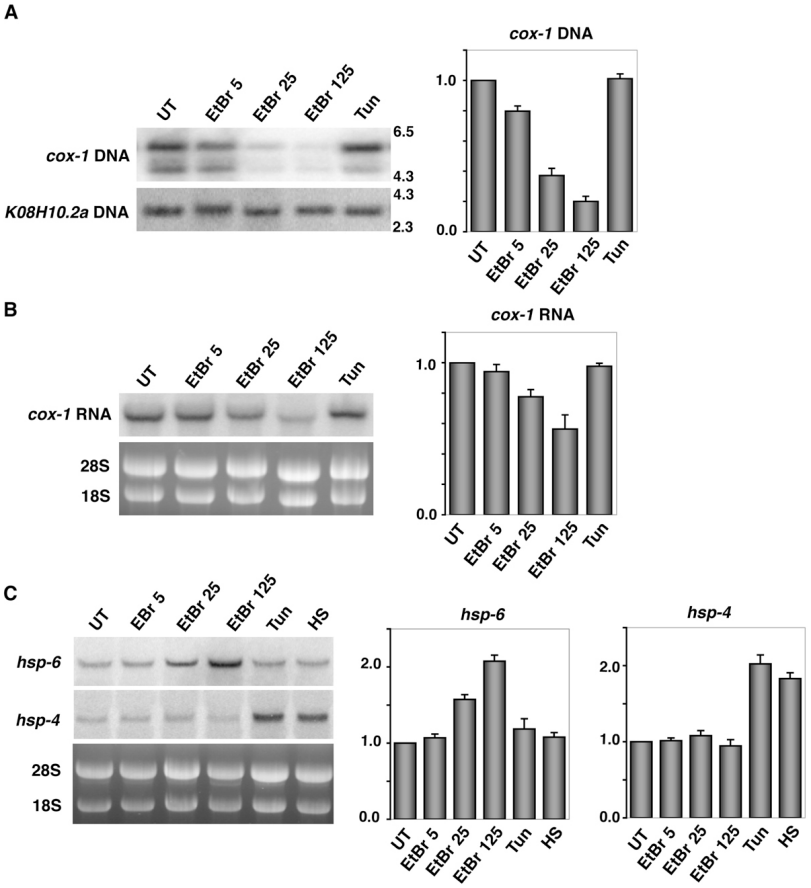

Fig. 1. Attenuated function of the mitochondrial genome induces mtHSP70 (*hsp-6*) mRNA. (A) Autoradiogram of a Southern blot of *Eco*RI-digested total DNA from animals raised in the indicated concentration of ethidium bromide (EtBr; μg/ml), or tunicamycin (Tun; 1 μg/ml). The blot was hybridized sequentially 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 radiolabled 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.

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

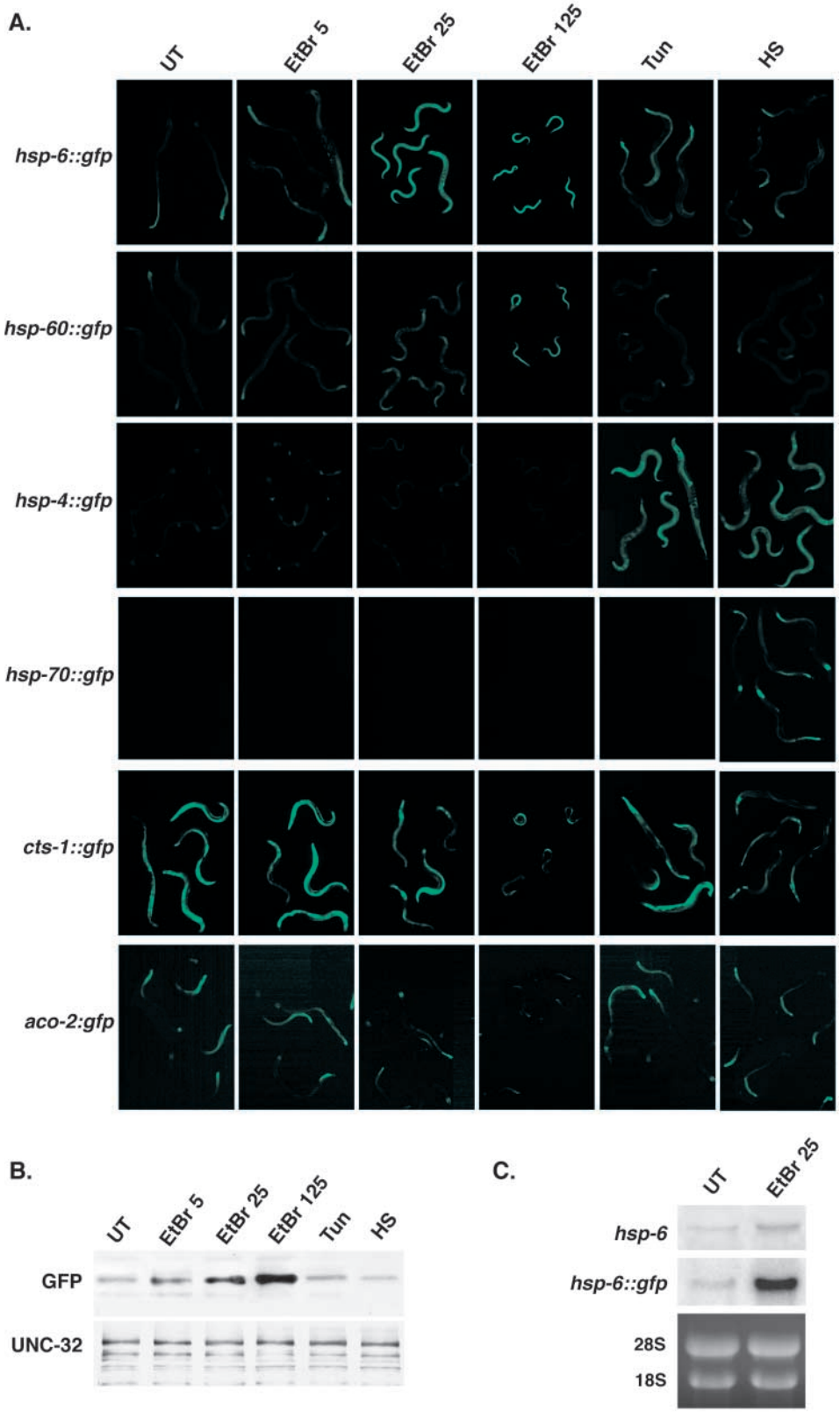

Fig. 2. Reporter genes activated by mitochondrial perturbation. (A) Fluorescence photomicrographs of untreated (UT), ethidium bromide (EtBr; μg/ml) or tunicamycin-treated (Tun; 1 μg/ml) or heat-shocked (HS) animals with reporter transgenes for mitochondrial chaperones (*hsp-6::gfp*, *hsp-60::gfp*), an 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.

(Fig. 2A,B). 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, mitochondrially 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 ($\Delta\Psi^{-}$) 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

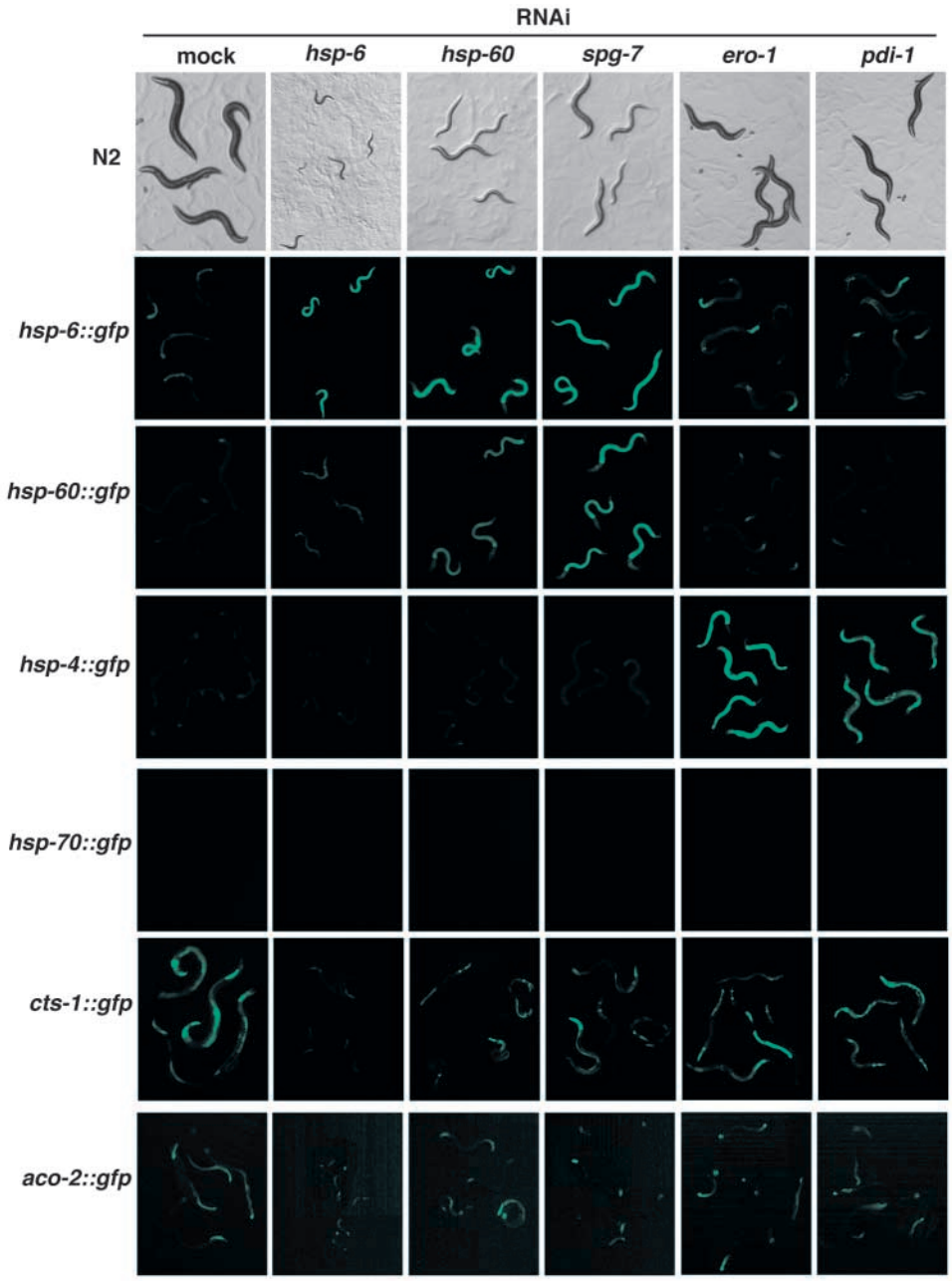

Fig. 3. Interference with genes that process mitochondrial proteins selectively activates mitochondrial chaperone genes. The top row of panels shows photomicrographs of wild-type (N2) animals exposed to the indicated RNAi; the panels below are photomicrographs of GFP fluorescence of *hsp-6::gfp*, *hsp-60::gfp*, *hsp-4::gfp*, *hsp-70::gfp*, *cts-1::gfp* and *aco-2::gfp* animals exposed to mock RNAi or RNAi of the indicated genes.

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

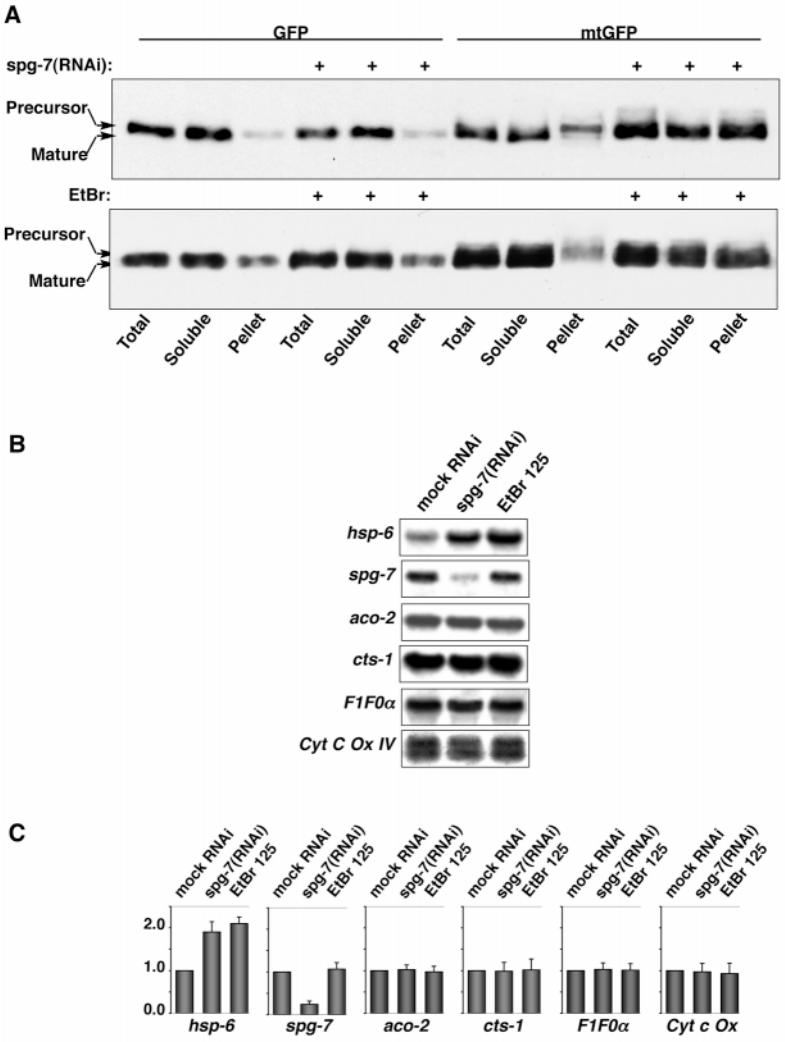

Fig. 4. Interference with *spg-7* (predicted to encode a mitochondrial protease) or ethidium bromide treatment perturbs protein processing in the mitochondria and activate the endogenous *hsp-6* gene. (A) Immunoblot of GFP in detergent extracts from transgenic animals expressing conventional GFP (a cytoplasmic 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 fractions (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.

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 *hsp-6* and *hsp-60* reporter (Table 1). Activation was apparent from the earliest larval stages and was also observed in the embryo in some cases. Ten of these 32 genes encode proteins predicted to play a role in expression of the mitochondrial genome. These include seven mitochondrial ribosomal proteins, a mitochondrial tRNA synthetase, a mitochondrial translation elongation factor and a mitochondrial DNA helicase. The activation of the mitochondrial chaperones by RNAi of genes in this class is consistent with the observations made on animals raised on ethidium bromide, which also impairs mitochondrial genome

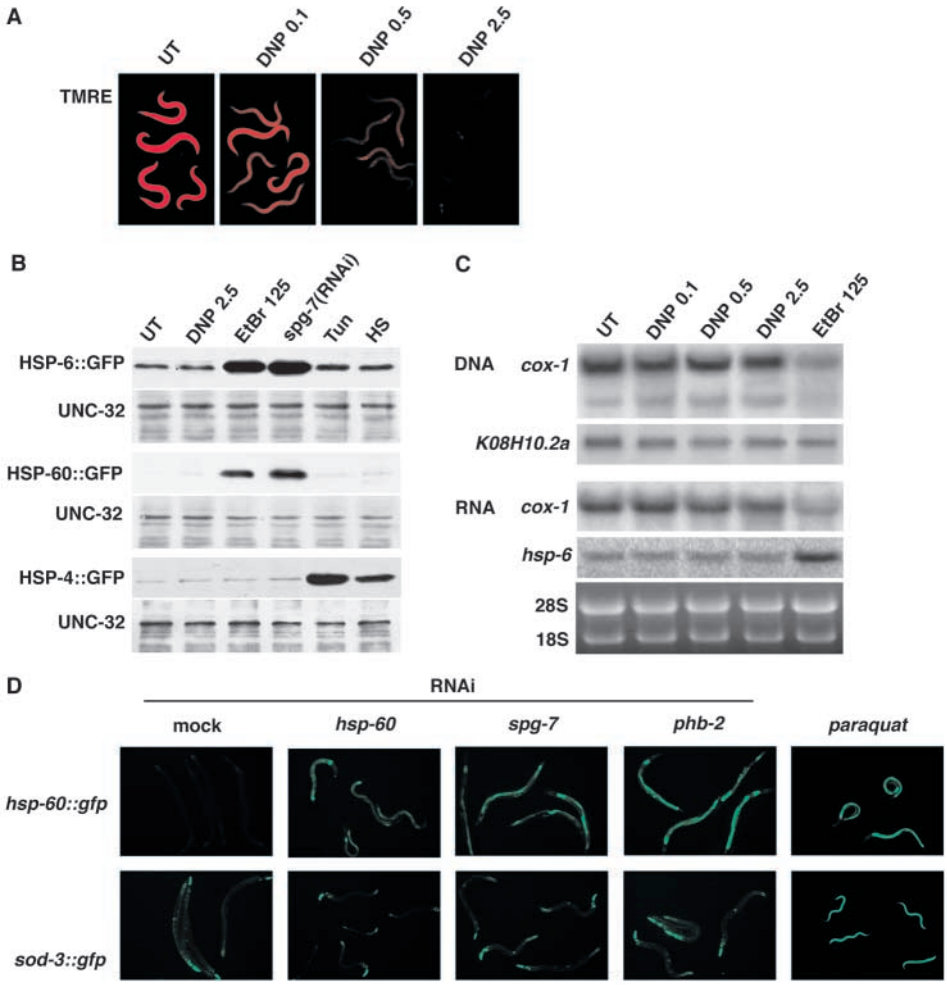

Fig. 5. Activation of mitochondrial chaperone genes can be uncoupled from depolarization of the mitochondrial membrane potential and the accumulation of reactive oxygen species. (A) Fluorescence photomicrographs of animals cultured on plates containing the indicated concentration of dinitrophenol (DNP, mM) and subsequently exposed to the fluorescent dye tetramethylrhodamine ethyl ester (TMRE). (B) Immunoblot of GFP (upper panel) or UNC-32 (lower panel) in lysates from untreated (UT), dinitrophenol (DNP; mM), ethidium bromide (EtBr, 125 μg/ml), *spg-7(RNAi)*, tunicamycin (Tun; 1 μg/ml) treated or heat shocked (HS) animals with *hsp-6::gfp*, *hsp-60::gfp* and *hsp-4::gfp* reporters. (C) Autoradiogram of a northern blot or Southern blot of total RNA or DNA from untreated animals (UT), dinitrophenol (DNP; mM) or ethidium bromide (EtBr, 125 μg/ml) treatment. The blots were hybridized to a radiolabeled fragment from the *C. elegans* mitochondrially encoded *cox-1* gene or nuclear *hsp-6* or *KO8H10.2a* genes. (D) Fluorescent photomicrographs of *hsp-60::gfp* and *sod-3::gfp* transgenic animals following RNAi of genes involved in mitochondrial protein processing or exposure to the toxin Paraquat (2 mM).

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 mitochondrially 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 stochiometry 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, *rls-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 1. Activators and non-activators of the mitochondrial UPR

Activators, Chr I

No.	Gene name	Description	Class
1	Y18H1A.6, *pif-1*	Mitochondrial DNA helicase	Mitochondrial DNA maintenance
2	Y47G6A_247.f, *spg-7*	Mitochondrial protease	Membrane, proteolysis
3	R12E2.12	Mitochondrial ribosomal protein S6	Mitochondrial protein synthesis
4	C43E11.4	Mitochondrial elongation factor EF-TU	Mitochondrial protein synthesis
5	ZK973.10	NADH-coQ oxidoreductase subunit	Mitochondrial membrane, multimeric
6	D1007.6	Cytosolic ribosomal protein (rps-10)	Cytoplasmic protein synthesis
7	D1007.12	Cytosolic ribosomal protein (rpl-24)	Cytoplasmic protein synthesis
8	B0261.4	Mitochondrial ribosomal protein L47	Mitochondrial protein synthesis
9	T19B4.4	Dnj-21, DNAJ homologue	Chaperone, unknown localization
10	T09B4.9	Tim44p homologue	Mitochondrial membrane, multimeric
11	T08B2.8	Mitochondrial ribosomal protein L23	Mitochondrial protein synthesis
12	T10E9.7	NADH-coQ oxidoreductase subunit	Mitochondrial membrane, multimeric
13	F22D6.4	NADH-coQ oxidoreductase subunit	Mitochondrial membrane, multimeric
14	WO1A8.4	NADH-coQ oxidoreductase subunit	Mitochondrial membrane, multimeric
15	R06C7.7, *Rls-1*	Polycomb group	Transcription repressor
16	F52A8.5	Mitochondrial glycine cleavage system protein H	Mitochondrial, multimeric
17	C25A1.7, *lrs-2*	Mitochondrial isoleucyl-tRNA transferase	Mitochondrial protein synthesis
18	D2030.4	NADH-coQ oxidoreductase subunit	Mitochondrial membrane, multimeric
19	F27D4.1	Electron transfer flavoprotein α-subunit	Mitochondrial matrix, multimeric
20	C54G4.8	Cytochrome c1	Mitochondrial membrane, multimeric
21	F43G9.1	Isocitrate dehydrogenase subunit	Mitochondrial, multimeric
22	K07A12.3	ATP synthase subunit gamma	Mitochondrial membrane, multimeric
23	F25H5.6	Mitochondrial ribosomal subunit L54	Mitochondrial protein synthesis
24	F26E4.6	Cytochrome c oxidase subunit	Mitochondrial membrane, multimeric
25	F26E4.9	Cytochrome c oxidase subunit	Mitochondrial membrane, multimeric
26	F59C6.5	NADH-coQ oxidoreductase subunit	Mitochondrial membrane, multimeric
27	B0511.8	Mitochondrial ribosomal protein S30	Mitochondrial protein synthesis
28	H28O16.1	ATP synthase α-chain (F1F0α)	Mitochondrial membrane, multimeric
29	C01A2.3	Cytochrome oxidase biogenesis;oxa1p homologue	Mitochondrial membrane, protein processing
30	W09C5.8	Cytochrome c oxidase subunit IV	Mitochondrial membrane, multimeric
31	Y92H12BR.8	Mitochondrial ribosomal protein L15	Mitochondrial protein synthesis
32	K11B4.1	Mitochondrial ribosomal protein S27	Mitochondrial protein synthesis

Non-activators, Chr I

No.	Gene name	Description	Class
1	Y71F9B.9	Similar to mitochondrial Hydroxyacyl-coA dehydrogenase	Soluble, homodimer
2	C32E8.9	Enoyl-coA hydratase	Soluble, homoexamer
3	T09B4.8	Mitochondrial alanine-glyoxilate aminotransferase	Soluble, monomeric
4	T08B2.7	Enoyl-coA hydratase	Soluble, homoexamer
5	K06A5.6	Acyl-coA dehydrogenase homologue	Soluble, homotetramer
6	C55B7.4	Acyl-coA dehydrogenase homologue	Soluble, homotetramer
7	T10E9.9	Acyl-coA dehydrogenase homologue	Soluble, homotetramer
8	W02D3.6	ATP-ADP transporter	Membrane, monomeric
9	W02D3.2	Mitochondrial dihydroorotate dehydrogenase	Membrane, monomeric
10	C37A2.3	Acyl-coA dehydrogenase homologue	Soluble, homotetramer
11	C30F12.7	Isocitrate dehydrogenase subunit	Soluble, multimeric
12	F13G3.7	Mitochondrial carrier	Membrane, monomeric
13	T25G3.4	Mitochondrial glycerol 3-phosphate dehydrogenase homologue	Soluble, homodimer
14	F27D4.5	Mitochondrial 2-oxoisovalerate dehydrogenase beta subunit	Soluble, multimeric
15	F30F8.2	Mitochondrial glutaminase	Soluble, monomeric
16	F10D11.1	Sod-2	Soluble, homotetramer
17	F43G9.3	Mitochondrial carrier	Membrane, monomeric
18	T05F1.8	Mitochondrial carrier	Membrane, monomeric
19	C34B2.6	Similar to lon proteases	Membrane, proteolysis
20	C34B2.7	Succinate dehydrogenase complex subunit	Membrane, multimeric
21	T26E3.7	ATP synthase alpha chain	Membrane, multimeric
22	F39B2.11	Metataxin homolog	Membrane, protein import
23	Y71F9B.9	Similar to mitochondrial hydroxyacyl-coA dehydrogenase	Soluble, homodimer
24	C32E8.9	Enoyl-coA hydratase	Soluble, homohexamer
25	T09B4.8	Mitochondrial alanine-glyoxilate aminotransferase	Soluble, monomeric
26	T08B2.7	Enoyl-coA hydratase	Soluble, homohexamer
27	K06A5.6	Acyl-coA dehydrogenase homologue	Soluble, homotetramer
28	C55B7.4	Acyl-coA dehydrogenase homologue	Soluble, homotetramer
29	T10E9.9	Acyl-coA dehydrogenase homologue	Soluble, homotetramer
30	W02D3.6	ATP-ADP transporter	Membrane, monomeric

Chr III

No.	Gene name	Description	Class
1	F54h12.1a, *aco-2*	Mitochondrial aconitase	Soluble, monomeric
2	F20H11.3, *mdh-1*	Mitochondrial malate dehydrogenase	Soluble, homodimer/tetramer
3	H14A12.2a, *fum-1*	Mitochondrial fumarase	Soluble, homotetramer
4	T20G5.2, *cit-1*	Mitochondrial citrate synthase	Soluble, monomeric

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).

References for this Table can be found in the supplemental data (see Table S1; http:// jcs. biologists. org/ supplemental/).

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 RNAi 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 coordinately upregulate 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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