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Ubiquitin-Like Protein 5 Positively Regulates Chaperone Gene Expression in the Mitochondrial Unfolded Protein Response

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ABSTRACT

Perturbation of the protein-folding environment in the mitochondrial matrix selectively upregulates the expression of nuclear genes encoding mitochondrial chaperones. To identify components of the signal transduction pathway(s) mediating this mitochondrial unfolded protein response (UPR^{mt}), we first isolated a temperature-sensitive mutation (*zc32*) that conditionally activates the UPR^{mt} in *C. elegans* and subsequently searched for suppressors by systematic inactivation of genes. RNAi of *ubl-5*, a gene encoding a ubiquitin-like protein, suppresses activation of the UPR^{mt} markers *hsp-60::gfp* and *hsp-6::gfp* by the *zc32* mutation and by other manipulations that promote mitochondrial protein misfolding. *ubl-5* (RNAi) inhibits the induction of endogenous mitochondrial chaperone encoding genes *hsp-60* and *hsp-6* and compromises the ability of animals to cope with mitochondrial stress. Mitochondrial morphology and assembly of multi-subunit mitochondrial complexes of biotinylated proteins are also perturbed in *ubl-5*(RNAi) worms, indicating that UBL-5 also counteracts physiological levels of mitochondrial stress. Induction of mitochondrial stress promotes accumulation of GFP-tagged UBL-5 in nuclei of transgenic worms, suggesting that UBL-5 effects a nuclear step required for mounting a response to the threat of mitochondrial protein misfolding.

PROTEIN chaperones are essential to the biogenesis of newly synthesized proteins and to the degradation of misfolded and mis-assembled proteins (Bukau and Horwich 1998; Horwich *et al.* 1999; Hartl and Hayer-Hartl 2002). Homeostasis requires a balance between the load of newly synthesized unfolded proteins or existing misfolded proteins and the levels of chaperones in the various cellular compartments. This balance is maintained by signal transduction pathways that respond to fluctuations in unfolded and misfolded protein load and appropriately activate genes encoding chaperones targeted to the stressed compartment. Such pathways are referred to as unfolded protein responses (UPR).

First identified in bacteria exposed to elevated temperatures, signaling pathways responsive to unfolded/misfolded proteins were subsequently recognized in the cytosol of all cells where they came to be known as the heat-shock response (Lindquist and Craig 1988). The effectors of activated gene expression in the heat-shock response are well characterized. In bacteria a specific σ-factor, σ32, is stabilized by the accumulation of misfolded cytoplasmic proteins, an event that correlates with reduced binding of σ32 to chaperones. As a consequence, RNA polymerase is directed to the promoters of genes encoding chaperones whose enhanced expression restores balance to the protein-folding environment in the cytosol (Bukau 1993; Biaszczak *et al.* 1999). In eukaryotes, specific transcription factors, heat-shock factors, are activated by the accumulation of unfolded/misfolded proteins in the cytosol (Wu 1995; Cotto and Morimoto 1999) and these bind to and activate hundreds of genes involved in adapting to the stress of protein misfolding in the eukaryotic cytosol (Murray *et al.* 2004).

A conceptually similar signal transduction pathway couples the stress of protein misfolding in the endoplasmic reticulum (ER) to activation of a gene expression program upregulating ER chaperones and other components of the secretory machinery. Signaling in this ER unfolded protein response (UPR^{er}) is initiated by ER transmembrane proteins whose lumenal domains sense the compartment-specific stress signal and whose cytoplasmic domains propagate the signal to the nucleus to activate genes that elicit a compartment-specific adaptation (Patil and Walter 2001; Harding *et al.* 2002; Kaufman *et al.* 2002).

The mitochondrial matrix presents a third discrete compartment for protein folding in eukaryotic cells. Most mitochondrial proteins are synthesized in the cytosol and are imported into the organelle across the outer and inner membrane in an unfolded state

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(Neupert 1997). A much smaller number of very abundant proteins are synthesized in the mitochondrial matrix. Compartment-specific chaperones assist in the translocation of cytosolic proteins into the organelle and subsequently in the folding of both imported and locally synthesized proteins (Voos and Rottgers 2002). Many mitochondrial proteins are proteolytically processed and further assembled into multi-subunit functional complexes of defined stoichiometry.

There are two major classes of matrix chaperones: Hsp70 homologs and Hsp60/Hsp10 homologs (of bacterial GroE) (Neupert 1997; Voos and Rottgers 2002). Together with an assortment of proteases (*e.g.*, SPG7, ClpP) (Langer *et al.* 2001) and more specific assembly factors (*e.g.*, Opa1, Prohibitin) (Nijtmans *et al.* 2000), these chaperones constitute a protein-handling machinery that plays an essential role in mitochondrial biogenesis and maintenance. Loss-of-function mutations in mitochondrial chaperones and other components of the aforementioned machinery are associated with a variety of mainly neurological diseases (Lindholm *et al.* 2004). The phenotypic convergence of these mutations and the slowly progressive nature of some of the associated diseases suggest that alterations of the protein-folding environment in the mitochondria might also contribute to sporadic diseases of aging (Beal 2005). This has generated an interest in the mechanisms by which cells cope with protein misfolding in the mitochondria.

Mitochondrial chaperones are encoded by nuclear genes that are selectively activated by genetic and pharmacological manipulations that impair the protein-folding environment in the mitochondrial matrix. Interfering with the expression of the mitochondrial genome, which is predicted to lead to accumulation of unassembled imported precursors of multi-subunit mitochondrial complexes, activates mitochondrial chaperone-encoding genes in *rho*$^{-}$ mammalian cells (Martinus *et al.* 1996). Overexpression of a folding-impaired matrix enzyme, mutant ornithine transcarbamylase, activates genes encoding the mitochondrial chaperones Hsp60/Hsp10 and the protease ClpP in mammalian cells (Zhao *et al.* 2002). In the nematode *Caenorhabditis elegans* inactivation of mitochondrial chaperones, proteases, or assembly factors selectively upregulates *hsp-60* and *hsp-6*, encoding, respectively, mitochondrial Hsp60 and Hsp70 proteins (Yoneda *et al.* 2004). Together these observations point to the existence of a signal transduction pathway that responds to the stress of unfolded/misfolded proteins in the mitochondrial matrix and selectively activates genes encoding mitochondrial chaperones. Here we describe a screen for genes encoding putative components of such a mitochondrial unfolded protein response (UPR^{mt}) and report on the identification of one such gene whose product appears to function at a nuclear step in the response.

MATERIALS AND METHODS

Transgenic and mutant *C. elegans* strains: Strains containing the mitochondrial misfolded protein stress reporters *hsp-6::gfp(zcIs13) V* (SJ4100) and *hsp-60::gfp(zcIs9) V* (SJ4058) and the ER stress reporters *hsp-4::gfp(zcIs4) V* (SJ4005) and *hsp-4::gfp(zcIs4) V; upr-1(zc6) X* (SJ6) have been previously described (Calfon *et al.* 2002; Yoneda *et al.* 2004). The *myo-3::gfp^{mt}* plasmid expressing a mitochondrially localized green fluorescent protein (GFP) (GFP^{mt}) with a cleavable mitochondrial import signal peptide (Labrousse *et al.* 1999; a gift of A. van der Bliek, University of California at Los Angeles) was integrated stably, generating *myo-3::gfp^{mt}(zcIs14)* (SJ4103), and a control line expressing similar levels of cytoplasmic GFP, *myo-3::gfp(zcIs21)*, was created using the same *myo-3* promoter (SJ4157). The *gfp^{mt}*-expressing fragment was excised from *myo-3::gfp^{mt}* and inserted downstream of the *ges-1* promoter to create a stable transgenic line expressing GFP^{mt} in the adult intestinal cell, *ges-1::gfp^{mt}(zcIs17)* (SJ4143). The control line *ges-1::gfp(zcIs18)* (SJ4144), expressing similar levels of cytoplasmic GFP, has been previously described (Urano *et al.* 2002). Strains expressing UBL-5 fused to GFP at the last residue were prepared by amplifying the ~1750-bp genomic region containing the promoter and coding sequence of *C. elegans ubl-5* using the primers F46F11.SalI.3S 5′-ATAAGTC GACTCTCCAGAAGAAACTTCACG-3′ and F46F11.BamHI.4AS 5′-TGATGGATCCTTGGTAGTAGAGCTCGAAATTGAATC-3′ and ligating the fragment into the *Sal*I–*Bam*HI sites of pPD95.77 to create UBL-5.pPD95.77.V1 and injecting N2 animals with the plasmid to create *ubl-5::gfp(zcIs19) X* (SJ4151). In a second strain, *ubl-5^{Cb}::gfp(zcIs22) I (SJ4153)*, the *C. elegans ubl-5* coding sequence was replaced by the synonymous coding sequence of *C. briggsae*; a 463-bp *Bsa*BI–*Sac*I fragment of *C. briggsae* genomic DNA encoding UBL-5 was recovered by PCR using the primers UBL-5.brig.1S (5′-CAAGAAAAATGATCGAAAT CACAGTGAATGATCGTC-3′) and UBL-5.brig.2AS (5′-GCT CATTGATAATAGAGCTCGAAGTTGAATCCC-3′) and used to replace the homologous *C. elegans* fragment to create UBL-5.brig1.pPD.95.77, which was injected into N2 animals. The ability of the transgene to rescue the *ubl-5*(RNAi) phenotype was assessed by two methods: (1) rescue of the growth defect in a hypersensitive *eri-1(mg366) IV* background obtained by crossing the SJ4153 and GR1373 strains and segregating *ubl-5^{Cb}::gfp(zcIs22)* transgenic and nontransgenic *eri-1(mg366) IV* F_2 animals from the cross and (2) rescue of the defect in *hsp-60::gfp* expression in a compound *ubl-5^{Cb}::gfp(zcIs22) I; hsp-60::gfp(zcIs9) V; zc32 II* strain obtained by crossing SJ4153 and SJ52 strains and segregating progeny homozygous at all three loci.

The *zc32 II* mutant strain was isolated following EMS mutagenesis of *hsp-60::gfp(zcIs9)*. The F_1 progeny of mutagenized animals were placed four per plate and their progeny (the F_2 generation) were screened by inverted fluorescent microscopy for induction of the GFP reporter at 25°. The mutation was backcrossed into the N2 background to eliminate the reporter and other adventitial mutations creating the strain (SJ60) and was subsequently reconstituted with the *hsp-60::gfp(zcIs9) V* (SJ52) or the *hsp-6::gfp(zcIs13) V* (SJ71) transgene to be used in the RNA interference (RNAi) screen described below.

RNAi procedures: RNAi of genes on chromosome I was carried out by sequential feeding of an arrayed library of genomic fragments obtained from the United Kingdom Human Genome Mapping Project Resource Center (Cambridge, UK) as described (Fraser *et al.* 2000). Isopropyl-β-D-thiogalactopyranoside (IPTG, 1 mM) was added to the bacterial growth media to induce transcription of the double-stranded RNA. Mock RNAi feeding was conducted with *Escherichia coli*

transformed with the same plasmid, but lacking an insert; they, too, were cultured in the presence of 1 mM IPTG.

cDNA-based RNAi-feeding plasmids directed toward *C. elegans* and *C. briggsae ubl-5* were constructed by reverse transcriptase PCR amplification of the coding sequence from whole-animal RNA. The *C. elegans* primers used were F46F11. Kpn.1S (5′-GGCCGGTACCATGATTGAAATCACAGTAAAC GATC-3′) and F46F11.2AS (5′-GAAATGAATTCTGATGAAT CATTGG-3′) and the *C. briggsae* cDNA was amplified using the same UBL-5.brig.1S and UBL-5.brig.2AS primers described above. The inserts were ligated into the pPD129.36 plasmid.

The screen for genes whose inactivation by RNAi interferes with *hsp-60::gfp* expression was conducted by placing four SJ52 L4 larvae of the *zc32 II; hsp-60::gfp(zcIs9) V* genotype on 60-mm plates seeded with *E. coli* strains carrying an RNAi-feeding plasmid and allowing their offspring to develop at the permissive temperature for 72 hr and then switching the plates to the nonpermissive temperature of 25° and observing, 24 hr later, the effects of the RNAi on growth, viability, fertility, and the expression of the *hsp-60::gfp*. At this point, the plate was populated with both F_1 and developing F_2 progeny of the original four animals, allowing us to score the phenotype across three generations of RNAi exposure.

The timing of exposure to RNAi was varied to manipulate the level of gene inactivation, which enabled us to overcome lethal or growth-suppressive phenotypes associated with complete inactivation. Placing embryonated eggs purified from gravid animals on RNAi plates restricts exposure to the RNAi to the later stages of embryonic development and often bypasses the lethality associated with earlier inactivation of some genes. The phenotype was scored in the F_0 animal. This design was used to study the interactions of *zc32* with RNAi of mitochondrial chaperones (Figure 2) to measure the effect of *ubl-5*(RNAi) on induction of the mitochondrial chaperones (Figure 3, C and D), on mitochondrial morphology in *myo-3::gfp*^{mt} animals (Figure 4A), and on the assembly of protein complexes in the mitochondrial matrix (Figure 5B) and to study the effect of RNAi of mitochondrial chaperones on UBL-5::GFP localization (Figure 6). In other experiments we measured the phenotype by assessing the effect of gene inactivation on the F_1 progeny that develop on RNAi-feeding plates (Figure 3, A and B; Figure 4, B and C).

The potential confounding effects of dilution on the outcome of double RNAi experiments were controlled by similar dilution of single RNAi-expressing bacteria with bacteria transformed with the empty RNAi expression plasmid pPD129.36.

Pharmacological treatment: Ethidium bromide (Sigma, St. Louis) was dissolved in water at 10 mg/ml and added to agar plates to a final concentration of 50 μg/ml and tunicamycin was used at 1 μg/ml as previously described (Calfon *et al.* 2002; Yoneda *et al.* 2004).

Microscopy and image analysis: Low-resolution images of live animals were obtained with an inverted dissecting microscope equipped with epifluorescent illumination (Zeiss Stemi SV 11 Apo) as described (Yoneda *et al.* 2004). Higher-resolution images were obtained by mounting animals on slides using a digital CCD camera attached to a Zeiss Axiophot 100 microscope. Where indicated, the samples were fixed (3.7% paraformaldehyde, 10 min at room temperature followed by 100% methanol, 5 min at −20°) and exposed to a 1:1000 dilution of H33258 dye to stain the nuclei. Images were constituted from composites of digital photographs obtained under identical exposure conditions.

Image analysis (Figure 6) was conducted by calculating the mean pixel intensity over the intestinal nuclei and an equal surface of cytoplasm using Volocity software for Mac OS X (Improvision, Coventry, UK). The measurements are reported as the mean nuclear/cytoplasmic ratio ±SEM of 20 nuclei from five animals of each genotype. Statistical analysis was performed by the unpaired two-tailed Student's *t*-test.

Northern blots, immunoblots, and detection of biotinylated protein by ligand blot: 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* and *hsp-60* were previously described (Yoneda *et al.* 2004). Detection of GFP and BiP (by antibodies reactive with the tetra-peptide: His-Asp-Glu-Leu-COOH, anti-HDEL) by immunoblotting was as previously described (Marciniak *et al.* 2004; Yoneda *et al.* 2004).

Biotinylated mitochondrial proteins were detected following reducing SDS–PAGE and blotting to a nitrocellulose membrane that was reacted with horseradish-peroxidase (HRP)-conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA, catalog no. 016-030-084) and the signal revealed by enhanced chemiluminescence, as previously described (Baumgartner *et al.* 2001). Subcellular fractionation of worm lysates and purification of mitochondria was achieved by equilibrium density centrifugation as described (Jonassen *et al.* 2002). Size fractionation of mitochondrial complexes by velocity gradient centrifugation was achieved by layering the Triton X-100 soluble proteins on a 10–40% v/v glycerol gradient in buffer A (20 mM HEPES, pH 7.9, 50 mM NaCl, 0.1 mM EDTA, 1.5 mM MgCl2), centrifuging the sample for 18 hr at 28,500 × *g* in a Beckman SW50 rotor, and blotting the individual fractions for their content of biotinylated proteins as described above.

RESULTS

Compartment-specific UPRs are predicted to share certain underlying principles. Developmental programs and physiological stimuli drive organelle biogenesis and specify its level of activity and thereby influence the load of unfolded proteins that confront chaperones in a particular compartment. The balance between unfolded/misfolded "client" protein load and available chaperones determines the level of stress in the compartment and the level of signaling in the afferent limb of the compartment-specific UPR (Figure 1A). Signaling in the afferent limb activates target genes encoding chaperones and other components of its efferent limb, restoring homeostasis to the organelle. Reporters linked to target gene promoters can be used to monitor signaling in the afferent limb of a UPR. Activation of the UPR is achieved by compromising the protein-folding environment in the compartment through pharmacological manipulations or by genetic inactivation of UPR effectors. In considering a screen for genes that constitute the afferent limb of a UPR, it is important to keep in mind that signaling may be suppressed by mutations that block signal transduction (interesting mutations) but also by mutations that reduce unfolded/misfolded ("client") protein load (trivial, upstream suppressors).

We had previously established transgenic lines of *C. elegans* containing green fluorescent protein reporters driven by the UPR^{mt}-inducible *hsp-6* and *hsp-60* promoters (Yoneda *et al.* 2004). Inactivation of genes that

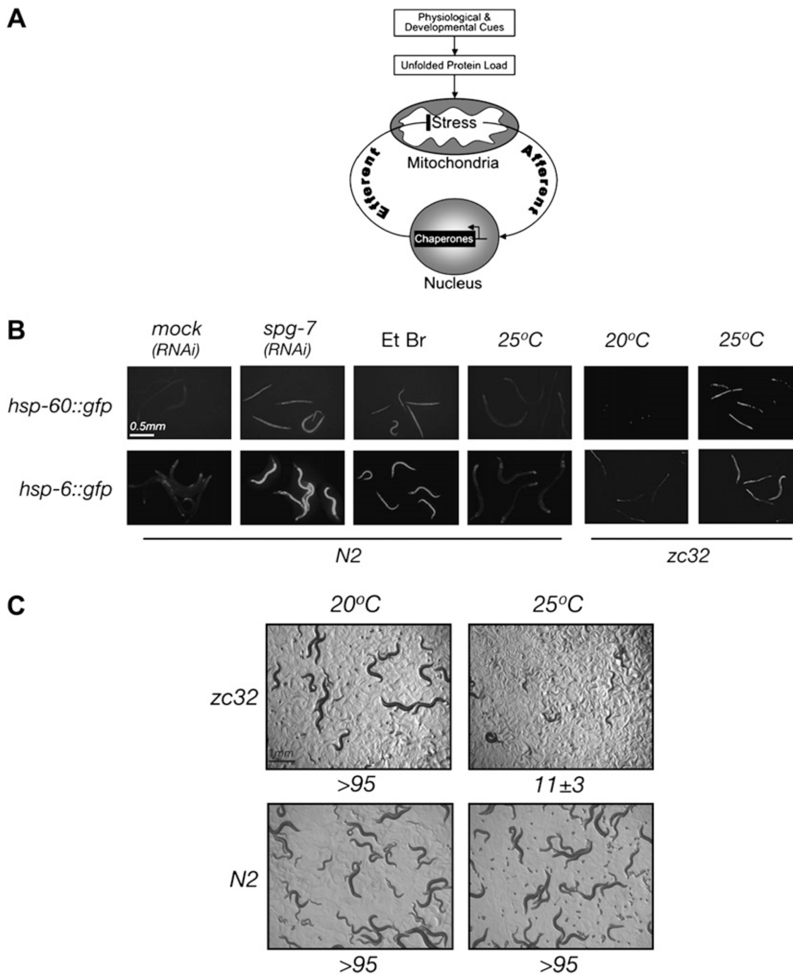

Figure 1.—(A) Hypothesized components of a mitochondrial UPR. Physiological and developmental cues impose an unfolded protein load on the mitochondria. The resultant physiological stress activates the afferent limb of the UPR^{mt}, increasing expression of genes encoding mitochondrial chaperones. The latter serve as the pathway's efferent limb that restores homeostasis to the organelle. A mutation such *zc32* (see below), which activates the afferent limb by causing more mitochondrial stress, may achieve this by compromising the UPR^{mt}'s efferent limb or by imposing an increased burden of unfolded/misfolded proteins. A gene whose inactivation diminishes the activity of the afferent limb (*ubl-5*, see below) may function in propagating the stress signal or may suppress the pathway upstream by diminishing the load of unfolded/misfolded proteins that enter the organelle. (B) Activation of the UPR^{mt} by a temperature-sensitive mutation, *zc32*. Shown are fluorescent micrographs of animals transgenic for a GFP reporter linked to the UPR^{mt}-inducible promoters of the mitochondrial chaperone-encoding genes *hsp-6* and *hsp-60*. Developing wild-type (N2) embryos were exposed to *spg-7*(RNAi) or to ethidium bromide (Et Br), which induce the UPR^{mt}. Mutant *zc32* embryos were allowed to develop to adulthood at the permissive or nonpermissive temperature. (C) Photomicrographs of progeny of wild-type (N2) and *zc32* mutant adults that had developed at the permissive (20°) or nonpermissive (25°) temperature. The mean (±SEM) number of progeny per mutant hermaphrodite reaching the L4 stage is indicated below the photomicrograph.

signal in the UPR^{mt} is predicted to diminish the activity of these *hsp-6::gfp* and *hsp-60::gfp* reporters and serves as a criterion for the identification of components of this signal transduction pathway. However, to recognize animals impaired in signaling the UPR^{mt}, the pathway needs to be robustly activated in the first place. The UPR^{mt} can be activated by RNAi of numerous genes whose products encode mitochondrial chaperones, proteases, and even components of the mitochondrial gene expression program (Yoneda *et al.* 2004; Hamilton *et al.* 2005), but the toxicity of such manipulations and the incomplete penetrance of RNAi limit the utility of this approach in wide-scale genetic screens. Therefore, as a first step toward a screen for genes that function in the UPR^{mt} we sought to identify a mutation that activates the pathway.

Isolating a mutation that conditionally activates the UPR^{mt}: The *hsp-60::gfp* reporter has a faint basal activity but is robustly and specifically induced by manipulations that activate the UPR^{mt} (Yoneda *et al.* 2004) (Figure 1B). We screened F_2 progeny of EMS-mutagenized *hsp-60::gfp*(*zcIs9*) transgenic worms for individuals with constitutive activity of the reporter. Such individuals were readily identified (250 of 7500 haploid genomes screened), an observation consistent with the large number of (likely nuclear) genes required for mitochondrial homeostasis (Table I in Yoneda *et al.* 2004). However, none of the constitutive UPR^{mt}-active animals could be propagated as a mutant line. The latter observation is consistent with the requirement for mitochondrial integrity in development of the *C. elegans* germline (Tsang *et al.* 2001; Artal-Sanz *et al.* 2003).

A screen for temperature-sensitive (ts) mutations that conditionally activated the UPR^{mt} yielded a line, *zc32*, which robustly activated *hsp-60::gfp* and *hsp-6::gfp* at the nonpermissive temperature (25°) and weakly activated the reporters at the permissive temperature (20°) (Figure 1B). Larvae that hatched from mutant eggs placed at the nonpermissive temperature developed into slightly uncoordinated, *hsp-60::gfp*-active adults with a compromised germline and very few progeny (Figure 1C); the latter is consistent with the detrimental role of mitochondrial stress on germline development. The ts mutation of *zc32* segregated as simple Mendelian recessive mapping to a small region on chromosome II flanked by the markers R12C12 (−0.63 cM) and C56E9 (−0.17 cM) (see supplemental data at http://www.genetics.org/supplemental/). The phenotype was also

uncovered by placing the mutant allele in *trans* to a deficiency (mnDf96, CGC strain SP788), suggesting that the mutation has significant loss-of-function features. The interval in question contains three genes whose products are predicted to function in mitochondria; however, the coding sequence of all three was identical in *zc32* and N2 animals. Furthermore, RNAi inactivation of none of the 116 predicted genes in the interval phenocopied the mutation in wild-type animals nor did such RNAi affect the mutant phenotype of *zc32* animals.

As the identity of the gene(s) responsible for the phenotype of *zc32* remained obscure, we sought further evidence that the mutation selectively compromises mitochondrial function. Therefore, we compared the ability of *zc32* animals to cope with mitochondrial stress or ER stress (as a reference) by inactivating genes whose products are important for either mitochondrial or ER homeostasis. At the permissive temperature, both wild-type and *zc32* larvae developed into adult animals, whether exposed to *spg-7*(RNAi) (a mitochondrial protease), *hsp-60*(RNAi) (a mitochondrial chaperone), *phb-2*(RNAi) (a mitochondrial membrane protein assembly factor), or *ire-1*(RNAi) (a component of the UPR^{er}, whose inactivation causes ER stress). At the nonpermissive temperature, *zc32* mutant animals were selectively impaired in their ability to develop under RNAi that induces further mitochondrial stress, but their ability to cope with ER stress was comparable to the wild type (Figure 2). The above observations present a genetic argument that *zc32* animals experience mitochondrial stress at the nonpermissive temperature and that they might be useful tools in identifying genes required for signaling the UPR^{mt}.

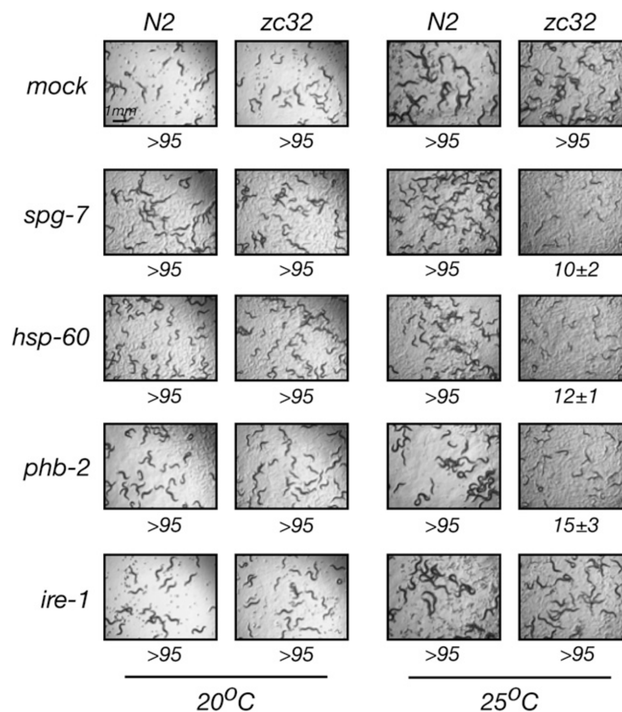

FIGURE 2.—The *zc32* mutation compromises the ability to cope with further mitochondrial stress. Shown are photomicrographs of plates with wild-type (N2) and *zc32* mutant animals that developed at the permissive (20°) and nonpermissive temperature (25°) from embryos exposed to mock RNAi or RNAi of genes that induce further mitochondrial stress (*spg-7*, *phb-2*, and *hsp-60*) or ER stress (*ire-1*). Photographs were taken 84 hr after seeding the RNAi plates with embryonated eggs. The fraction of animals that reached developmental stage ≥L4 (mean ±SEM) is indicated below the images.

***ubl-5*, a gene that functions in the UPR^{mt}:** Using an arrayed RNAi library (KAMATH *et al.* 2003), we sequentially inactivated annotated genes in the *C. elegans* genome in *zc32* animals, searching for genes whose RNAi inhibited the *hsp-60::gfp* reporter transgene at the nonpermissive temperature. To minimize the predicted negative affect of UPR^{mt} inactivation on development and fertility of *zc32* animals, we allowed embryonic development of the RNAi animals to proceed at the permissive temperature and switched the plates to the nonpermissive temperature for the final 24 hr before observing the animals. To identify the nonspecific effects of a given RNAi clone on animal health or reporter gene activity, we monitored, in parallel, the effects of RNAi on animals with a mutation, *upr-1(zc6) X*, that activates the UPR^{er} reporter *hsp-4::gfp* (CALFON *et al.* 2002). We sought genes whose RNAi selectively impaired the health of *zc32* animals while selectively diminishing *hsp-60::gfp* activity.

A systematic RNAi survey of all (2445) available genes on chromosome I identified numerous genes whose inactivation selectively impaired the health of *zc32* animals. All but one of these were associated with activation of the *hsp-60::gfp* reporter. As expected, most such genes encode known mitochondrial proteins whose inactivation promotes further mitochondrial misfolded protein stress (*i.e.*, they encode chaperones, proteases, subunits of protein complexes, or components of the mitochondrial transcription or translational apparatus) (YONEDA *et al.* 2004; HAMILTON *et al.* 2005). The selective negative effect of their inactivation on the health of *zc32* animals is readily explained by inability of the mutant animals to cope with further mitochondrial misfolded protein stress (Figure 2). RNAi of one gene, *lin-35*, reproducibly attenuated *hsp-60::gfp* expression but did not selectively compromise the health of *zc32* animals, suggesting that the encoded protein, a *C. elegans* homolog of the retinoblastoma gene product, is not implicated in the endogenous UPR^{mt}.

Inactivation of one gene, *ubl-5*, was associated both with decreased expression of the UPR^{mt} reporter genes (Figure 3A) and with impaired development of *zc32* animals (Figure 3B). The RNAi clone used in this screen contained a relatively large genomic fragment; therefore, to confirm that the phenotype observed was due to inactivation of *ubl-5* (and not other genes on the same

DNA fragment), we constructed a cDNA-based RNAi-feeding plasmid that contained only the coding region of *ubl-5*, which produced the same phenotype as the original genomic RNAi clone (Figure 3B, left). We also confirmed by Northern blotting the degradation of endogenous *ubl-5* mRNA in the *ubl-5*(RNAi) animals (data not shown).

To examine the scope of *ubl-5*'s role in the UPR^{mt}, we compared the activity of *hsp-60::gfp* in wild-type and *ubl-5*(RNAi) animals in which mitochondrial stress had been induced by inactivation of the mitochondrial chaperone HSP-60, protein assembly factor PHB-2, or the mitochondrial protease SPG-7. In each case, *ubl-5* (RNAi) diminished the expression of the *hsp-60::gfp* reporter, as measured by GFP immunoblot (Figure 3C). Furthermore, *ubl-5*(RNAi) also lowered levels of stress-induced expression of endogenous *hsp-6* and *hsp-60* mRNA, an effect that was evident at both 72 and 96 hr after hatching in animals developing on *ubl-5*(RNAi) plates (Figure 3D).

To determine if the inhibitory effects of *ubl-5*(RNAi) on the UPR^{mt} are a consequence of diminished expression of functional UBL-5, we sought to rescue the RNAi phenotype using a transgene resistant to the RNAi effects of *C. elegans ubl-5*. To this end, we exploited the fact that the homologous gene from *C. briggsae* encodes an identical protein; however, sequence divergence at the nucleic acid level was predicted to negate the effects of heterologous RNAi. This notion was supported by the observation that RNAi of *C. briggsae ubl-5* had no phenotype in *C. elegans* (Figure 3, A and B, right). A UBL-5:: GFP-expressing transgene was constructed using the *C. briggsae ubl-5* coding sequence (designated *ubl-5*^{Cb}*::gfp*). The *C. briggsae* transgene was immune to the *C. elegans* coding sequence *ubl-5*(RNAi), whereas expression of the synonymous *C. elegans*-based transgene *ubl-5*^{Ce}*::gfp* was inhibited in tissues susceptible to RNAi effects (supplemental Figure S1A at http://www.genetics.org/supplemental/).

To determine if UBL-5^{Cb}-GFP is a functional protein, we examined its ability to rescue the phenotype

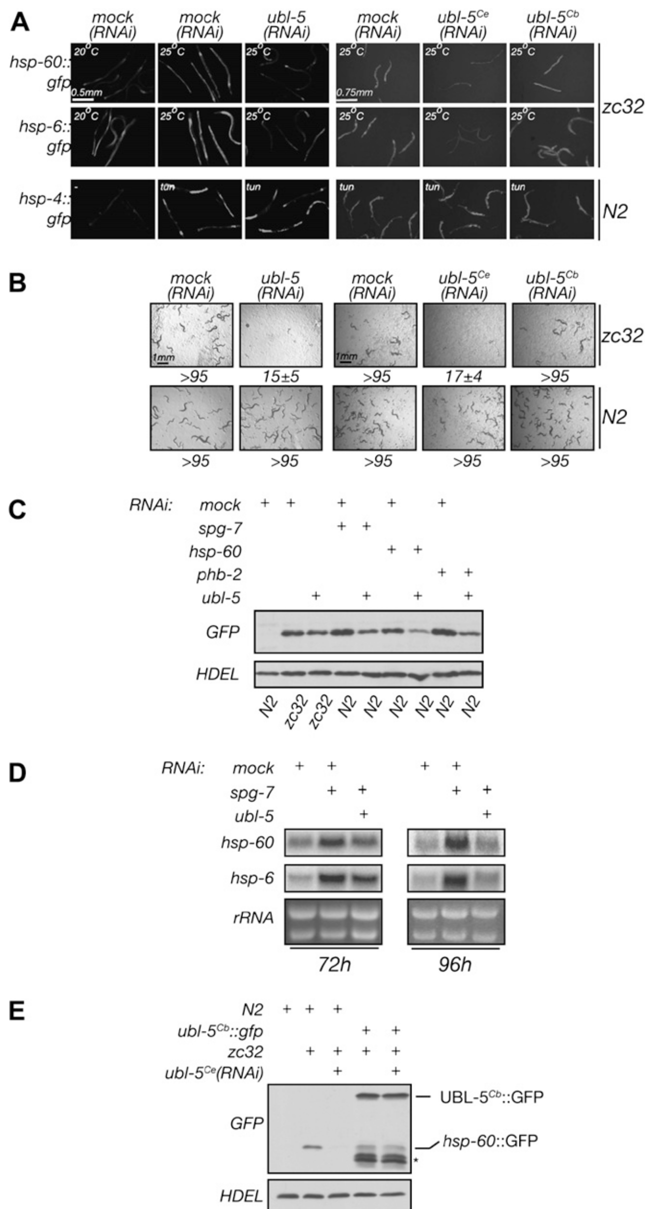

FIGURE 3.—Inactivation of *ubl-5* compromises signaling in the UPR^{mt}. (A) Fluorescent photomicrographs of wild-type (N2) and *zc32* mutant animals transgenic for the UPR^{mt} reporters *hsp-6::gfp* and *hsp-60::gfp* or for the UPR^{er} reporter *hsp-4::gfp*. Animals developed from founders placed on mock RNAi or *ubl-5*(RNAi) plates at 20° and were shifted, where indicated, to the nonpermissive temperature (25°) for 24 hr before analysis. The animals in the left panels were exposed to genomic-based RNAi whereas those on the right were exposed to cDNA-based RNAi constructs derived from *C. elegans* mRNA (*Ce*) or *C. briggsae* mRNA (*Cb*). Where indicated, the *hsp-4::gfp* reporter was activated by exposure to the ER-stress-inducing drug tunicamycin [note that *ubl-5*(RNAi) does not affect the induction of *hsp-4::gfp*]. (B) Photomicrographs of progeny of wild-type (N2) or *zc32* founder animals that developed on mock or *ubl-5*(RNAi) plates maintained for 16 hr at the permissive temperature of 20° (to allow egg laying by the founder) and shifted to the nonpermissive temperature of 25° for 3 days. The number of F_1 progeny that reached developmental stage ≥L4 (mean ±SEM)/F_0 hermaphrodite is indicated below the images. (C) Immunoblot of GFP reporter in *hsp-60::gfp* transgenic wild-type, *zc32* mutant, or animals exposed to the indicated mitochondrial stress-inducing RNAi in the presence or absence of *ubl-5*(RNAi). The anti-HDEL blot, which detects *C. elegans* BiP, serves as a loading control. (D) Northern blot of endogenous *hsp-60* and *hsp-6* RNA in animals that developed on mock RNAi, *spg-7*(RNAi) (to induce mitochondrial stress), and a combination of *spg-7*(RNAi) and *ubl-5*(RNAi). Samples were processed 72 and 96 hr after seeding the RNAi plates with eggs. Ribosomal RNAs stained with ethidium bromide serve as a loading control. (E) Immunoblot of GFP, as in C. The GFP protein encoded by the *hsp-60::gfp(zcIs9)* transgenic UPR^{mt} reporter (hsp-60::GFP) and the UBL-5Cb::GFP fusion protein encoded by the rescuing transgenic allele of *ubl-5*^{Cb}*::gfp(zcIs22)* are indicated. The asterisk marks an immunoreactive protein fragment found in *ubl-5*^{Cb}*::gfp(zcIs22)* animals, which is likely an *in vitro* proteolytic fragment of the fusion protein. The anti-HDEL blot, which detects *C. elegans* BiP, serves as a loading control.

associated with loss of endogenous UBL-5 expression; the marked decrease in fertility of *ubl-5*(RNAi) observed in hypersensitive *eri-1 (mg366) IV* mutant animals maintained at 20° was corrected by the *ubl-5^{Cb}::gfp* transgene (supplemental Figure 1B at http://www.genetics.org/supplemental/). Next we determined if UBL-5^{Cb}::GFP can rescue the defect in the UPR^{mt} imposed by *C. elegans ubl-5*(RNAi). We bred the rescuing transgene into a background containing the *zc32* mutation (to activate the UPR^{mt}) and the *hsp-60::gfp* transgene (to report on the activation). The GFP protein expressed by the reporter transgene is smaller than the UBL-5-GFP fusion protein expressed from the rescuing transgene and the two are readily distinguished by their mobility on SDS–PAGE. *C. elegans ubl-5*(RNAi) markedly diminished the expression of the reporter in N2 animals, as noted above (Figure 3C); however, the inhibitory effect of *ubl-5*(RNAi) on the UPR^{mt} was rescued by the UBL-5^{Cb}-GFP transgene (Figure 3E). These experiments implicate UBL-5 protein in the UPR^{mt}.

***ubl-5*(RNAi) adversely affects the protein-folding environment in the mitochondria:** While the observations noted above indicate that *ubl-5*(RNAi) attenuates signaling in the UPR^{mt}, they do not identify the step in the process at which the gene acts. If UBL-5 is active in the UPR^{mt} afferent limb, *ubl-5*(RNAi) is predicted to enhance mitochondrial misfolded protein stress and to promote intrinsic defects in mitochondrial function and structure. However, if *ubl-5*(RNAi) suppresses the UPR^{mt} upstream by reducing the load of unfolded/misfolded proteins in the mitochondrial matrix, the mitochondria of *ubl-5*(RNAi) animals would not be expected to exhibit intrinsic defects in function or structure (see Figure 1A).

To address this issue, we began by examining mitochondrial morphology, making use of *myo-3::gfp^{mt}* transgenic animals whose body-wall muscles express GFP tagged with a mitochondrial import signal (GFP^{mt}). In wild-type *myo-3::gfp^{mt}* transgenic animals, the GFP^{mt} signal presents a pattern of neatly stacked arrays of mitochondria aligned with the muscle fibers (Labrousse *et al.* 1999) (Figure 4A). The mitochondria of *spg-7*(RNAi), *hsp-60*(RNAi), and *phb-2*(RNAi) animals are thicker and often fragmented and have brighter GFP^{mt} signals than animals exposed to mock RNAi treatment or to *ire-1*(RNAi) (which, by causing ER stress, serves as a control for nonspecific stressful effects of mitochondrial perturbations). These findings are consistent with the hypothesis that inactivation of genes required for protein folding and assembly in the mitochondrial matrix indirectly alters organelle morphology (although a direct role for *hsp-60, spg-7,* and *phb-2* in maintaining mitochondrial morphology cannot be excluded). Importantly, animals raised on *ubl-5*(RNAi) have a GFP^{mt} pattern that resembles mitochondrially stressed animals (Figure 4A). The latter observation is consistent with compromised mitochondrial homeostasis in the *ubl-5*(RNAi) animals and does not support upstream suppression of the UPR^{mt} by *ubl-5* inactivation.

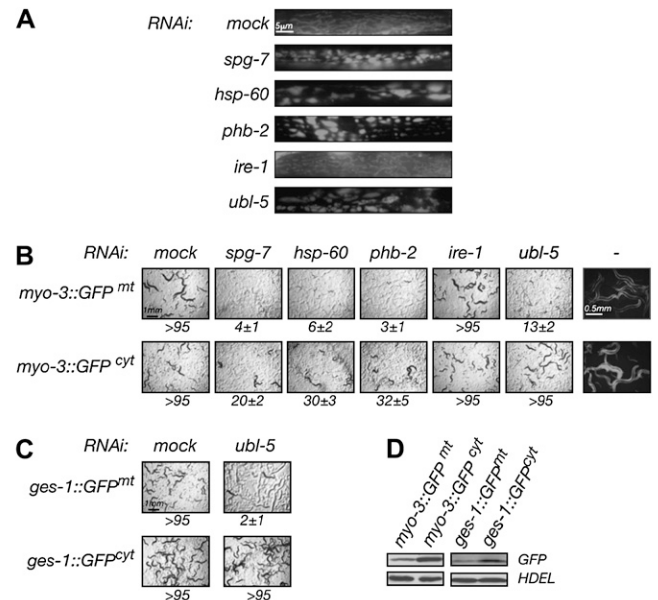

Figure 4.—Inactivation of *ubl-5* selectively compromises an animal's ability to cope with mitochondrial stress. (A) High-magnification fluorescent photomicrographs of mitochondria labeled with a GFP with a cleavable mitochondrial import signal (GFP^{mt}) expressed from a *myo-3::gfp^{mt}* transgene. Animals developed to adulthood from embryos exposed to the indicated RNAi. Note the irregular morphology of the mitochondria in animals exposed to RNAi that induces mitochondrial stress (*spg-7, hsp-60, phb-2*) or inactivates *ubl-5* and the normal morphology of the mock and *ire-1*(RNAi) animals. (B) Photomicrographs of plates populated by progeny (F_1) of *myo-3::gfp^{mt}* or *myo-3::gfp^{cyt}* animals subjected to the indicated RNAi. The F_0 animals had been removed from the plate. The number of F_1 progeny that reached a developmental stage ≥L4 (mean ±SEM)/F_0 hermaphrodite ($n = 4$) is indicated below the images. The panels at the far right are fluorescent photomicrographs of transgenic animals. (C) Photomicrographs of progeny produced by the F_1 generation of mock and *ubl-5*(RNAi) transgenic animals expressing GFP in mitochondria (*ges-1::gfp^{mt}*) or cytoplasm (*ges-1::gfp^{cyt}*) of the intestinal cells. (D) Immunoblot of GFP and the anti-HDEL loading control in test and control pairs of *myo-3::gfp^{mt}* and *myo-3::gfp^{cyt}* or *ges-1::gfp^{mt}* and *ges-1::gfp^{cyt}* transgenic animals.

Whereas adult N2 animals that developed from larvae raised on *ubl-5*(RNAi) had a roughly normal brood size, *zc32* embryos raised to adulthood at the nonpermissive temperature on *ubl-5*(RNAi) plates had very few progeny (Figure 3B). To determine if these negative interactions of *ubl-5*(RNAi) extend to other conditions associated with enhanced mitochondrial stress, we took advantage of the observation that high-level expression of mitochondrially targeted GFP (*myo-3::gfp^{mt}*) markedly attenuates an animal's ability to cope with further mitochondrial stress whereas expression of even higher levels of cytoplasmic GFP (*myo-3::gfp^{cyt}*) is less perturbing (Figure 4B). These observations are consistent with the fact that GFP is slow to fold and requires significant assistance by chaperones (Wang *et al.* 2002). Thus,

evidence of a selective negative interaction with a transgene expressing GFP^{mt} suggests that a given genetic lesion is associated with mitochondrial stress. We found that *ubl-5*(RNAi) adversely affected growth and development of *myo-3::gfp^{mt}* animals but had no such negative effect on *myo-3::gfp^{cyt}* animals expressing GFP in the cytosol (Figure 4, B and D). Furthermore, *ubl-5*(RNAi) compromised fertility of *ges-1::gfp^{mt}* animals expressing GFP^{mt} in their intestinal cells, but had no such negative effect on *ges-1::gfp^{cyt}* animals expressing higher levels of cytosolic GFP (Figure 4, C and D).

To further examine the effect of *ubl-5* inactivation on the protein-folding environment in the mitochondrial matrix, we followed the fate of marker mitochondrial proteins that normally assemble into multi-subunit complexes in the mitochondrial matrix. As antisera to *C. elegans* mitochondrial proteins were not available, we exploited the fact that some abundant mitochondrial proteins are naturally tagged with a covalently bound biotin moiety and can be detected by their reactivity to enzyme-linked streptavidin following denaturing SDS–PAGE and ligand blotting. In mammalian mitochondria, the α-subunits of 3-methylcrotonyl-CoA carboxylase (αMCC) and propionyl-CoA carboxylase (αPCC) account for most of the biotinylated proteins detected by this ligand-blot assay (Baumgartner *et al.* 2001). In *C. elegans*, too, a ligand blot using HRP-linked strepavidin yielded a major labeled species of ~75 kDa, consistent with the predicted size of αPCC (F27D9.5, predicted 79.7 KDa), αMCC (F32B6.2, predicted 73.7 kDa), or both. The biotinylated species were enriched in the mitochondrial fraction (Figure 5A) and were distinct in size from the major *E. coli* biotinylated proteins detected by the same assay (data not shown) (as worms are fed *E. coli*, it was important to deal with the potentially confounding effects of contaminating bacterial biotinylated proteins on this assay).

Both αPCC and αMCC assemble into high-molecular-weight complexes in the mitochondrial matrix (Chloupkova *et al.* 2000), and velocity gradient centrifugation showed a peak of biotinylated proteins with a predicted mass of ~800 kDa, consistent with the expected mass of a dodecamer of α- and β-subunits of MCC or PCC (Figure 5B, top). The peak of biotinylated protein was shifted toward lighter fractions in animals in which mitochondrial protein folding was compromised by *hsp-60*(RNAi) and a similar shift was also observed in *myo-3::gfp^{mt} animals*, consistent with impaired complex formation or turnover in stressed animals. In unstressed animals, *ubl-5*(RNAi) did not markedly affect the distribution of biotinylated proteins in the gradient (although a trend toward a lighter peak was noted; data not shown). However, compounding the stress-inducing *myo-3::gfp^{mt}* transgene with *ubl-5*(RNAi) shifted the peak to lighter fractions (Figure 5B, bottom-most panel), indicating that loss of UBL-5 adversely affects the protein-folding environment in mitochondrially stressed worms. These experiments support the hypothesis that *ubl-5*(RNAi) leads to a state of enhanced mitochondrial misfolded protein stress by compromising the counteractive response to such stress.

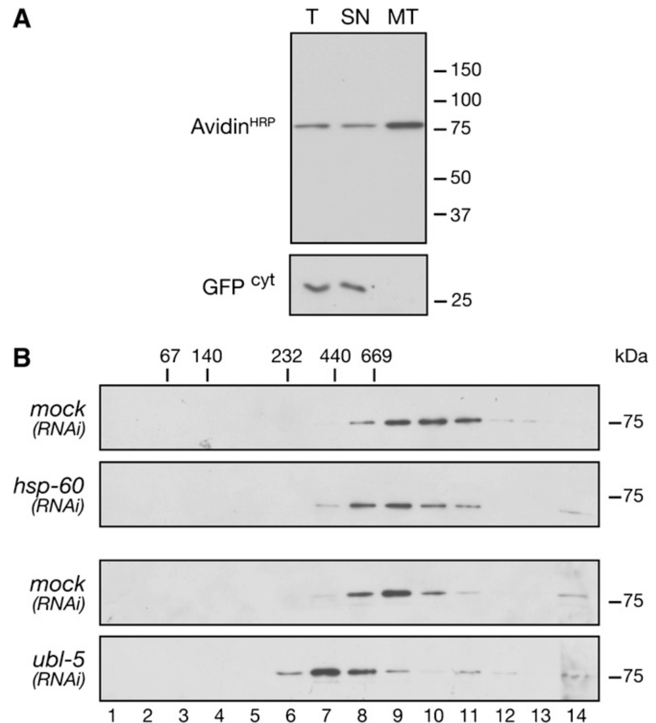

Figure 5.—Inactivation of *ubl-5* perturbs formation of protein complexes in the mitochondria. (A) Detection of an ~75-kDa biotinylated protein(s) in fractionated worm extracts by strepavidin–HRP ligand blotting. Total worm extract (T), postmitochondrial supernatant (SN), and mitochondrial pellet (MT) are shown. GFP^{cyt} expressed from the *myo-3* promoter serves as a marker for a cytoplasmic protein. (B) Distribution of an ~75-kDa biotinylated mitochondrial protein(s) in fractions of glycerol gradient prepared from animals exposed to mock RNAi, *hsp-60*(RNAi), and *ubl-5*(RNAi). (Top) Unstressed animals. (Bottom) Mitochondrially stressed *myo-3::gfp^{mt}* animals. The migration of complexes of the indicated size in this gradient is shown. The final fraction (14) also contains the pellet.

Mitochondrial stress is associated with increased levels of UBL-5 in the nucleus: To gain further insight into how UBL-5 might contribute to signaling in the UPR^{mt}, we sought to localize the protein in unstressed and stressed animals. Unlike other ubiquitin-like proteins whose C termini engage in isopeptide bond formation with various targets, UBL-5 remains covalently unattached (Lüders *et al.* 2003). A high-resolution NMR structure of human UBL-5 indicates that despite a high level of overall structural similarity to ubiquitin and SUMO, the C terminus of UBL-5 is unavailable for isopeptide bond formation (McNally *et al.* 2003). Furthermore, the presence of an uncleaved C-terminal epitope tag on UBL-5 is compatible with rescue of the lethal phenotype associated with deletion of the endogenous gene in *Schizosaccharoumyces pombe* (Yashiroda

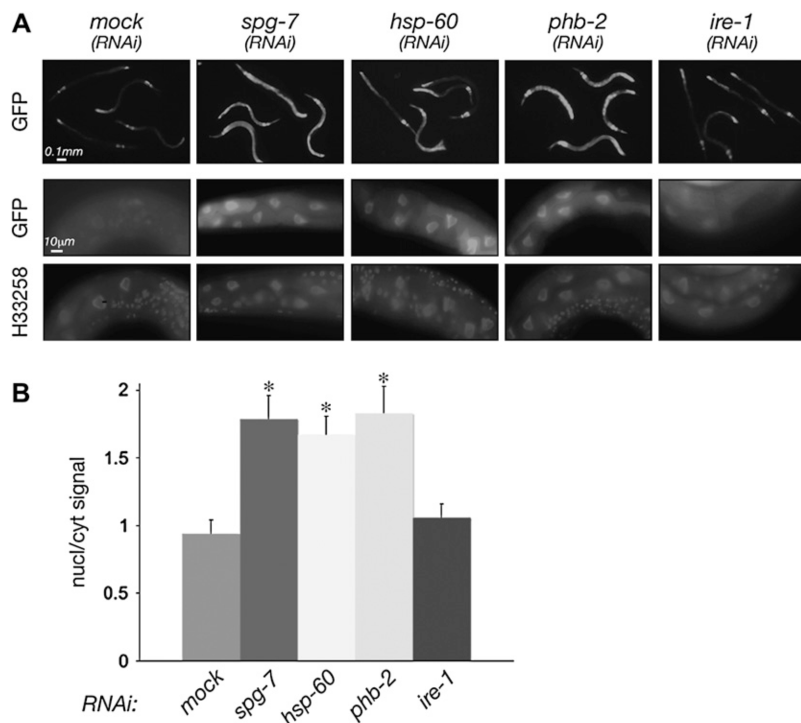

FIGURE 6.—The stress of protein misfolding in the mitochondrial matrix promotes nuclear localization of UBL-5::GFP. (A) Fluorescent photomicrographs of *ubl-5::gfp* transgenic animals that developed from larvae while exposed to the indicated RNAi. Shown are low magnification (top) and high-magnification (bottom) fluorescent micrographs of the GFP channel and the H33258 nuclear stain. Note that the large polyploid intestinal nuclei stain brightly with H33258 in all samples and with UBL-5::GFP in the stressed samples. (B) The relative intensity of the cytosolic and nuclear UBL–GFP signal in animals subjected to the indicated genetic manipulations. The mean ±SEM ratio of signal from the nuclear and cytosolic regions of the intestinal cell is plotted ($N = 20$, * $P < 0.05$, unpaired two-tailed Student's *t*-test, compared with mock sample).

and TANAKA 2004). Therefore, we studied transgenic animals in which UBL-5 protein, expressed from its endogenous promoter, was tagged by GFP at the C terminus, *ubl-5::gfp*.

Several integrated transgenic lines of *ubl-5::gfp* were produced and they exhibited similar distribution of the transgene reporter. UBL-5::GFP was expressed at low levels in most tissues of the adult, with slightly more conspicuous staining in the intestine, especially its posterior segment, and in the pharynx. Viewed at high magnification, the UBL-5::GFP signal appeared to be distributed throughout the intestinal cell (Figure 6A). Mitochondrial stress, induced by *spg-7*(RNAi), *hsp-60* (RNAi), or *phb-2*(RNAi), increased reporter levels, whereas induction of ER stress by *ire-1*(RNAi) did not (Figure 6A). In the mitochondrially stressed animals, the UBL-5::GFP signal was notably concentrated in the nuclei, as revealed by the overlap between the GFP signal and the H33258 DNA stain (Figure 6A) and by measurements of the ratio of the nuclear-to-cytosolic fluorescence in individual intestinal cells (Figure 6B). These stress-dependent changes in UBL-5::GFP expression were most conspicuous in intestinal cells in which *hsp-60* and *hsp-6* activation by mitochondrial stress is also most apparent (YONEDA *et al.* 2004). These observations are consistent with UBL-5 acting at a nuclear step of the UPR^{mt}.

DISCUSSION

Using an unbiased method of RNAi-mediated sequential gene inactivation, we screened *C. elegans* chromosome I for genes whose loss of function interferes with the UPR^{mt}. Inactivation of *ubl-5*, a gene encoding a small ubiquitin-like protein, was discovered to attenuate both UPR^{mt} reporters (*hsp-60::gfp*, *hsp-6::gfp*) and endogenous target genes of the response. Genetic, morphological, and biochemical evidence indicated that *ubl-5*(RNAi) compromises the protein-folding environment in the mitochondria and adversely affects mitochondrial morphology. These observations are most consistent with UBL-5's participation in the afferent limb of the UPR^{mt}, which couples misfolded protein stress to the activation of nuclear-encoded mitochondrial chaperones. Diminished activity of this afferent limb leads to a relative deficiency in chaperone expression and to higher levels of mitochondrial stress in *ubl-5*(RNAi) animals.

Mitochondrial stress leads to accumulation of UBL-5::GFP in nuclei, a finding consistent with a role for the protein in executing a late step in activated gene expression in the UPR^{mt}. The increase in the nuclear UBL-5::GFP signal reflects in part an increase in protein levels, which correlates to transcriptional activation in stressed animals (data not shown). More importantly, mitochondrial stress promotes a net accumulation of the protein in the nucleus (evident after adjusting for differences in UBL-5::GFP protein levels in unstressed and stressed animals). These observations suggest that UBL-5 is a target of both transcriptional and post-transcriptional signals in mitochondrially stressed cells. Components of the analogous UPR^{er} are also regulated both post-transcriptionally and transcriptionally (HARDING *et al.* 2000; CALFON *et al.* 2002), suggesting that this is

a general mechanism for amplifying stress responses. We expect that the identification of other components of the UPR^{mt} will help elucidate the signals that convey UBL-5 to the nucleus in stressed cells.

UBL-5 is a highly conserved protein with homologs in all known eukaryotes. The budding yeast homolog Hub1p has been implicated in polarized morphogenesis, a process that appears to depend on interactions with proteins that function at the bud neck (Dittmar *et al.* 2002). In fission yeast, by contrast, Hub1p is nuclear, forms a complex with the splicing factor Snu66, and plays an essential role in pre-mRNA splicing (Wilkinson *et al.* 2004; Yashiroda and Tanaka 2004). The involvement of UBL-5 in splicing is also consistent with reports of the formation of a complex between the cdc2-like kinases (CLKs) and mammalian UBL-5; CLKs are known to phosphorylate SR proteins that interact with pre-mRNA (Kantham *et al.* 2003).

We did not observe a general defect in pre-mRNA splicing in *ubl-5*(RNAi) animals and RNAi of CLK homologs in *C. elegans* did not phenocopy *ubl-5*(RNAi) (data not shown). These negative results are not definitive; RNAi may be less effective at eliminating gene function than the temperature-sensitive alleles of *S. pombe HUB1* in which the general splicing defect was observed (Wilkinson *et al.* 2004; Yashiroda and Tanaka 2004). Therefore, the nuclear localization of UBL-5::GFP remains consistent with a role for UBL-5 in a regulated splicing event required for activated gene expression in the UPR^{mt}. But it is also possible that UBL-5 is implicated in other aspects of gene expression, such as regulated transcription. The latter possibility is favored by the observations that UBL-5 interacts physically with a transcription factor whose encoding gene is required for signaling the UPR^{mt} in worms (C. M. Haynes, unpublished observations).

A unifying explanation for the diversity of observations made of cells and organisms lacking UBL-5 may not be required, as the protein may contribute to processes lacking a shared biological theme. The observation that *hub1*Δ yeast are respiratory competent suggests that the protein's role in the UPR^{mt} might not be conserved in budding yeast (a species in which a UPR^{mt} has not been demonstrated to exist). Nor do we know if UBL-5's role in the UPR^{mt} is conserved in other metazoans. However, in regard to the latter, it is intriguing to note that *UBL-5* mRNA is relatively abundant in mitochondria-rich human tissues, such as heart, skeletal muscle, liver, and kidney (Friedman *et al.* 2001), and that polymorphisms in *UBL-5* have been linked to the metabolic syndrome of obesity and diabetes (Jowett *et al.* 2004), a syndrome in which mitochondrial homeostasis plays an important role.

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