Mitochondria are the main oxygen consumers in cells and as such are the primary organelle affected by hypoxia. All hypoxia pathology presumably derives from the initial mitochondrial dysfunction. An early event in hypoxic pathology in C. elegans is disruption of mitochondrial proteostasis with induction of the mitochondrial unfolded protein response (UPR\textsuperscript{mt}) and mitochondrial protein aggregation. Here in C. elegans, we screen through RNAs and mutants that confer either strong resistance to hypoxic cell death or strong induction of the UPR\textsuperscript{mt} to determine the relationship between hypoxic cell death, UPR\textsuperscript{mt} activation, and hypoxia-induced mitochondrial protein aggregation (HIMPA). We find that resistance to hypoxic cell death invariably mitigated HIMPA. We also find that UPR\textsuperscript{mt} activation invariably mitigated HIMPA. However, UPR\textsuperscript{mt} activation was neither necessary nor sufficient for resistance to hypoxic cell death and vice versa. We conclude that UPR\textsuperscript{mt} is not necessarily hypoxia protective against cell death but does protect from mitochondrial protein aggregation, one of the early hypoxic pathologies in C. elegans.

Cell Death and Disease (2021)12:711 ; https://doi.org/10.1038/s41419-021-03979-z
Activation of the UPR mt by three RNAis targeting mitochondrial proteins reduced HIMPA and protected from hypoxic death [6]. However, loss of function of atfs-1, a gene which is thought to be essential for UPR mt activation, also decreased HIMPA while atfs-1 gain-of-function mutants increased HIMPA [6]. Thus, whether the UPR mt promotes HIMPA or inhibits it is unclear with the relatively small number of UPR mt activators examined. Here, we make use of the wide variety of C. elegans RNAis and mutants that have been shown to be hypoxia protective, or to induce the UPR mt to address three fundamental questions about the relationship between mitochondrial proteostasis and hypoxic injury. Is activation of the UPR mt a necessary component of hypoxia protective mechanisms? Is UPR mt activation itself hypoxia protective? Does the UPR mt promote or reduce HIMPA?

RESULTS

UPR mt activation not necessary for hypoxia resistance
We have previously performed a forward genome-wide screen in Caenorhabditis elegans for genes, whose inactivation by RNAi conferred a hypoxia resistance phenotype (HypR) and identified about 200 such genes [9]. To explore the hypothesis that activation of the UPR mt might be a common or even invariant component of the mechanism underlying hypoxia resistance of these RNAis, we screened through all the HypR RNAis that we could recover from the library and confirm their identity by sequence. Hundred and sixty-one of the HypR RNAi were confirmed and tested for activation of the mitoUPR under normoxic conditions as assessed by the fluorescence levels of the mitoUPR transcriptional reporter hsp-6::GFP (hsp-6 is the ortholog of human mitochondrial chaperone HSP70 and is a canonical component of the UPR mt) [28–31]. RNAi knockdown of only five genes reproducibly induced the mitoUPR reporter (Fig. 1A). Thus, mitoUPR activation, at least as assessed by the hsp-6 reporter, is by no means necessary for hypoxia resistance. The five RNAis that induced the mitoUPR were against pept-1, sulc-2, C33F10.12, pdha-1, and R04F11.2 (Fig. 1A and Supplementary Table S1). pept-1 encodes an oligopeptide transmembrane transporter, localized to the apical plasma membrane in the intestine [32]. Reduction of function of pept-1 has been found to reduce protein synthesis rates and levels of essential amino acids [5, 33]. A pept-1 loss of function mutant was recently isolated in a screen for hypoxia resistant mutant worms, and its essential role in normal rates of protein synthesis was confirmed [5]. How loss of function of pept-1 activates the UPR mt is unclear although recently the UPR mt has been shown to regulate mitochondrial protein synthesis that would rely on amino acids, whose transport depends on pept-1. On the other hand, sulc-2, C33F10.12, pdha-1, R04F11.2 all encode mitochondrial proteins that form or interact with multi-subunit proteins, where perturbations in stoichiometry have been shown to induce the UPR mt [28, 30, 34]. Inactivation of these five genes by RNAi led to a range of UPR mt reporter induction (Fig. 1B, C), from 2.3 to 13.7-fold that had no correlation with the previously reported levels of hypoxia resistance where pept-1(RNAi) was most resistant and sulc-2(RNAi) least resistant to hypoxia [9]. atfs-1 is a transcription factor required for UPR mt activation [35, 36]. The induction of hsp-6::GFP by the five UPR mt-activating HypR RNAis was fully suppressed by an atfs-1 loss-of-function mutation (Fig. 1D, E), indicating that the induction of hsp-6::GFP by the five Hyp RNAis is acting through the canonical UPR mt pathway.

Hypoxia induces mitochondrial protein aggregation reporter (HIMPA) [6, 26]. We found that inactivation of all five UPR mt-activating HypR genes strongly lowered HIMPA as assessed by the ucr-11::GFP mitochondrial protein aggregation reporter [6] (Fig. 1F, G). As shown previously, atfs-1(lf) also reduced the aggregate levels; only pept-1 (RNAi) significantly reduced aggregates further in the atfs-1(lf) background indicating that at least part of the effect of pept-1(RNAi) is independent of atfs-1 (Fig. 1H). atfs-1(lf) did not suppress the hypoxia resistance of any of the five RNAis; thus, neither atfs-1 function nor UPR mt activation is essential to their mechanisms of hypoxia resistance (Fig. 1I). These results suggest that while resistance to hypoxic cell death and HIMPA may be mechanistically associated and that a functional UPR mt may be critical for aggregate formation, the UPR mt is not required for protection from hypoxia and in general activation of the UPR mt does not appear to commonly accompany a hypoxia-resistant phenotype in C. elegans.

UPR mt activation reduces HIMPA
To examine more broadly the role of the UPR mt in HIMPA, we tested 12 RNAis previously shown to activate the UPR mt (Supplementary Table S2) [37]. We first confirmed that all 12 RNAis did indeed activate the UPR mt as measured by atfs-1-dependent induction of the hsp-6::GFP transcriptional reporter (Fig. 2A, C). All 12 UPR mt-activating RNAis strongly reduced HIMPA (Fig. 2B). Unlike for the pept-1(RNAi), none of the 12 RNAis significantly reduced HIMPA further in the atfs-1(lf) background (Fig. 2D). Again, atfs-1(lf) paradoxically reduced HIMPA in the no RNAi empty vector condition but not to the same degree of reduction as the UPR mt RNAis. The mitochondrial translation inhibitors doxycycline and meclofenicol induce the UPR mt in an atfs-1-dependent manner (Supplementary Fig. S1) [26, 38]. Likewise, doxycycline and meclofenicol reduced HIMPA and did not further reduce the aggregate levels in atfs-1(lf) animals (Fig. 2E). Thus both genetic and pharmacological UPR mt-activators reduce HIMPA, while at the same time the UPR mt master regulator gene atfs-1 paradoxically promotes HIMPA, as previously reported [6].

Activation of UPR mt does not necessarily confer hypoxia resistance
We tested whether the 12 UPR mt-activating RNAis were protective from hypoxic organismal death like the five UPR mt-activating HypR RNAis and like doxycycline and meclofenicol [9, 26, 38]. Five RNAis (letm-1, pdhb-1, timm-17B1, tin-44, and tomm-22) produced significant hypoxia resistance (Fig. 3A–E); five RNAis (cco-1, dnpj-21, mrl-44, ldp-9, and wah-1) showed no significant effect on hypoxic sensitivity (Fig. 3F–J); 2 RNAis (dist-1, F15D3.6) produced hypersensitivity to hypoxia (Fig. 3K, L). Interestingly, unlike for the UPR mt-activating RNAis identified in our screen for strongly hypoxia resistant RNAis (Fig. 1), the relatively weak hypoxia resistance of these UPR mt-activating RNAis was atfs-1-dependent (Fig. 3M–Q). Thus, our data shows that activation of the UPR mt may in some genetic contexts confer atfs-1-dependent mild hypoxia protection, but this is by no means a necessary consequence of UPR mt activation.

TMRE stains mitochondrial aggregates
While examining the effect of hypoxia on mitochondrial membrane potential, we discovered that the voltage sensitive mitochondrial dye tetramethylrhodamine ethyl ester (TMRE) not only stained functional mitochondria, but also stained hypoxia-induced mitochondrial protein aggregates (Fig. 4A–C). This finding in the wild type N2 strain importantly demonstrates that aggregates occur in an animal with native mitochondrial proteins. We then compared the aggregates visualized with TMRE to those visible with the ucr-11::GFP reporter. The TMRE-stained aggregates strongly colocalized with UCR-11::GFP aggregates (Fig. 4D–F). Besides demonstrating importantly that the UCR-11::GFP aggregates are not an artifact of an overexpressed and GFP-tagged protein, TMRE staining can be used in all C. elegans strains to detect mitochondrial aggregates and potentially in other models of hypoxic injury.

HIMPA blocked by hypoxia resistance without UPR mt activation
We have previously identified two distinct mechanisms, the insulin/IGF-receptor pathway and the translation machinery, that
regulate cytoplasmic proteostasis and profoundly modulate hypoxic survival. The insulin/IGF-receptor pathway has been shown to strongly regulate hypoxic sensitivity, aging, and proteostasis in *C. elegans* [12, 23, 39, 40]. *daf-2* encodes the *C. elegans* insulin/IGF receptor, and *daf-2* reduction-of-function mutants are highly resistant to hypoxic injury via a mechanism that requires the FOXO transcription factor DAF-16 [12, 23]. To test whether the *daf-2* pathway affected HIMPA, we built the *daf-2* (e1370; gcl-46[ucr-11p::GFP]) strain, confirmed that it was strongly hypoxia resistant (Fig. 5A), and then scored for HIMPA. *daf-2*
Fig. 1 Only a small subset of hypoxia resistant RNAis activate the UPRmt. A Fluorescence screen of RNAis for UPRmt activation. hsp-6p::GFP induction was quantified for 161 Hyp RNAi clones with the functional categories as originally assigned [9]. Fold induction (mean) of GFP fluorescence is relative to empty vector (EV) RNAi (L4440) (n = 10). Expression of GFP over 2-fold (blue dash line) of empty vector is considered to be positive. Five positive hits confirmed in 2nd screen are shown in red. B Examples of hsp-6p::GFP expression induced by knockdown of the five positive RNAi clones. zcs13(hsp-6p::GFP) worms were placed onto RNAi bacteria from egg and imaged as young adults 3 days later. Bar, 0.1 mm. C Quantification of GFP expression by corresponding RNAis as in B. Data (mean ± SD) were obtained from four independent experiments, n = 30 animals; ***p < 0.001 with pooled data shown and p-values were calculated using unpaired t-test. D Examples of zcs13 [hsp-6p::GFP] GFP expression induced by knockdown of positive five RNAi clones in atfs-1(tm4919) background. Worms were placed onto RNAi bacteria from egg and imaged 3 days later. E Quantification of GFP expression by corresponding RNAis in atfs-1(tm4919) background. Data (mean ± SD) were obtained from five independent experiments, n = 30 animals; ***p < 0.001. with pooled data shown and p-values were calculated using unpaired t-test. F Examples of HIMPA in body wall muscles after hypoxia of indicated RNAi treated gcIs46 worms. Green, UCR-11::GFP. Arrow, UCR-11::GFP aggregate. Bar, 2 μm. G Quantification of aggregate numbers as in F. n = 15 animals from three independent experiments; ***p < 0.001, with pooled data shown. P-values were calculated using unpaired t-test. H Hypoxia resistance is maintained in atfs-1(If) background. Wild-type (N2) or atfs-1(tm4919) treated with indicated RNAis and hypoxic survival was scored. n = 9 plates of worms (>100 worms/plate) from three independent trials. *p < 0.05, unpaired t-test.

Fig. 2 UPRmt activation Reduces HIMPA. A UPRmt-activating RNAis as assessed by hsp-6p::GFP expression. hsp-6p::GFP induction was quantified for 12 RNAis previously shown to induce the UPRmt. Fold induction of GFP fluorescence is the mean fluorescence relative to empty vector RNAi (L4440) (n = 10). Data were obtained from 10–36 individual animals for all panels; mean ± SD; **p < 0.01, ****p < 0.0001, unpaired t-test. B UCR-11::GFP HIMPA reduced by the UPRmt activating RNAis. C UPRmt activation is partially or completely blocked in the atfs-1(If) animals. D atfs-1(If) reduces aggregates and suppresses a further reduction in aggregates by UPRmt-activating RNAis. E Mitochondrial translation inhibitors (doxycycline and meclocycline) reduce HIMPA in a non-additive manner along with atfs-1(If). **p < 0.01, ***p < 0.001, ****p < 0.0001, n or ns not significant versus EV @ Bonferroni-corrected p < 0.01, unpaired t-test.
Fig. 3  Activation of UPR(mt) does Not Necessarily Produce Hypoxia Resistance. The twelve UPR(mt)-activating RNAis are tested for hypoxic sensitivity in the wild type background A–L and in the atfs-1(lf) mutant background M–Q; significance @ p < 0.0001, F-test, simultaneous nonlinear regression; data for each point is from 3 to 12 independent trials with at least 100 animals/trial, mean ±/− sem. A–E Five RNAis produced significant hypoxia resistance; F–J Five RNAis have no significant effect on hypoxic sensitivity; and K, L Two RNAis produced significant hypersensitivity to hypoxia. M–Q The hypoxia resistance of the five RNAis is suppressed by atfs-1(lf).
strong enrichment for hypoxia resistance, where daf-2 effect of UPR mt activation (Supplementary Fig. S2).

Our study shows that a hypoxia resistant phenotype in *C. elegans* is not commonly accompanied by UPR mt activation and that protective mechanisms of UPR mt activation is not always hypoxia protective. In other words, UPR mt activation is neither necessary nor sufficient for protection from hypoxic cell death in *C. elegans*. However, among RNAis selected solely for their ability to induce the UPR mt we did find a strong enrichment for hypoxia resistance, where five of the twelve UPR mt-activating RNAis conferred hypoxia resistance. By comparison, in a screen of over 16,000 RNAis, only 199 were found to reproducibly produce hypoxia resistance [9]. While these twelve are a small sample and the whole genome RNAi screen deliberately ignored weak hypoxia resistance phenotypes, this approximately 30-fold enrichment for hypoxia resistance suggests that UPR mt activation may provide hypoxia protection in certain genetic backgrounds.

We also examined the effect of hypoxia resistance and UPR mt activation on HIMPA. We found that resistance to hypoxic death and resistance to HIMPA were invariably associated. All RNAis or mutants that were resistant to hypoxic death reduced HIMPA. Importantly, the strongly hypoxia resistant daf-2, tars-1, and rars-1 mutants or RNAis block HIMPA without activating the UPR mt. Thus, UPR mt activation is neither necessary for resistance to hypoxia nor for abrogation of HIMPA. We also found that all UPR mt-activating RNAis as well as doxycycline and meclocycline, which strongly activate the UPR mt, brought hypoxia-induced mitochondrial aggregates to near background levels. To be clear, both hypoxia-resistant and non-hypoxia resistant UPR mt-activating RNAis strongly reduced HIMPA. The effect of the RNAis on activation of the UPR mt was mostly if not completely dependent on a functional atfs-1 gene. This was to be expected as hsp-6 activation is dependent on ATF-1 [34, 36].

The reduction in the aggregates by both UPR mt activation and atfs-1 loss-of-function is a puzzling paradox, albeit consistent with our previously published results [6]. We previously showed that atfs-1 RNAi and the atfs-1(tm4919) loss-of-function mutant reduced HIMPA and an atfs-1 gain-of-function mutant increased aggregates [6]. We had also previously shown that cco-1, letm-1, and F02A9.4 RNAis reduced HIMPA, but we attributed this effect to their hypoxia resistance or reduction in the levels of their corresponding proteins that might form the aggregates themselves [6]. We found here that of these three only letm-1 RNAi reproducibly conferred hypoxia resistance yet all three RNAis, indeed all of the UPR mt-activating RNAis, strongly reduced HIMPA. Thus, clearly hypoxia resistance is not necessary for this effect. To
reconcile that both atfs-1(II) and atfs-1-dependent UPR\textsuperscript{mt} activation reduce aggregates, we hypothesize that ATFS-1 functions both to inhibit HIMPA by activating the UPR\textsuperscript{mt} and to promote HIMPA through some other unknown mechanism (Fig. 7). ATFS-1 could both function to reduce aggregates by activation of its downstream UPRmt targets, and in the absence of UPRmt activation function to promote HIMPA through an unknown mechanism. The fact that the level of activation of the hsp-6::GFP transcriptional reporter does not correlate with the ability to suppress hypoxia-induced aggregates (Fig. 2A, B) suggests that the HSP-6 chaperone levels are not directly responsible for the effect of the UPR\textsuperscript{mt} on aggregation, or that a very low threshold of hsp-6 induction suppresses aggregation fully. Which atfs-1-regulated protein(s) might be responsible for promoting HIMPA is unknown; ATFS-1 has been shown to control the synthesis of a large number of proteins involved in mitochondrial proteostasis and mitochondrial function as well as some non-mitochondrial proteins [34, 41, 42]. Likewise, cytoplasmic and ER proteostasis pathways regulate the UPR\textsuperscript{mt} and mitochondrial proteostasis [42]. Thus, ATFS-1 may function in concert with one of those non-UPR\textsuperscript{mt} pathways to regulate HIMPA directly.

An important technical advance of this work is the discovery that TMRE stains HIMPA. TMRE is a lipophilic cationic dye that accumulates in the matrix of viable mitochondrial, where the membrane potential is highly negative relative to the intermembrane space [43, 44]. Besides membrane potential driven accumulation, TMRE has been shown to bind to both surfaces of the inner membrane; however, whether it is binding to lipids, proteins, or both at the inner mitochondrial membrane is unclear [43]. The aggregates appear to be in the matrix of the mitochondria although the resolution is inadequate to be certain. Other than UCR-11::GFP which strongly colocalizes with the TMRE-stained aggregates, we have no empirical evidence for what composes the TMRE-aggregates. In principle, TMRE can bind non-specifically to hydrophobic and negatively charged surfaces. Thus, denatured mitochondrial proteins, lipids, and even nucleic acids are reasonable candidates. Regardless of the nature of the binding target, the discovery that TMRE binds to hypoxia-induced mitochondrial aggregates rules out that HIMPA is an artifact of the GFP reporter overexpression. TMRE can be used to examine for HIMPA in any hypoxic/ischemic model without the need for expression of a tagged protein, electron microscopy, or immunofluorescence.

In summary, resistance to hypoxic organismal death and resistance to hypoxia-induced mitochondrial protein aggregation are strongly correlated. This suggests that HIMPA may be an early hypoxic pathology along the pathway to eventual hypoxic cell death. However, it is important to emphasize that we have not directly measured any aspect of mitochondrial function to determine whether HIMPA correlates with abnormalities in mitochondrial function. On the other hand, activation of the UPR\textsuperscript{mt} is neither necessary nor sufficient for hypoxia resistance but may weakly promote hypoxic survival in certain contexts. Activation of the UPR\textsuperscript{mt} did invariably reduce HIMPA whether or not it conferred resistance to hypoxic death. This effect of the UPR\textsuperscript{mt} favors a model, where the UPR\textsuperscript{mt} directly abrogates
mitochondrial protein aggregation during hypoxia. Future investigation will continue to define the breadth of mechanisms that protect cells from hypoxic death, to define the components of the UPR mt that inhibit HIMPA, and the ATFS-1-regulated mechanism that promotes it.

**MATERIALS AND METHODS**

*C. elegans* strains and culture methods

*C. elegans* strains were cultured and maintained at 20 °C on NGM agar with *OP50 E. coli* food unless otherwise noted [45]. The N2 (Bristol) strain was the standard wild-type strain from the Caenorhabditis Genetics Center (CGC, University of Minnesota). The mitochondrial UPR transcriptional reporter strain SJ4100 (*zcIs13 [hsp-6p::GFP]*) was obtained from the CGC. *gcIs46 [ucr-11::GFP]* and *atfs-1(tm4919);gcIs46* were generated as previously described [6]. *atfs-1 (tm4919);zcIs13, daf-2(e1370);gcIs46, rars-1(gc47);gcIs46*, and *tars-1(gc53);gcIs46* were generated in this study by routine genetic crosses and confirmation by phenotypes and/or PCR genotyping.

**Hypoxic incubations and hypoxic death**

Synchronized young adult worms were subjected to hypoxia as described previously except that hypoxic incubation temperature was 26–26.5 °C [12, 46]. Briefly, each plate of worms was washed into a 1.5 ml tube with 1 ml of M9 buffer (22 mM KH2PO4, 22 mM Na2HPO4, 85 mM NaCl, and 1 mM MgSO4) and after the worms settled by gravity, all but 100 μl of M9 was removed. The tubes were then placed in the anaerobic chamber (Forma Scientific, Waltham, MA, USA) for incubation times ranging from 10 to 34 h as indicated. Oxygen tension was always ≤0.3%. Following the hypoxic incubation unless otherwise noted, worms were transferred using glass Pasteur pipettes onto seeded NGM plates and recovered at 20 °C for 24 h. Normoxic incubations were otherwise identical except performed in a 26.5 °C room air incubator. Organismal death was scored as previously described [46]. Briefly, animals were scored as dead if pharyngeal pumping, spontaneous and evoked movement (touching with a platinum wire) were...
not observed. For the mitochondrial aggregates assay, synchronized worms were kept on NGM plates and placed into anaerobic chamber for 12 h and then mounted for confocal imaging on glass slides [6].

RNAi experiments

The bacterial RNAi feeding strain L4440 carrying the empty RNAi vector strain was from theahringer C. elegans RNAi library (MRC Gene service, Swindon, UK) and was used as the negative control (Empty Vector-EV) for all RNAi experiments [47]. Bacterial strains containing RNAi plasmids were cultured and induced with 0.1% [lactose in 100 mg/ml ampicillin for 24 h at 23 °C as described previously [9]. Worms were synchronized on RNAi plates for 3.5 days (N2) or 4.5 days (ras-1(gc477)) until reaching adulthood; worms not reaching adulthood were excluded.

Mitochondrial UPR reporter and screening

SJ4100 (zcb13; hsp-6p:GFP) was used as a reporter for mitochondrial UPR activation [28]. Animals were synchronized from eggs by bleeding gravid adults, allowed to develop to adulthood, and then mounted on agar pads for imaging. All UPR reporter determinations were performed with worms exposed only to normoxic conditions. Briefly, a minimum of ten worms were mounted onto an agar pad on a standard microscope slide and imaged on a Zeiss axioskope 2 at ×10 magnification. Using ImageJ software (https://imagej.nih.gov/ij/), each worm was outlined and the fluorescence intensity within the area was measured and background intensity was then subtracted to obtain the total intensity/worm as previously described [26].

Chemical treatment and TMRE staining. Concentrated stocks of doxycycline (225 mM) and mecloxycline (294 mM) in DMSO (Dimethyl sulfoxide) (Sigma-Aldrich Corp., St. Louis, MO, USA) were diluted to a final concentration of 100 µM with nuclease-free, deionized water and 300 µl was spread evenly onto a NGM plate seeded with OP50 onto which were placed synchronized eggs allowed to develop into 1-day-old adults. Plates containing TMRE (ThermoFisher, Waltham, MA, USA) were made the same way as described above with 300 µl at a final concentration of 5 µM spread onto the plate. For TMRE staining, 1-day-old synchronized worms were transferred onto the TMRE-containing plates for 2 h before the plates were placed into the anaerobic chamber for 12 h for hypoxic exposure.

Aggregate imaging and counting

Images of UCR-11::GFP and TMRE staining were acquired with confocal microscopy as previously described [6, 26]. Briefly, paralysis was produced by mounting worms in a solution of 50 mM levamisole (Sigma-Aldrich Corp., St. Louis, MO, USA) in M9 prior to imaging. Images were acquired from at least 5–10 randomly selected worms at 1024 × 1024 resolution using a 63× objective with 8 × zoom producing a 23.07 × 23.07 µm image (defined as one high power field, HPF). All images were acquired as a ten slice Z-stack with scan speed of 800–1800 Hz and flattened as a maximum intensity projection prior to analysis. The wavelength of the laser to capture the signal for GFP and TMRE signals was 500–530 nm and 620–700 nm, respectively. Aggregates were counted per HPF by an observer blinded to condition.

Statistics

Two-sided unpaired or paired, as appropriate, t-tests were used for statistical comparisons and all variances were of a similar magnitude except for death versus hypoxic exposure time curves (Fig. 3), where simultaneous curve-fitting was performed [48, 49]. Simultaneous curve-fitting forced the test RNAi death curve fit and the L4440 empty vector control curve fit to the same hypoxic exposure time producing 50% death (LT50) and t-tests were performed for statistical significance of the change in variance of the LT50 versus fitting each curve independently. Statistics were calculated using GraphPad Prism 6.01 (San Diego, CA, USA) for simultaneous curve-fitting, t-tests, or Excel 2007 for t-tests (Microsoft, Redmond, WA). Values are expressed as mean ± SD of at least three independent experiments. A minimum p value of <0.05 was considered significant and was Bonferroni-corrected for multiple comparisons where appropriate. Sample sizes for technical replicates for number of animals was based on the variability from animal to animal for fluorescence-based assays and was always ≥10 or a minimum of 30 animals, where a percent of the population was scored. Based on our previous experience with hypoxic death population assays biological replicates were at least three per condition, and if significant variability was observed among replicates more were performed until clear reproducibility was achieved. In terms of randomization, strains were not randomized as they are genetically identical and raised under environmentally identical conditions.

DATA AVAILABILITY

The raw data from the screen of the RNAi strain set are available upon request. All other data generated and analyzed during this study are included in the published article. The unique C. elegans strains generated for this study are available upon request.

REFERENCES

1. Carreau A, El Hafyi-Rahbi B, Matejuk A, Grillon C, Kieda C. Why is the partial oxygen pressure of human tissues a crucial parameter? Small molecules and hypoxia. J Cell Mol Med. 2011;15:1239–53.
2. Liu S, Shi H, Liu W, Fusushi T, Timmins GS, Liu KJ. Interstitial pO2 in ischemic penumbra and core are differentially affected following transient focal cerebral ischemia in rats. J Cereb Blood Flow Metab. 2004;24:343–9.
3. Storey KB, Storey JM. Metabolic rate depression: the biochemistry of mammalian hibernation. Adv Clin Chem. 2010;52:77–108.
4. Poyton RO, Ball KA, Castello PR. Mitochondrial generation of free radicals and hypoxic signaling. Trends Endocrinol Metab. 2009;20:332–40.
5. Itani OA, Zhong X, Tang X, Scott BA, Yan JY, Filbotte S, et al. Coordinate regulation of ribosome and IRNA biogenesis controls hypoxic injury and translation. Curr Biol. 2021;31:128–37. e5
6. Kaufman DM, Wu X, Scott BA, Itani OA, Van Gilst MR, Bruce JE, et al. Ageing and hypoxia cause protein aggregation in mitochondria. Cell Death Differ. 2013;20:1730–8.
7. Mao XR, Kaufman DM, Crowder CM. Nicotinamide mononucleotide adenyllyl-transferase promotes hypoxic survival by activating the mitochondrial unfolded protein response. Cell Death Dis. 2016;7:e2113.
8. Scott B, Sun CL, Mao X, Yu C, Vohra BP, Milbrandt J, et al. Role of oxygen consumption in hypoxia protection by translation factor depletion. J Exp Biol. 2013;216:2283–92.
9. Mabon ME, Mao X, Jiao Y, Scott BA, Crowder CM. Systematic identification of gene activities promoting hypoxic death. Genetics 2009;181:483–96.
10. Mabon ME, Scott BA, Crowder CM. Divergent mechanisms controlling hypoxic survival and lifespan by the DAF-2/Insulin/IGF-receptor pathway. PLoS ONE. 2009;4:e7937.
11. Anderson LL, Mao X, Scott BA, Crowder CM. Survival from hypoxia in C. elegans by inactivation of acinaroY-IRNA synthetases. Science 2009;323:630–3.
12. Scott BA, Avidan MS, Crowder CM. Regulation of hypoxic death in C. elegans by the insulin/IGF receptor homolog DAF-2. Science 2002;296:2388–91.
13. Heimburger T, Hog J, Gupta P, Murphy CT. PQM-1 controls hypoxic survival via regulation of lipid metabolism. Nat Commun. 2020;11:4627.
14. Iarong NN, Achim BE, Miller DL. Fasting prevents hypoxia-induced defects of proteostasis in C. elegans. PLoS Genet. 2019;15:e1008242.
15. Doshi S, Price E, Landis J, Barot U, Sabatella M, Lans H, et al. Neurpeptide signaling regulates the susceptibility of developing C. elegans to anoxia. Free Radic Biol Med. 2018;131:197–208.
16. Pena S, Sherman T, Brooksos PS, Nehrke K. The mitochondrial unfolded protein response protects against anoxia in caenorhabditis elegans. PLoS ONE. 2016;11:e0150989.
17. Ladage ML, King SD, Burks DJ, Quan DL, Garcia AM, Azad RK, et al. Glucose or altered ceramide biosynthesis mediate oxygen deprivation sensitivity through novel pathways revealed by transcriptome analysis in Caenorhabditis elegans. G3 2016;6:1349–60.
18. LaMacchia JC, Frazier HN, 3rd, Roth MB. Glycogen fuels survival during hypoxia cause protein aggregation in mitochondria. Cell Death Differ. 2013;20:1730–8.
19. Fawcett EM, Hoyt JM, Johnson JK, Miller DL. Hypoxia disrupts proteostasis in Caenorhabditis elegans. Aging Cell. 2015;14:92–101.
20. Filbotte SJ, Jablonski AM, Kollar RG. Oxygen sensing neurons and neuropeptides regulate survival after anoxia in developing C. elegans. PLoS ONE. 2014;9:e101102.
21. Menzu V, Howell KS, Gentina S, Epstein S, Riezman I, Fornallaz-Mulhauser M, et al. Protection of C. elegans from anoxia by HYL-2 ceramide synthase. Science 2009;324:381–4.
22. Mendenhall AR, LaRue B, Padilla PA. Glyceroldehyde-3-phosphate dehydrogenase mediates anoxia response and survival in Caenorhabditis elegans. Genetics 2006;174:173–87.

J. Yan et al.
24. Nystul TG, Goldmark JP, Padilla PA, Roth MB. Suspended animation in C. elegans requires the spindle checkpoint. Science 2003;302:1038–41.
25. Jiang H, Guo R, Powell-Coffman JA. The Caenorhabditis elegans hif-1 gene encodes a bHLH-PAS protein that is required for adaptation to hypoxia. Proc Natl Acad Sci USA. 2001;98:7916–21.
26. Kaufman DM, Crowder CM. Mitochondrial proteostatic collapse leads to hypoxic injury. Curr Biol. 2015;25:2171–6.
27. Naresh NU, Haynes CM. Signaling and regulation of the mitochondrial unfolded protein response. Cold Spring Harb Perspect Biol. 2019;11:a033944.
28. Yoneda T, Benedetti C, Urano F, Clark SG, Harding HP, Ron D. Compartment-specific perturbation of protein handling activates genes encoding mitochondrial chaperones. J Cell Sci. 2004;117:4055–66.
29. Benedetti C, Haynes CM, Yang Y, Harding HP, Ron D. Ubiquitin-like protein 5 positively regulates chaperone gene expression in the mitochondrial unfolded protein response. Genetics 2006;174:229–39.
30. Haynes CM, Petrova K, Benedetti C, Yang Y, Ron D. ClpP mediates activation of a mitochondrial unfolded protein response in C. elegans. Dev Cell. 2007;13:467–80.
31. Duriex J, Wolff S, Dillin A. The cell non-autonomous nature of electron transport chain-mediated longevity. Cell. 2011;144:79–91.
32. Meissner B, Boll M, Daniel H, Baumeister R. Deletion of the intestinal peptide transporter affects insulin and TOR signaling in Caenorhabditis elegans. J Biol Chem. 2004;279:36739–45.
33. Geillinger KE, Kuhlmann K, Eisenacher M, Gesbert P, Meyer HE, Daniel H, et al. Intestinal amino acid availability via PEPT-1 affects TORK1/2 signaling and the unfolded protein response. J Proteome Res. 2014;13:3685–92.
34. Nargund AM, Fiorese CJ, Pellegrino MW, Deng P, Haynes CM. Mitochondrial and nuclear accumulation of the translation factor ATF5-1 promotes OXPHOS recovery during the UPR(mt). Mol Cell. 2015;58:123–33.
35. Haynes CM, Yang Y, Blais SP, Neubert TA, Ron D. The matrix peptide exporter HAF-1 signals a mitochondrial UPR by activating the transcription factor ZC376.7 in C. elegans. Mol Cell. 2010;37:529–40.
36. Nargund AM, Pellegrino MW, Fiorese CJ, Baker BM, Haynes CM. Mitochondrial import efficiency of ATF5-1 regulates mitochondrial UPR activation. Science 2012;337:587–90.
37. Bennett CF, Vander Wende H, Simko M, Klum S, Barfield S, Choi H, et al. Activation of the mitochondrial unfolded protein response does not predict longevity in Caenorhabditis elegans. Nat Commun. 2014;5:3483.
38. Sun CL, Zhang H, Liu M, Wang W, Crowder CM. A screen for protective drugs against delayed hypoxic injury. PLoS ONE. 2017;12:e0176061.
39. Kenyon CJ. The genetics of ageing. Nature 2010;464:504–12.
40. Walther DM, Kasturi P, Zheng M, PInkert S, Vecchi G, Ciryam P, et al. Widespread proteome remodeling and aggregation in aging C. elegans. Cell 2015;161:919–32.
41. Shpilka T, Du Y, Yang Q, Melber A, Uma Naresh N, Lavelle J, et al. UPR(mt) scales mitochondrial network expansion with protein synthesis via mitochondrial import in Caenorhabditis elegans. Nat Commun. 2021;12:479.
42. Anderson NS, Haynes CM. Folding the mitochondrial UPR into the integrated stress response. Trends Cell Biol. 2020;30:428–39.
43. Scaduto RC Jr, Grotjohann LW. Measurement of mitochondrial membrane potential using fluorescent rhodamine derivatives. Biophys. J. 1999;76:469–77.
44. Teodor J, Machado IF, Castela AC, Rolo AP, Palmeira CM. The evaluation of mitochondrial membrane potential using fluorescent dyes or a membrane-permeable cation (TPP(−)) electrode in isolated mitochondria and intact cells. Methods Mol. Biol. 2020;2184:197–213.
45. Brenner S. The genetics of Caenorhabditis elegans. Genetics 1974;77:71–94.
46. Sun CL, Kim E, Crowder CM. Delayed innocent bystander cell death following hypoxia in Caenorhabditis elegans. Cell Death Differ. 2014;21:557–67.
47. Karnath RS, Fraser AG, Dong Y, Poulin G, Durbin R, Gotta M, et al. Systematic functional analysis of the Caenorhabditis elegans genome using RNAi. Nature 2003;421:231–7.
48. Waud DR. On biological assays involving quantal responses. J Pharmacol Exp Ther. 1972;183:577–607.

ACKNOWLEDGEMENTS
We thank Barbara Scott for her technical contributions to the work.

AUTHOR CONTRIBUTIONS
C.S., M.V.G., and C.M.C. conceived the study. J.Y., C.S., and S.S. performed the experiments. C.S., M.V.G., J.Y., and C.M.C. wrote the manuscript with input from all co-authors.

FUNDING
The work was supported by National Institute of Neurological Disorders and Stroke R01NS109088 (C.M.C., and M.V.G.), R01NS100350 (C.M.C.) and R01GM129034 (M.V.G.).

ETHICS STATEMENT
There is no requirement for ethical approval of this work as it did not involve the study of humans, human material, or vertebrate animals.

COMPETING INTERESTS
The authors declare no competing interests.

ADDITIONAL INFORMATION
Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41419-021-03979-z.

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