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RESEARCH ARTICLE

Role of oxygen consumption in hypoxia protection by translation factor depletion

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SUMMARY

The reduction of protein synthesis has been associated with resistance to hypoxic cell death. Which components of the translation machinery control hypoxic sensitivity and the precise mechanism has not been systematically investigated, although a reduction in oxygen consumption has been widely assumed to be the mechanism. Using genetic reagents in *Caenorhabditis elegans*, we examined the effect on organismal survival after hypoxia of knockdown of 10 factors functioning at the three principal steps in translation. Reduction-of-function of all 10 translation factors significantly increased hypoxic survival to varying degrees, not fully accounted for by the level of translational suppression. Measurement of oxygen consumption showed that strong hypoxia resistance was possible without a significant decrease in oxygen consumption. Hypoxic sensitivity had no correlation with lifespan or reactive oxygen species sensitivity, two phenotypes associated with reduced translation. Resistance to tunicamycin, which produces misfolded protein toxicity, was the only phenotype that significantly correlated with hypoxic sensitivity. Translation factor knockdown was also hypoxia protective for mouse primary neurons. These data show that translation factor knockdown is hypoxia protective in both *C. elegans* and mouse neurons and that oxygen consumption does not necessarily determine survival; rather, mitigation of misfolded protein toxicity is more strongly associated with hypoxic protection.

Key words: hypoxia, ischemia, translation factors, *C. elegans*.

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INTRODUCTION

The sensitivity of metazoan cells to injury from reduced oxygen (hypoxia) varies greatly among cell types and organisms. Exposure to severe hypoxia for only minutes is sufficient to kill human central nervous system neurons or myocardial myocytes, while human skeletal myocytes can survive hours of complete ischemia. Cancer cells are typically hypoxia resistant, a trait that may contribute to their tumorigenicity and metastatic potential (Brahimi-Horn et al., 2007; Wouters and Koritzinsky, 2008; Kim et al., 2009; Rockwell et al., 2009). At an organismal level, perhaps best studied are certain hibernating animals that become profoundly resistant to hypoxia during hibernation. In both cancer cells and hibernating animals, suppression of protein translation rate is associated with adaptation to hypoxia (van den Beucken et al., 2006; Arimoto et al., 2008; Spriggs et al., 2010; Storey and Storey, 2010).

Protein translation is a highly energy-consuming process, accounting for a significant proportion of total cellular oxygen consumption (Rolfe and Brown, 1997). The lower oxygen consumption that accompanies a reduction in global translation rate has been assumed to be the primary mechanism whereby translational suppression protects from hypoxic injury. However, translational suppression not only reduces oxygen consumption but also has other potentially protective effects. One such effect is improved protein homeostasis by decreased synthesis of nascent polypeptides that are a source of misfolded proteins (Guerriero and Brodsky, 2012).

In *Caenorhabditis elegans* (Maupas 1900), we have performed screens for genes whose mutant phenotype is improved organismal survival after hypoxia (Scott et al., 2002; Anderson et al., 2009; Mabon et al., 2009). Each of these screens has identified genes known to regulate protein homeostasis. Most recently, in a forward mutagenesis screen, *gc47*, a partial reduction-of-function allele of *rars-1*, which encodes a cytoplasmic arginyl-tRNA synthetase, was isolated and identified based on its strong hypoxia-resistant phenotype (Anderson et al., 2009). *rars-1(gc47)* animals have an ~50% reduction in global translation rate consistent with the essential role of aminoacyl-tRNA synthetases such as RARS-1 in protein translation. The discovery that RARS-1 along with other aminoacyl-tRNA synthetases (Anderson et al., 2009) and a few implicated translation factors (Mabon et al., 2009) strongly control hypoxic survival motivated a more systematic study of the effect of translation factor knockdown on hypoxic injury. In order to establish mammalian relevance and therapeutic potential, we first tested the ability of translation factor knockdown to protect cultured mouse hippocampal neurons. We then tested the effect on hypoxic survival of a diverse set of *C. elegans* translation factors to determine whether hypoxia resistance was a general feature of translational suppression, regardless of which factor was inhibited. We then asked whether hypoxia resistance by translation factor knockdown was necessarily associated with reduced oxygen consumption or whether other known effects of translational suppression might be more tightly associated with hypoxic survival.

Our hypothesis was that hypoxia protection produced by translation factor knockdown is not solely due to a reduced rate of oxygen consumption.

MATERIALS AND METHODS

Lentiviral shRNA, hippocampal neuron culture and hypoxia

Coding sequences of translation factors/modulators were cloned into the FCIV shuttle lentiviral vector [ubiquitin promoter–shRNA-IRES-enhanced yellow fluorescent protein (YFP) (Venus)] (Araki et al., 2004). Each clone was confirmed by nucleotide sequencing. Viral packaging took place in HEK293T cells using Fugene reagent to transfect cells with a cocktail of plasmids: small hairpin RNA (shRNA) construct, Δ8.9 and pVSV-g. Transfection efficiency was confirmed by monitoring expression of co-transcribed YFP 48 h post transfection. Once transfection was confirmed, HEK293T medium was collected. After a brief centrifugation, 20 μl of each lentivirus-containing media (~10^3 particles μl^{-1}) was directly added into neuronal cultures. Primary P0 mouse hippocampal neurons were cultured for 5 days on poly-*d*-lysine-coated 4-well dishes in neurobasal medium supplemented with B27, L-glutamine and 5-fluoro-2′-deoxyuridine (FUDR) to prevent growth of glia. After 5 days, the neurons were infected with lentivirus carrying each shRNA or control luciferase shRNA. Seven days post infection, cultures were examined for co-transcribed YFP expression to confirm successful transduction. Cultures were then exposed to 7 h of hypoxia (<0.3% O_2) at 37°C. Cells were allowed to recover for 20 h in normoxia at 37°C and scored for death by an ethidium homodimer assay (Invitrogen, Grand Island, NY, USA). Cultures were then fixed and stained with Tuj1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) to count total neurons. Survival data (100×alive neurons/total neurons) from three wells (≥10 high power fields) were pooled as one trial.

Caenorhabditis elegans strains and culture conditions

The wild-type strain for all experiments was N2 (var. Bristol) (Brenner, 1974). Strains were maintained at 20°C on nematode growth medium (NGM) agar seeded with OP50 bacteria (Stiernagle, 2006). *rars-1(gc47)* was generated in our laboratory (Anderson et al., 2009) and was outcrossed prior to testing; the genotypes were confirmed by PCR.

RNA interference

The gene target of all RNA interference (RNAi) vectors was confirmed by sequencing. One-generation feeding RNAi was performed as described previously (Timmons, 2006b; Mabon et al., 2009). Briefly, two gravid adult worms were left on agar RNAi plates with 2-day-old RNAi bacterial lawns for 3 h to obtain 30–50 eggs per plate. RNAi plates were composed of NGM agar supplemented with 50 μg ml^{-1} carbenicillin (catalog no. C1389, Sigma-Aldrich, St Louis, MO, USA) and 1 mmol l^{-1} isopropyl β-D-1-thiogalactopyranoside (IPTG) (catalog no. I6758, Sigma-Aldrich) and seeded with the appropriate RNAi bacterial strain cultured in 2xYT with 50 μg ml^{-1} carbenicillin, 10 μg ml^{-1} tetracycline and 0.8 mmol l^{-1} IPTG. Unless otherwise specified, animals were grown on RNAi plates from embryos to young adulthood, 1 day past the L4 stage and then phenotyped. Feeding with bacteria transformed with the L4440 empty vector was used as a negative control for all RNAi experiments (Timmons, 2006a).

Hypoxic killing assays

Hypoxic killing assays were performed as described previously (Scott et al., 2002) except at 26°C and without buffer change. Briefly, synchronized 1-day-post-L4 well-fed adults were transferred from agar plates to 1.5 ml polypropylene tubes with 1 ml of M9 buffer (Stiernagle, 2006). After the worms were pelleted by gravity, buffer was removed to reach a volume of 100 μl and the tubes were placed in the hypoxic chamber ([O_2]<0.3%, ~26°C) for 20 h except for delayed death assays (10 h hypoxic incubation). In preliminary experiments, dissolved oxygen concentration in the M9 buffer with worms was found to be initially 15.6±0.05 vol% (mean ± s.e.m. of five replicates) outside of the hypoxic chamber and dropped exponentially to 0.06±0.04 vol% within 1 h in the hypoxic chamber and to below the level of detection by 1.5 h. After the hypoxic incubation, the worms were transferred back to NGM agar plates and recovered in air at 20°C for 24 h prior to scoring as alive (any spontaneous or evoked behavior) or dead. One trial consisted of triplicate tubes with ~50 worms per tube. For delayed death, worms were scored for death every 24 h after placing on recovery plates up to 96 h of recovery.

FRAP assays

Fluorescence recovery after photobleaching (FRAP) assays were adapted from previous studies (Syntichaki et al., 2007; Kourtis and Tavernarakis, 2009). *js115*;*oxIs34*[P*unc-64*::open Stx P*myo*-2::GFP], which expresses pharyngeal green fluorescent protein (GFP) (Richmond et al., 2001), was synchronized on RNAi plates and grown at 20°C to the adult stage. Worms were mounted and immobilized on a 5% agarose pad in a drop of 0.10 μm microspheres (catalog no. 00876, Polysciences, Warrington, PA, USA) and imaged/photobleached with a 40× objective. Prior to photobleaching, an image of each worm was taken at an exposure time just below saturation and these camera settings were used for that particular worm for the remainder of the experiment. The worm was then photobleached for 10 to 13 min to reduce the initial emission intensity to 20 to 30% of the pre-bleach value. All worms on a given day were photobleached for the same number of minutes. An immediate post bleach image was taken, and the worm was then returned to the particular RNAi plate for 24 h at 20°C. After the 24 h recovery period, dead worms or worms showing signs of damage were not analyzed, and healthy worms were remounted and imaged with exposure times identical to the pre-bleach image. ImageJ (National Institutes of Health, Bethesda, MD, USA) was used to quantify the GFP emission intensities that were then normalized to the pre-bleach image defined as 100%. Relative recovery for each worm was calculated according to the GFP emission intensities as follows: (recovery–bleach)/(prebleach–bleach). A minimum of six worms per RNAi were tested.

For FRAP with cycloheximide (catalog no. C7698, Sigma-Aldrich), 1 day post L4 adults were picked to plates containing various concentrations of cycloheximide and allowed to incubate for 2 h. Plates were prepared by spreading the appropriate concentration of cycloheximide (from a stock solution of 50 mg ml^{-1} in 70% ethanol) onto NGM and then seeding with L4440 that was concentrated 10-fold and resuspended in M9 containing the appropriate cycloheximide dilution. The FRAP assays were then performed as above except that the worms were recovered on plates with the respective concentration of cycloheximide. Comparison of the FRAP values with the addition of water *versus* 70% ethanol at the volume needed for the highest [cycloheximide] showed no effect of this concentration of ethanol on FRAP.

Oxygen consumption assays

Synchronized 1-day-post-L4 adult worms grown on L4440 or RNAi-expressing bacteria were washed three times with M9 buffer to

remove bacteria. Approximately 3000 worms were transferred in M9 buffer to three respirometer cells (1000 worms per cell in 1 ml M9) (Strathkelvin Model 929, Glasgow, UK); oxygen consumption values were found to be linear over a range of worm numbers from 1000 to 7500. Oxygen consumption was measured for 20–30 min with continuous stirring at 20°C. The oxygen consumption rate was calculated from the slope of the linear portion of the plot, which was between 250 and 30 μmol l^{-1} dissolved oxygen. The worms were then removed, resuspended in 500 μl of 1% sodium dodecyl sulfate and sonicated, and debris was removed by centrifugation for 10 min at 17,000 ***g***. Protein concentration was determined in duplicate by direct spectrophotometry at 280 nm (ND-1000, Nanodrop Technologies, Wilmington, DE, USA) with bovine serum albumin used as a standard. Oxygen consumption values were normalized to protein concentration, which was found to be linear with the number of worms over a range from at least 1000 to 7500. Bacteria was found not to contribute to oxygen consumption by three control experiments: (1) oxygen consumption of heat-killed worms (0.22±0.05 nmol min^{-1}, O_2 consumption not normalized to biomass) was not significantly different than buffer control (0.12±0.22 nmol min^{-1}, mean ± s.d., P=0.42, unpaired t-test); (2) oxygen consumptions of worms fed live OP50 bacteria, no OP50 bacteria for 3 h or heat-killed OP50 bacteria were similar (10.14, 10.34 and 9.2 nmol min^{-1} mg^{-1}, respectively); and (3) oxygen consumption of worms treated with 100 mmol l^{-1} sodium azide for 2 h was not significantly different than buffer control (0.18±0.09 *versus* 0.12±0.22 nmol min^{-1}, not normalized to biomass, mean ± s.d., P=0.63, unpaired t-test).

For oxygen consumption assays with cycloheximide, worms were transferred with M9 into 15 ml conical polypropylene tubes, rinsed once, the supernatant removed, and then incubated in 500 μl of various concentrations of cycloheximide for 2 h. The worms were then rinsed twice with 8 ml of M9, and the solution was changed back to 1 ml at the original cycloheximide concentration and transferred to the respirometer for oxygen consumption measurement followed by protein quantification as described above.

Lifespan and paraquat assays

Lifespan was defined as the time from laid embryo to death. Worms that died of 'unnatural causes' such as crawling up the side of the Petri dish or bagging (internal hatching of larvae) were censored and were removed from further analysis. Median lifespans were calculated from Kaplan–Meier survival curves of 50 worms per condition and curves were compared for statistical difference in median lifespan using the Mantel–Cox log-rank test (GraphPad Prism 5.0, GraphPad Software, La Jolla, CA, USA). Paraquat assays were performed and analyzed identically to lifespan, except 50 worms per condition were transferred to plates containing 4 mmol l^{-1} paraquat on day 7 after egg laying.

Tunicamycin assays

The tunicamycin growth arrest assay has been published previously (Anderson et al., 2009). Briefly, eggs were laid on the various RNAi plates containing 1 μg ml^{-1} tunicamycin; 14–81 eggs were scored per trial. The fraction of worms reaching the adult stage was scored 4 days later. Growth was at 20°C.

RESULTS

Knockdown of translation factors in mouse neurons can protect from hypoxic death

In our previous screens, we have found that reduction-of-function of multiple aminoacyl tRNA synthetases and a few translation initiation factors strongly protected *C. elegans* from hypoxia. To assess the relevance of our findings in *C. elegans* to hypoxic injury of mammalian neurons, we tested whether knockdown of the mouse homolog of five of the implicated translation factors protect cultured mouse hippocampal neurons. Knockdown of four out of five translation factors provided significant protection of the hippocampal neurons from hypoxic death (Fig. 1A). Unlike in *C. elegans*, shRNA knockdown of Rars (arginyl-tRNA synthetase) was uniformly neurotoxic, and the effect on hypoxic sensitivity was not tested. Some Nars (asparaginyl-tRNA synthetase) and Yars (tyrosyl-tRNA synthetase) shRNA constructs at higher viral titers were also toxic, but at lower titers were protective without gross toxicity. Knockdown of Ei2b2 (eukaryotic translation initiation factor 2B, subunit 2 beta) and Gars (glycyl-tRNA synthetase) were each significantly protective without evidence of toxicity. For all of the protective constructs, the level of knockdown was greater than fourfold (Fig. 1B).

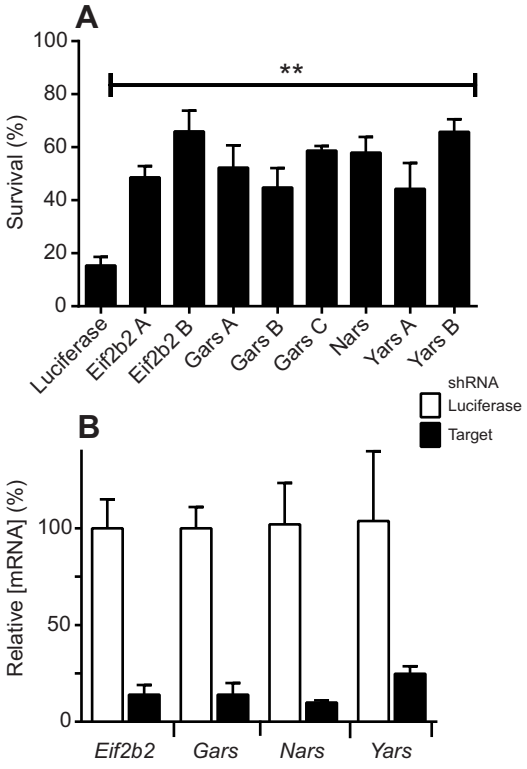

Fig. 1. Survival of mouse hippocampal neurons after hypoxia with translation factor knockdown. (A) Survival of mouse hippocampal neuron cultures after hypoxia when infected with lentiviruses expressing shRNAs targeting translation factors. At least two distinct lentiviral constructs (designated by an A, B or C suffix) were effective for three of the four genes without overt toxicity; only one Nars targeting virus was effective without toxicity. Data are means ± s.e.m. of a minimum of three independent trials (**P<0.01 *versus* luciferase virus control, one-way ANOVA with Dunnett's multiple comparison correction). (B) qRT-PCR shows >4-fold knockdown for all four translation factor transcripts. Gene names: *Ei2b2*, eukaryotic translation initiation factor 2B, subunit 2 beta; *Gars*, glycyl-tRNA synthetase; *Nars*, asparaginyl-tRNA synthetase; *Yars*, tyrosyl-tRNA synthetase.

Knockdown of multiple translation factors in *C. elegans* protects from organismal hypoxic death

In order to examine systematically the efficacy of translation factor knockdown against hypoxic injury, we returned to the *C. elegans*

Table 1. *Caenorhabditis elegans* translation machinery genes studied

Gene name	Gene product
eef-2	EEF2 translation elongation factor
erfa-3	ERF3A translation termination factor
fars-1	Phenylalanyl-tRNA synthetase
ife-2	EIF4E cap binding protein
ifg-1	IEF4G cap binding protein
iftb-1	EIF2beta translation initiation factor
nars-1	Asparaginyl-tRNA synthetase
rars-1	Arginyl-tRNA synthetase
rpl-6	Large ribosomal subunit L6 protein
rps-15	Small ribosomal subunit S15 protein

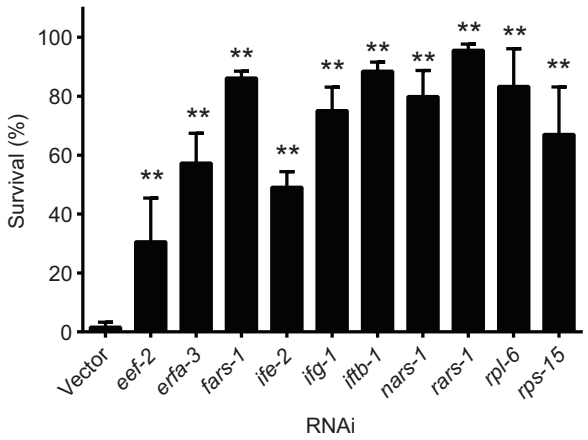

Fig. 2. Organismal hypoxic survival of *C. elegans* after RNAi knockdown of the various translation genes. Wild-type *C. elegans* were grown from embryo to adult on the particular RNAi bacteria, exposed to hypoxia at 26°C for 20 h, recovered for 24 h, and scored for organismal survival. Data are means ± s.e.m. of a minimum of three independent trials with at least 70 animals per trial (**P<0.01 *versus* vector control, one-way ANOVA with Dunnett's multiple comparison correction).

model. We knocked down translation factors that mediate all three major steps in translation: initiation, elongation and termination (Table 1). Knockdown of all 10 translation factors produced significant hypoxia resistance although the level of resistance varied considerably (Fig. 2).

Variations in hypoxia protection not fully accounted for by the degree of translational suppression

We assumed that the level of hypoxia resistance provided by each RNAi was proportional to the degree of translational suppression. To test this assumption, we compared protein translation rates in *C. elegans* pharyngeal myocytes after treatment with the various RNAis using FRAP (Syntichaki et al., 2007; Kourtis and Tavernarakis, 2009). The FRAP assay uses animals expressing GFP; recovery of fluorescence after photobleaching is a measure of new protein production. We first validated the FRAP method by showing that it fell dose-dependently with the translational inhibitor cycloheximide (Fig. 3A). Also, FRAP estimates of translation rates for *rars-1*(RNAi) agreed well with previous estimates derived from ^{35}S methionine incorporation (54% of control by FRAP *versus* 52% by ^{35}S) (Anderson et al., 2009). However, the translational rates as measured by FRAP did not fully explain the hypoxia resistance of the translation factor RNAis. For example, *iftb-1* and *rps-15* RNAi reduced FRAP least of all of the RNAis but both produced strong hypoxia survival (88.5 and 67%, respectively) (Fig. 3B). In contrast, knockdown of the translation factor gene *erfa-3* produced the strongest reduction in FRAP, but one of the weakest hypoxic survival phenotypes. Correlation of FRAP with hypoxic survival did not reach significance (Fig. 3C).

Variations in hypoxia protection not fully accounted for by oxygen consumption

A longstanding and widely stated explanation for the hypoxia resistance of cells or organisms with reduced translation is an energetic one, that is, reduced oxygen consumption allows cells to withstand hypoxia until oxygen is restored (Hochachka et al., 1996; Liu and Simon, 2004; Fähling, 2009; Wheaton and Chandel, 2011). To test this hypothesis, we measured oxygen consumption and hypoxia resistance in cohorts of worms with translation factor knockdown. As for FRAP, we verified that oxygen consumption varied reliably with cycloheximide-mediated translational suppression (Fig. 4A). The correlation of FRAP with oxygen consumption for various cycloheximide concentrations was strong (r=0.85; data not shown). We then measured oxygen consumption for all 10 translation factor RNAis (Fig. 4B). Most RNAis did produce significant reductions in oxygen consumption; some RNAis, such as those targeting *eef-2*, *ifg-1* and *rpl-6*, strongly reduced oxygen consumption to levels similar to the maximum reduction seen with cycloheximide. However, *rars-1* and *ife-2* RNAi produced no significant reduction in oxygen consumption. *rars-1(gc47)*, a reduction-of-function allele shown previously to have a translation rate similar to that of *rars-1*(RNAi), had only a 26% reduction in oxygen consumption (Fig. 4B). Overall as for FRAP, the correlation of hypoxic survival with reduced oxygen consumption did not reach statistical significance (Fig. 4C). However with the exclusion of *rars-1* and *eef-2*, the hypoxic survival to oxygen correlation for the rest of the RNAis was highly significant (r=–0.93, P=0.003).

We have previously shown that *rars-1*(RNAi) applied only after hypoxia protects from hypoxia (Anderson et al., 2009). We reproduced that result here (Fig. 5A,C). This result is inconsistent with a mechanism of hypoxia protection where reduced oxygen consumption during the hypoxic exposure is entirely responsible for the protection. Given that the hypoxia resistance of other translation factors correlated well with oxygen consumption, we considered the possibility that knockdown of these factors might not be protective after hypoxia. We tested this hypothesis for *iftb-1*(RNAi), which produces a level of hypoxia resistance similar to *rars-1*(RNAi) but with a much stronger reduction in oxygen consumption. Unlike *rars-1*(RNAi), *iftb-1*(RNAi) provided no survival benefit when applied only during recovery after hypoxia (Fig. 5B,C). The lack of efficacy of *iftb-1*(RNAi) after hypoxia could be due to slower kinetics of functional knockdown so that the level of IFTB-1 protein is not reduced quickly enough to improve recovery. We tested this hypothesis by exposure to either *iftb-1* or *rars-1* RNAi for only 1 day just prior to hypoxic incubation and then recovery on non-RNAi for the usual 24 h. With this brief RNAi exposure, both *iftb-1*(RNAi) and *rars-1*(RNAi) provided a similar level of modest but significant hypoxia resistance compared with the empty vector control (Fig. 5D). Thus, the kinetics of functional knockdown by *iftb-1*(RNAi) appears similar to that of *rars-1*(RNAi). We conclude that the mechanisms of protection produced by knockdown of RARS-1 and IFTB-1 fundamentally differ.

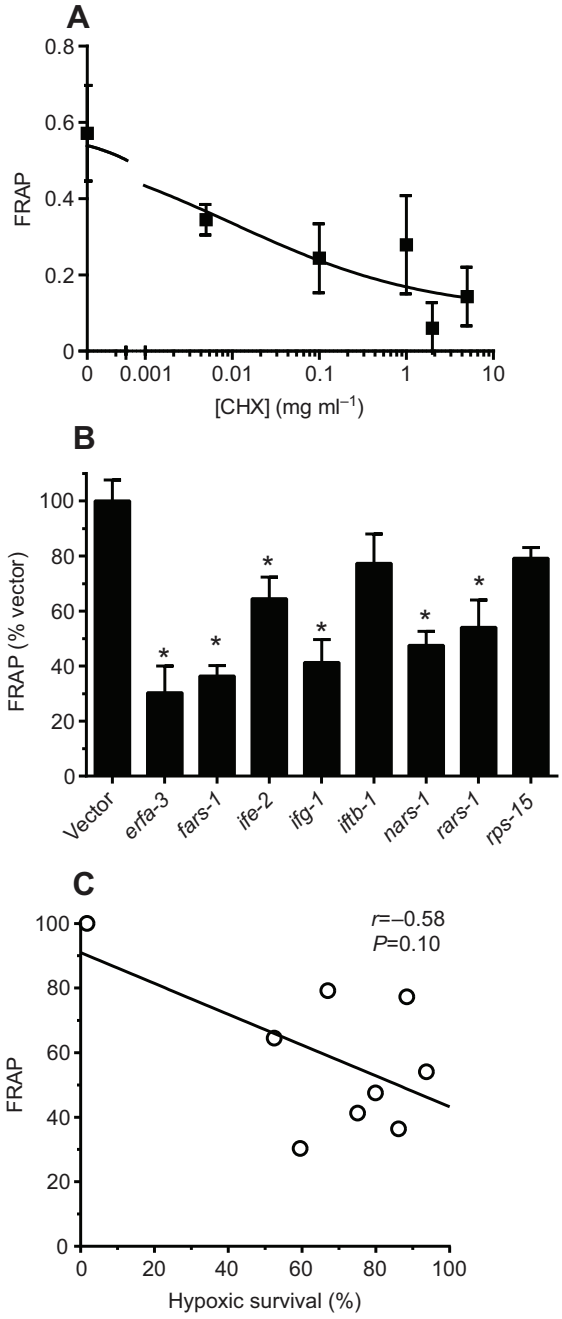

Fig. 3. Correlation of translational suppression with hypoxia resistance for *C. elegans* translation factor knockdown. (A) Adult animals were incubated on cycloheximide plates for 2 h, at which time they were removed for photobleaching then returned to the same cycloheximide plates for 24 h before the fractional fluorescent recovery after photobleaching (FRAP) was measured. (B) FRAP normalized to L4440 vector control values for young adult animals exposed to RNAi from embryo to adult. Data are means ± s.e.m. of six to 22 animals (**P*<0.05 *versus* vector control, one-way ANOVA with Dunnett's multiple comparison correction). (C) Correlation of FRAP with hypoxic survival for the various RNA conditions is not significant.

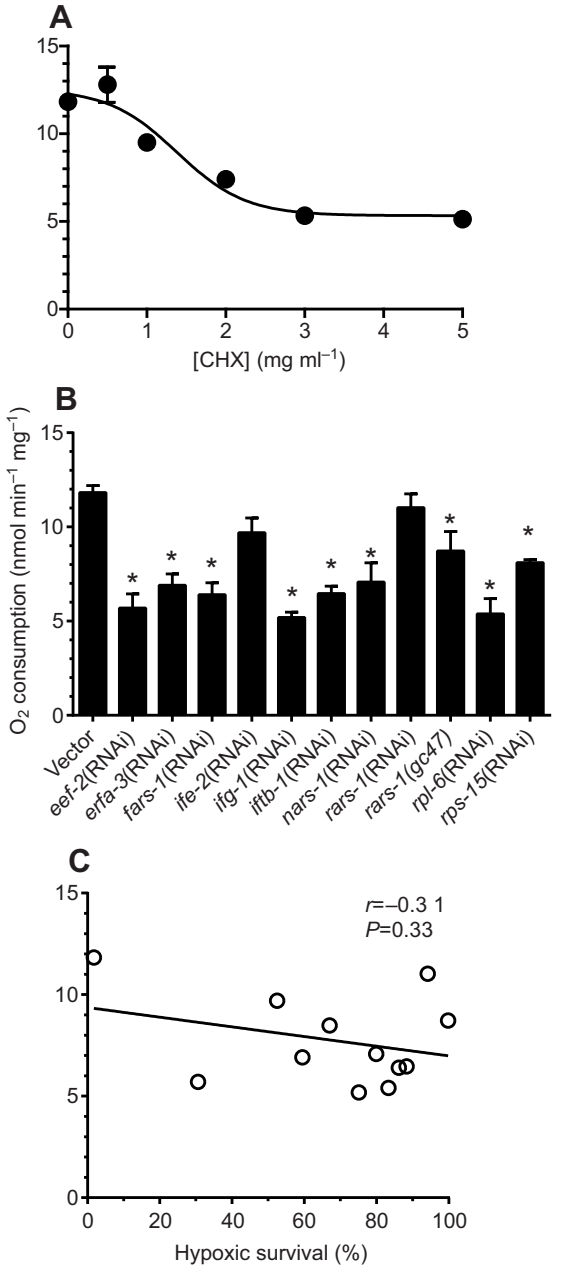

Fig. 4. Correlation of oxygen consumption with hypoxia resistance for translation factor knockdown/mutant animals. (A) One-day-post-L4 adult animals were incubated on cycloheximide plates for 24 h, at which time oxygen consumption was measured. Worms were then lysed and protein concentration measured for normalization. Data are means ± s.e.m. of a minimum of three independent trials; error bars are smaller than the data points in most cases. (B) Oxygen consumption for young adult animals exposed to RNAi from embryo to adult. Data are means ± s.e.m. of a minimum of three independent trials (**P*<0.05 *versus* vector control, one-way ANOVA with Dunnett's multiple comparison correction). (C) Correlation of oxygen consumption with hypoxic survival for the various RNAi conditions is not significant.

Hypoxia resistance and lifespan are not correlated

Knockdown or mutation of a number of translation factors has been found to increase longevity of *C. elegans* (Curran and Ruvkun, 2007; Hansen et al., 2007; Hipkiss, 2007; Pan et al., 2007; Syntichaki et al., 2007; Rogers et al., 2011). We have shown that long-lived *C. elegans* mutants of the insulin/IGF receptor homolog *daf-2* are highly hypoxia resistant (Scott et al., 2002) and that *rars-1(gc47)* has a significantly prolonged lifespan (Anderson et al., 2009). Thus, we hypothesized that lifespan and hypoxia resistance would covary with translation factor knockdown. As previously shown for the *rars-*

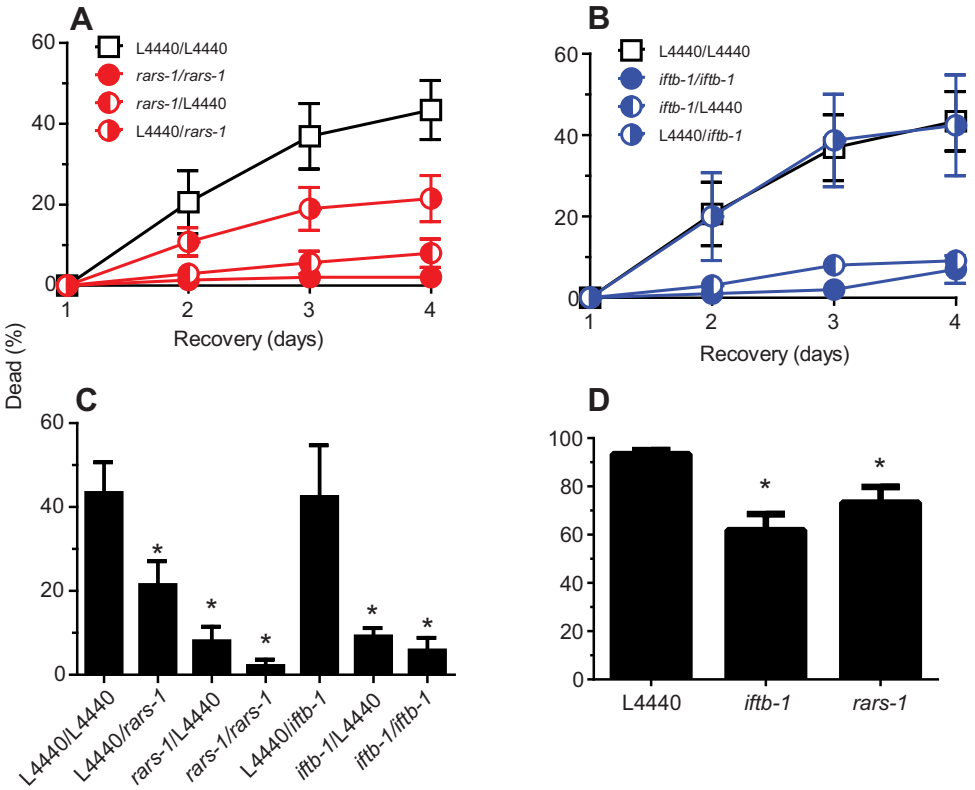

Fig. 5. Effect of post-hypoxia RNAi knockdown of *rars-1* and *iftb-1* on survival. (A) Wild-type N2 were grown under four RNAi conditions: (1) on L4440 empty vector from egg to adult and before and after a 10 h hypoxic exposure (L4440/L4440); (2) on *rars-1*(RNAi) bacteria from egg to adult and before and after hypoxic exposure (*rars-1/rars-1*); (3) on *rars-1*(RNAi) from egg to adult and before hypoxia with L4440 after hypoxia during recovery (*rars-1*/L4440); (4) on L4440 from egg to adult and before hypoxia with *rars-1*(RNAi) after hypoxia during recovery (L4440/*rars-1*). Death was scored after each day of recovery. (B) N2 incubated with *iftb-1*(RNAi) before and after, only before, or only after a 10 h hypoxic exposure. (C) Percentage dead after 4 days of recovery under the various conditions. Data are means ± s.e.m. of at least five independent trials per condition (*P<0.05 *versus* L4440/L4440 by two-tailed *t*-test). (D) Short exposure to *iftb-1* and *rars-1* RNAi prior to hypoxia. Animals were grown to adults on OP50 bacteria then exposed for 24 h to empty vector (L4440), *iftb-1* RNAi, or *rars-1* RNAi then washed free of bacteria, incubated for 20 h in hypoxia, recovered for 24 h on OP50 bacteria and then scored. Data are means ± s.e.m. of three independent trials/condition (*P<0.05 *versus* L4440 by two-tailed *t*-test).

1(gc47) mutation (Anderson et al., 2009), *rars-1*(RNAi) did result in a significant increase in lifespan (Fig. 6F,I). Likewise, *ifg-1* and *rps-15* RNAi animals were long-lived (Fig. 6C,H,I). However, the five other RNAis did not significantly increase lifespan (Fig. 6A,B,D,E,G,I). The overall correlation of the lifespan and hypoxic survival phenotypes was not significant (Fig. 6J). Lifespan also failed to correlate with FRAP or oxygen consumption (Fig. 6K,L).

Resistance to hypoxia and paraquat are not correlated

Reactive oxygen species are generated upon reoxygenation after hypoxia and can promote cell death (Allen and Bayraktutan, 2009; Niizuma et al., 2009; Raedschelders et al., 2012). Resistance to reactive oxygen species in *C. elegans* has been associated with translational suppression (Syntichaki et al., 2007; Wang et al., 2010). Thus, we hypothesized that the protective effect against hypoxia of translation factor knockdown was secondary to resistance to oxidative damage. To test this hypothesis, we measured the survival of RNAi-treated worms on agar plates containing the reactive oxygen species generator paraquat. Six of the eight translation factor RNAis tested produced a significant increase in survival on paraquat; *erfa-3* and *iftb-1* RNAis did not (Fig. 7A). The correlation of the paraquat and hypoxic survival phenotypes was not significant (Fig. 7B). However, the correlation of paraquat survival with lifespan was highly significant (Fig. 7C).

Resistance to hypoxia and protein misfolding stress are strongly correlated

Hypoxia produces a significant stress on intracellular protein folding homeostasis and ultimately results in an increase in misfolded proteins (Koumenis and Wouters, 2006; Ge et al., 2007; Rzymski and Harris, 2007; Mao and Crowder, 2010). Misfolded proteins can directly or indirectly promote cell death (Dobson, 2003; Powers et al., 2009). By measuring sensitivity to tunicamycin, which increases the level of misfolded proteins (Merksamer et al., 2008), we tested whether translation factor knockdown generally produces tunicamycin resistance and whether this resistance covaries with hypoxia resistance. All but one translation factor RNAi conferred significant resistance to tunicamycin (Fig. 8A). As shown previously (Anderson et al., 2009), *rars-1(gc47)* was strongly tunicamycin resistant. The correlation of resistance to tunicamycin with that to hypoxia was highly significant (Fig. 8B).

DISCUSSION

The data presented herein support four novel conclusions. First, in both mouse and worm, knockdown of multiple translation factors can improve hypoxic survival. Second, and quite surprisingly, the hypoxia protection produced by knockdown of translation factors in *C. elegans* is not explained entirely by the degree of reduction of translation rate. Third, reduction in oxygen consumption during

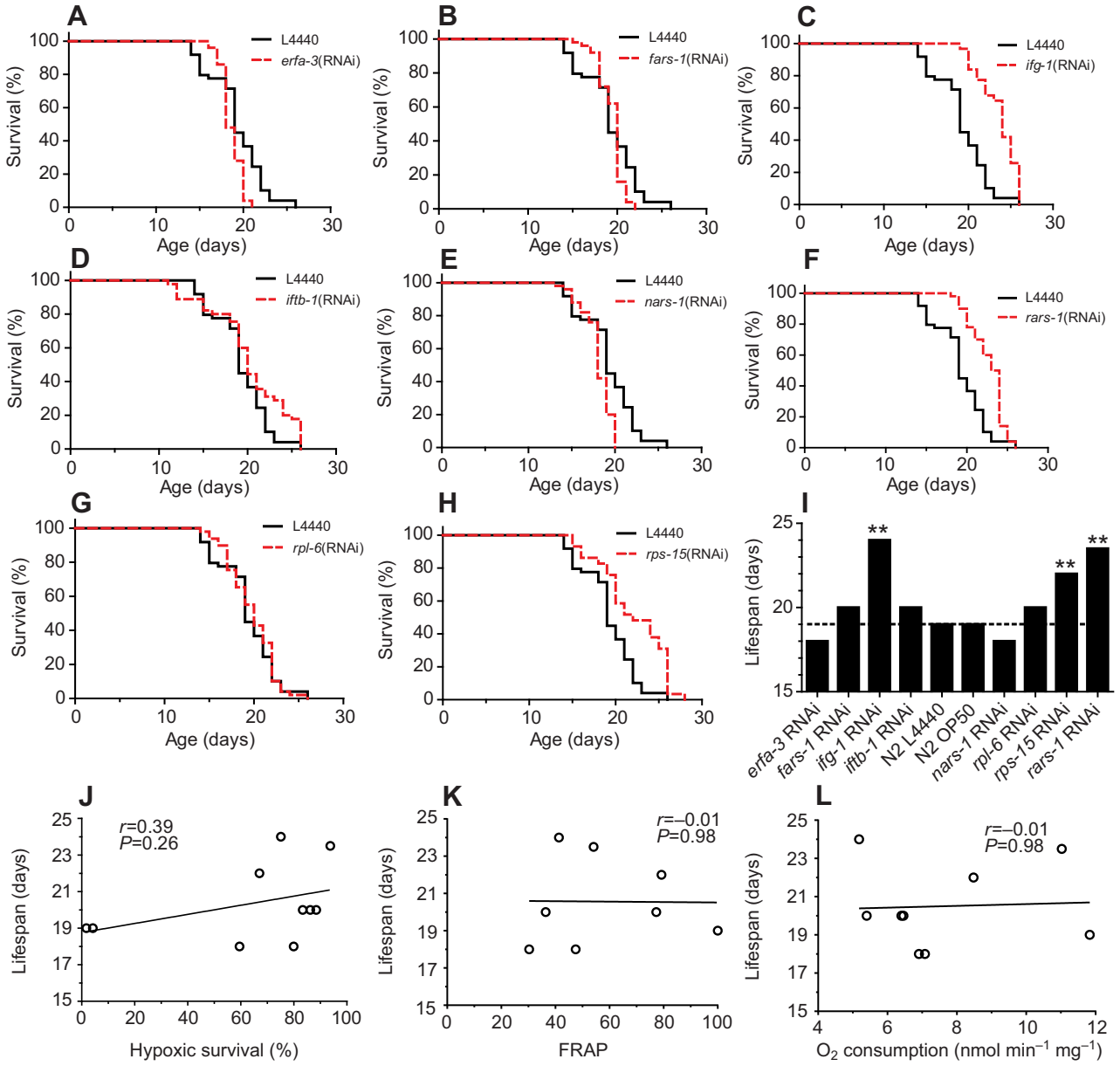

Fig. 6. Correlations of lifespans of translation factor knockdown animals with oxygen consumption, translation rate and hypoxia resistance. (A–H) Lifespan measured from egg onward. Fifty animals per condition were scored with RNAi applied from egg onward. (I) Median lifespan. $^{**}P<0.01$ log-rank test *versus* L4440 vector control. (J–L) Lifespan does not correlate with hypoxic survival, FRAP or oxygen consumption.

hypoxic exposure may be the primary mechanism of protection for knockdown of some translation factors but is not necessary for the improved survival produced by reduction-of-function of *rars-1* and perhaps of others. Finally, among the factors tested, the survival advantage conferred by translation factor knockdown is most highly correlated with resistance to the protein misfolding agent tunicamycin. We will discuss below the implications and potential limitations of these conclusions.

Knockdown of translation factors mediating all three steps of translation – initiation, elongation and termination – as well as a knockdown of two ribosomal subunits were protective against hypoxic death in *C. elegans*. After accounting for the effect on translation rate, there was clearly a large range of efficiencies for hypoxic protection with knockdown of the translation termination factor ERFA-3 having decidedly the weakest protective effect for the level of translational suppression and IFTB-1 knockdown the strongest. IFTB-1 encodes eIF2β, which is one of three subunits of the eIF2 initiation factors. Given the strong correlation of resistance to misfolded protein stress and hypoxia, it is interesting to speculate that the highly effective hypoxic protection derived from *iftb-1* knockdown is related to a reduction in the levels of the eIF2:GTP:met-tRNAi ternary initiation complex. Reduction in this ternary complex not only reduces global translation rate, but can also increase initiation from non-AUG codons (Marintchev and Wagner, 2004) in a manner similar to and perhaps synergistic with that produced by activation of PERK, a component of the endoplasmic reticulum unfolded protein response (UPR^{ER}). Upon activation by unfolded protein stress such as that produced by

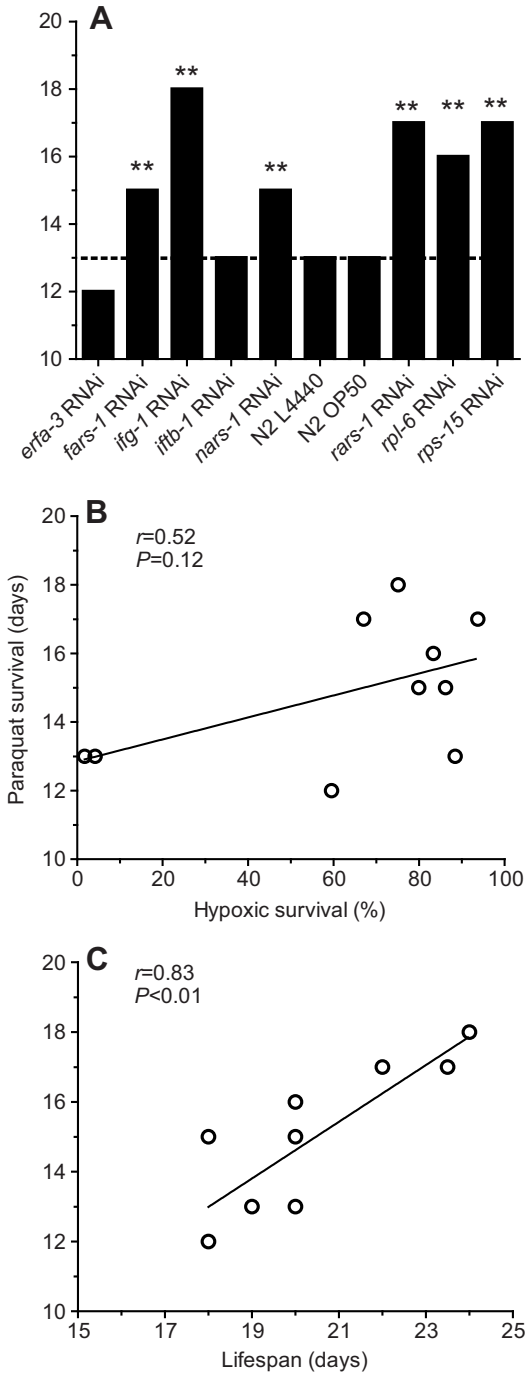

Fig. 7. Correlation of paraquat resistance with lifespan and hypoxic survival with translation factor knockdown. (A) Kaplan–Meier curves were measured on 50 worms per condition to calculate median duration of survival on 4 mmol l^{-1} paraquat agar plates. Worms were transferred to plates containing paraquat on day 7 after egg laying. **P<0.01 log-rank test. (B) Survival duration on paraquat does not correlate with hypoxic survival. (C) Survival duration on paraquat correlates significantly with lifespan.

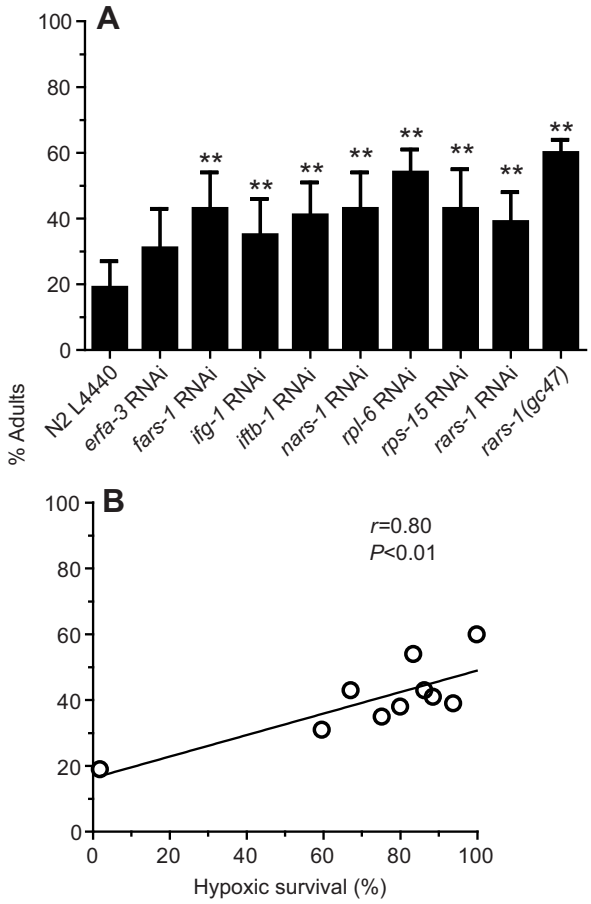

Fig. 8. Correlation of tunicamycin resistance with hypoxic survival with translation factor knockdown or mutation. (A) Percentage of animals reaching adulthood 4 days post embryonic stage on plates containing 1 μg ml^{-1} tunicamycin. Data are means ± s.e.m. of six independent trials (**P<0.01, Fisher's exact test). (B) Tunicamycin resistance correlates significantly with hypoxia resistance.

hypoxia, PERK phosphorylates eIF2α and reduces global translation rate while paradoxically increasing the translation of certain transcripts that help to ameliorate misfolded protein stress (Vattem and Wek, 2004; Zhou et al., 2008). In mammalian cells, PERK minus cells are significantly hypersensitive to hypoxia, and mutation of the PERK-phosphorylated serine on eIF2α also increases hypoxic sensitivity (Bi et al., 2005). In *C. elegans*, hypoxia does induce PERK-dependent phosphorylation of eIF2α, but a PERK-1 loss-of-function mutant does not appear to be hypersensitive to hypoxia, at least in a wild-type background (Mao and Crowder, 2010). However, a reasonable hypothesis for the weakly protective effect of the translation termination factor ERFA-3 derives from the fact that eRF3 depletion should produce an increase in nascent polypeptides still attached to ribosomes as well as novel polypeptides produced by read-through of stop codons (Chauvin et al., 2005; Dever and Green, 2012). This relative increase in nascent polypeptides relative to mature protein as well as prolonged association with the translation complex would be expected to increase the level of misfolded proteins (Guerriero and Brodsky, 2012) and might mitigate the protective effects against hypoxia from the reduction in overall translation rates.

Prior to this work, the essentially universal and widely stated assumption was that the improved survival from translational suppression derived from reduced oxygen consumption (Hochachka et al., 1996; Liu and Simon, 2004; Fähling, 2009; Wheaton and Chandel, 2011). While reasonable, this assumption was not experimentally tested. Our data demonstrate that the oxygen consumption mechanism cannot explain the protection derived from knockdown of at least one translation factor, *rars-1*, where

knockdown does not significantly reduce oxygen consumption. As we cannot independently manipulate oxygen consumption and translation factor levels, it is unclear whether the oxygen consumption reductions produced by the knockdown of the other translation factors are essential to their protective phenotype. However, the strong correlation of hypoxic survival and oxygen consumption for knockdown of all but *rars-1* and *eef-2* suggests that the oxygen consumption mechanistic hypothesis could be correct for many translation factors. What is unique about the *rars-1* mechanism is unclear. Whatever the mechanism, it is capable of improving survival when applied after hypoxic survival.

A limitation of our results is the lack of anatomical expression or functional data for most of the *C. elegans* translation machinery genes studied. Thus, one possibility for the lack of correlation of oxygen consumption or FRAP with hypoxic survival is that the expression and, more importantly, the function of some of the translation factor genes might not be ubiquitous. For example, if *rars-1* were functionally important in only a specific cell type that strongly controls organismal hypoxic survival but that did not contribute greatly to organismal oxygen consumption, then reduction of *rars-1* function could strongly improve hypoxic survival without significantly altering oxygen consumption. In other words, *rars-1* might function at a cell-autonomous level to control translation rate and oxygen consumption and, secondarily, hypoxic survival of that cell, but at an organismal level *rars-1*-regulated hypoxic sensitivity might be cell non-autonomous. Without detailed cell-specific functional data for all three traits (hypoxic sensitivity, oxygen consumption and translation rate) for *rars-1* and the other translation factor genes, we cannot rule out cell-specific functions for some of these genes as an explanation for the lack of correlation of these traits at an organismal level.

The only effect of translation factor knockdown that was well correlated with the improved hypoxic survival for all genes was resistance to the protein misfolding agent tunicamycin. We previously observed that *rars-1(gc47)* was tunicamycin resistant and that mutations in components of the UPR^{ER} weakly suppressed the hypoxia resistance of *rars-1*(RNAi) (Anderson et al., 2009). We have also shown that brief non-lethal hypoxic exposure induces a protective response against hypoxia that requires an intact UPR^{ER} (Mao and Crowder, 2010). Based on these results, we previously hypothesized that reducing the function of *rars-1* and, by inference, of all other translation factors improves hypoxic survival by reducing the load of nascent polypeptides that would contribute to the misfolded protein load during the peri-hypoxic period (Anderson et al., 2009). However, our current results indicate that translational suppression does not solely determine the level of hypoxic protection.

An alternative hypothesis is that depletion of specific translation factors reduces translation machinery aggregation produced by hypoxia. Components of the translation machinery have been found to be aggregated after hypoxia and this aggregation may promote or reduce cell death depending on the experimental model (Jamison et al., 2008; Buchan and Parker, 2009). Further, depletion of specific translation factors has differential effects on translation machinery aggregation (Mokas et al., 2009). One reasonable hypothesis is that reduction of some translation factors such as RARS-1 strongly alters the level of translation machinery aggregation produced by hypoxia in a way that improves recovery after hypoxia and increases resistance to protein misfolding agents. This hypothesis remains to be tested.

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AUTHOR CONTRIBUTIONS

B.S. performed and analyzed the experiments shown in Figs 2–5 and helped write the manuscript. C.-L.S. performed and analyzed the experiments shown in Figs 6–8 and helped write the manuscript. X.M. performed and analyzed the experiments shown in Fig. 1 and helped write the manuscript. C.Y. helped perform and analyze the experiments shown in Fig. 8 and helped write the manuscript. B.V. helped design and analyze the experiments shown in Fig. 1 and helped write the manuscript. J.M. helped design and analyze the experiments shown in Fig. 1 and helped write the manuscript. C.M.C. wrote the manuscript, analyzed all data and helped design all experiments.

COMPETING INTERESTS

No competing interests declared.

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