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1 **Role of oxygen consumption in hypoxia protection by translation factor depletion**
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12 Running Title: Translation and hypoxic survival

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30 **SUMMARY**

31

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

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50 **INTRODUCTION**

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

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

69 In *C. elegans*, we have performed screens for genes whose mutant phenotype is
70 improved organismal survival after hypoxia (Scott et al., 2002; Anderson et al., 2009; Mabon et
71 al., 2009). Each of these screens has identified genes known to regulate protein homeostasis.
72 Most recently, in a forward mutagenesis screen, *gc47,* a partial reduction-of-function allele of
73 *rars-1*, which encodes a cytoplasmic arginyl-tRNA synthetase, was isolated and identified based
74 on its strong hypoxia resistant phenotype (Anderson et al., 2009). *rars-1* (*gc47*) animals have an
75 approximately 50% reduction in global translation rate consistent with the essential role of
76 aminoacyl-tRNA synthetases like RARS-1 in protein translation. The discovery that RARS-1 along
77 with other aminoacyl-tRNA synthetases (Anderson et al., 2009) and a few implicated translation
78 factors (Mabon et al., 2009) strongly control hypoxic survival motivated a more systematic

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79 study of the effect of translation factor knockdown on hypoxic injury. In order to establish
80 mammalian relevance and therapeutic potential, we first tested the ability of translation factor
81 knockdown to protect cultured mouse hippocampal neurons. We then tested the effect on
82 hypoxic survival of a diverse set of *C. elegans* translation factors to determine if hypoxia
83 resistance was a general feature of translational suppression, regardless of which factor was
84 inhibited. We then asked if hypoxia resistance by translation factor knockdown was necessarily
85 associated with reduced oxygen consumption or if other known effects of translational
86 suppression might be more tightly associated with hypoxic survival. Our hypothesis was that
87 hypoxia protection produced by translation factor knockdown is not solely due to a reduced
88 rate of oxygen consumption.
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91 **MATERIALS AND METHODS**

92 **Lentiviral shRNA, Hippocampal Neuron culture and hypoxia**

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

110

111 ***C. elegans*** **strains and culture conditions**

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

116 **RNAi**

117 The gene target of all RNAis was confirmed by sequencing. One-generation feeding RNAi was
118 performed as described previously (Timmons, 2006b; Mabon et al., 2009). Briefly, two gravid
119 adult worms were left on agar RNAi plates with 2 day old RNAi bacterial lawns for 3 hours to

120 obtain 30-50 eggs per plate. RNAi plates were composed of NGM agar supplemented with 50
121 μg ml^{-1} carbenicillin and 1 mM IPTG and seeded with the appropriate RNAi bacterial strain
122 cultured in 2xYT with 50 μg ml^{-1} carbenicillin, 10 μg ml^{-1} tetracycline and 0.8 mM IPTG. Unless
123 otherwise specified, animals were grown on RNAi plates from embryos to young adulthood,
124 one day past the L4 stage and then phenotyped. Feeding with bacteria transformed with the
125 L4440 empty vector was used as a negative control for all RNAi experiments (Timmons, 2006a).

126 **Hypoxic killing assays**

127 Hypoxic killing assays were performed as described previously (Scott et al., 2002) except at 26°
128 and without buffer change . Briefly, synchronized one day post L4 well-fed adults were
129 transferred from agar plates to 1.5 ml polypropylene tubes with 1 ml of M9 buffer (Stiernagle,
130 2006). After the worms pelleted by gravity, buffer was removed to 100 μl and the tubes placed
131 in the hypoxic chamber ([O2] < 0.3%, T ≈ 26°) for 20 hours except for delayed death assays (10
132 hour hypoxic incubation). In preliminary experiments, dissolved oxygen concentration in the
133 M9 buffer with worms was found to be initially 15.6 ± 0.05 vol% (mean ± sem of 5 replicates)
134 outside of the hypoxic chamber and dropped exponentially to 0.06 ± 0.04 vol% within 1 hour in
135 the hypoxic chamber and to below the level of detection by 1.5 hours. After the hypoxic
136 incubation, the worms were transferred back to NGM agar plates and recovered in air at 20° for
137 24 hours prior to scoring as alive (any spontaneous or evoked behavior) or dead. One trial
138 consisted of triplicate tubes with ≈ 50 worms/tube. For delayed death, worms were scored for
139 death every 24 hours after placing on recovery plates up to 96 hours of recovery.

140 **FRAP assays**

141 FRAP assays were adapted from Tavernarakis (Syntichaki et al., 2007; Kourtis and Tavernarakis,
142 2009). *js115*;*oxIs34*[P*unc-64*::open Stx P*myo*-2::GFP], which expresses pharyngeal GFP
143 (Richmond et al., 2001), was synchronized on RNAi plates and grown at 20° to the adult stage.
144 Worms were mounted and immobilized on a 5% agarose pad in a drop of 0.10 μm
145 microspheres (Polysciences, Inc catalog # 00876) and imaged/photobleached with a 40X
146 objective. Prior to photobleaching, an image of each worm was taken at an exposure time just
147 below saturation and these camera settings were used for that particular worm for the
148 remainder of the experiment. The worm was then photobleached for 10 to 13 minutes to

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149 reduce the initial emission intensity to 20 to 30% of the pre-bleach value. All worms on a given
150 day were photobleached for the same number of minutes. An immediate post bleach image
151 was taken, and the worm was then returned to the particular RNAi plate for 24 hours at 20°.
152 After the 24 hour recovery period, dead worms or worms showing signs of damage were
153 censored, and healthy worms were remounted and imaged with exposure times identical to the
154 pre-bleach image. Image J was used to quantify the GFP emission intensities that were then
155 normalized to the pre-bleach image defined as 100%. Relative recovery for each worm was
156 calculated according to (recovery – bleach) / (prebleach – bleach). A minimum of six worms per
157 RNAi were tested.

158 For FRAP with cycloheximide, one day post L4 adults were picked to plates containing
159 various concentrations of cycloheximide and allowed to incubate for 2 hours. Plates were
160 prepared by spreading the appropriate concentration of cycloheximide (from a stock solution of
161 50 mg ml^{-1} in 70% ethanol) onto NGM and then seeding with L4440 that was concentrated 10-
162 fold and resuspended in M9 containing the appropriate cycloheximide dilution. The FRAP
163 assays were then performed as above except that the worms were recovered on plates with
164 the respective concentration of cycloheximide. Comparison of the FRAP values with the
165 addition of water versus 70% ethanol at the volume needed for the highest [cycloheximide]
166 showed no effect of this concentration of ethanol on FRAP.

167 **Oxygen consumption assays**

168 Synchronized one day post L4 adult worms grown on L4440 or RNAi-expressing bacteria
169 were washed three times with M9 buffer to remove bacteria. Approximately 3000 worms were
170 transferred in M9 buffer to three respirometer cells (1000 worms/cell in 1 ml M9) (Strathkelvin
171 Model 929, Glasgow, UK); oxygen consumption values were found to be linear over a range of
172 worm numbers from 1000 to 7500. Oxygen consumption was measured for 20-30 minutes with
173 continuous stirring at 20°. The oxygen consumption rate was calculated from the slope of the
174 linear portion of the plot, which was between 250 -30 µmole l^{-1} dissolved oxygen. The worms
175 were then removed, resuspended in 500ul of 1% SDS, sonicated, and debris removed by
176 centrifugation for 10 minutes at 17,000g. Protein concentration was determined in duplicate
177 by direct spectrophotometry at 280 nm (ND-1000, Nanodrop Technologies, USA) with bovine

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178 serum albumin used as a standard. Oxygen consumption values were normalized to protein
179 concentration, which was found to be linear with the number of worms over a range from at
180 least 1000 – 7500 worms. Bacteria was found not to contribute to oxygen consumption by
181 three control experiments: 1) Oxygen consumption of heat killed worms (0.22 ± 0.05 nmoles
182 min^{-1} – O_2 consumption not normalized to biomass) was not significantly different than buffer
183 control (0.12 ± 0.22 nmoles min^{-1}, mean ± SD, p = 0.42, unpaired t-test); 2) the oxygen
184 consumptions of worms fed live OP50 bacteria, no OP50 bacteria for 3 hours, or heat-killed
185 OP50 bacteria were similar (10.14, 10.34, and 9.2 nmoles min^{-1} mg^{-1}, respectively); 3) Oxygen
186 consumption of worms treated with 100 mM sodium azide for two hours was not significantly
187 different than buffer control (0.18 ± 0.09 vs 0.12 ± 0.22 nmoles min^{-1} not normalized to
188 biomass, mean ± SD of , p = 0.63, unpaired t-test)
189 For oxygen consumption assays with cycloheximide, worms were transferred with M9
190 into 15 ml conical polypropylene tubes, rinsed once, the supernatant removed, then incubated
191 in 500 μl of various concentrations of cycloheximide for 2 hours. The worms were then rinsed
192 twice with 8 ml of M9, and the solution changed back to 1 ml at the original cycloheximide
193 concentration and transferred to the respirometer for oxygen consumption measurement
194 followed by protein quantification as described above.
195 **Lifespan and paraquat assays**
196 Lifespan was defined as the time from laid embryo to death. Worms that died of
197 "unnatural causes" such as crawling up the side of the petri dish or bagging (internal hatching
198 of larvae) were censored. Median lifespans were calculated from Kaplan-Meier survival curves
199 of 50 worms/condition and curves were compared for statistical difference in median lifespan
200 using Mantel-Cox log rank test (Graphpad Prism 5.0). Paraquat assays were performed and
201 analyzed identically to lifespan except 50 worms/condition were transferred to plates
202 containing 4 mM paraquat on day 7 after egg lay.
203 **Tunicamycin assays**
204 The tunicamycin growth arrest assay has been published previously (Anderson et al., 2009).
205 Briefly, eggs were laid on the various RNAi plates containing 1μg mL^{-1} tunicamycin; 14 – 81

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206 eggs/trial were scored. The fraction of worms reaching the adult stage was scored four days
207 later. Growth was at 20°.
208

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209 **RESULTS**

210 **Knockdown of translation factors in mouse neurons can protect from hypoxic death.** In our
211 previous screens, we have found that reduction-of-function of multiple aminoacyl tRNA
212 synthetases and a few translation initiation factors strongly protected *C. elegans* from hypoxia.
213 To assess the relevance of our findings in *C. elegans* to hypoxic injury of mammalian neurons,
214 we tested whether knockdown of the mouse homolog of five of the implicated translation
215 factors protect cultured mouse hippocampal neurons. Knockdown of four out of five translation
216 factors provided significant protection of the hippocampal neurons from hypoxic death (Figure
217 1A). Unlike in *C. elegans*, shRNA knockdown of Rars (arginyl-tRNA synthetase) was uniformly
218 neurotoxic, and the effect on hypoxic sensitivity was not tested. Some Nars (asparaginyl-tRNA
219 synthetase) and Yars (tyrosyl-tRNA synthetase) shRNA constructs at higher viral titers were also
220 toxic, but at lower titers were protective without gross toxicity. Knockdown of Ei2b2 (eukaryotic
221 translation initiation factor 2B, subunit 2 beta) and Gars (glycyl-tRNA synthetase) were each
222 significantly protective without evidence of toxicity. For all of the protective constructs, the
223 level of knockdown was greater than four-fold (Figure 1B).
224

225 **Knockdown of multiple translation factors in *C.* elegans protects from organismal hypoxic**
226 **death.** In order to examine systematically the efficacy of translation factor knock down against
227 hypoxic injury, we returned to the *C. elegans* model. We knocked down translation factors that
228 mediate all three major steps in translation: initiation, elongation, and termination (Figure 2A).
229 Knockdown of all ten translation factors produced significant hypoxia resistance although the
230 level of resistance varied considerably (Figure 2B).
231

232 **Variations in hypoxia protection not fully accounted for by the degree of translational**
233 **suppression.** We assumed that the level of hypoxia resistance provided by each RNAi was
234 proportional to the degree of translational suppression. To test this assumption, we compared
235 protein translation rates in *C. elegans* pharyngeal myocytes after treatment with the various
236 RNAis using fluorescence recovery after photobleaching (FRAP) (Syntichaki et al., 2007; Kourtis
237 and Tavernarakis, 2009). The FRAP assay uses animals expressing green fluorescent protein

238 (GFP); recovery of fluorescence after photobleaching is a measure of new protein production.
239 We first validated the FRAP method by showing that it fell dose-dependently with the
240 translational inhibitor cycloheximide (Figure 3A). Also FRAP estimates of translation rates for
241 *rars-1* RNAi agreed well with previous estimates derived from ^{35}S methionine incorporation (
242 54% of control by FRAP vs 52% by ^{35}S) (Anderson et al., 2009). However, the translational rates
243 as measured by FRAP did not fully explain the hypoxia resistance of the translation factor
244 RNAis. For example, *iftb-1* and *rps-15* RNAi reduced FRAP least of all of the RNAis but both
245 produced strong hypoxia survival (88.5% and 67% respectively). On the other hand, knockdown
246 of the translation factor gene *erfa-3* produced the strongest reduction in FRAP, but one of the
247 weakest hypoxic survival phenotypes. Expressing the data as the product of the hypoxic survival
248 and FRAP within the same cohorts (aliquots of worms from the same RNAi growth plates)
249 shows that the variation in hypoxic survival produced by translation factor knockdown is not
250 fully explained by the degree of translational suppression (Figure 3C). Correlation of FRAP with
251 hypoxic survival did not reach significance (Figure 3D).
252
253 **Variations in hypoxia protection not fully accounted for by oxygen consumption.** A
254 longstanding and widely stated explanation for the hypoxia resistance of cells or organisms with
255 reduced translation is an energetic one, that is, reduced oxygen consumption allows cells to
256 withstand hypoxia until oxygen is restored (Hochachka et al., 1996; Liu and Simon, 2004;
257 Fahling, 2009; Wheaton and Chandel, 2011). To test the hypothesis, we measured oxygen
258 consumption and hypoxia resistance in cohorts of worms with translation factor knockdown. As
259 for FRAP, we verified that oxygen consumption varied reliably with cycloheximide-mediated
260 translational suppression (Figure 4A). The correlation of FRAP with oxygen consumption for
261 various cycloheximide concentrations was strong (r=0.85 – data not shown). We then measured
262 oxygen consumption for all ten translation factor RNAis (Figure 4B). Most RNAis did produce
263 significant reductions in oxygen consumption; some RNAis such as those targeting *eef-2, ifg-1*,
264 and *rpl-6* strongly reduced oxygen consumption to levels similar to the maximum reduction
265 seen with cycloheximide. However, *rars-1* and *ife-2* RNAis produced no significant reduction in
266 O_2 consumption. The product of hypoxic survival and oxygen consumption for cohorts (aliquots

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267 of worms from the same RNAi growth plates) shows that *rars-1* and *eef-2* are outliers compared
268 to the rest of the RNAis (Figure 4C). *rars-1(gc47),* a reduction-of-function allele shown
269 previously to have a translation rate similar to that of *rars-1*(RNAi), had only a 26% reduction in
270 O_2 consumption (Figure 4B) and had a hypoxic survival x oxygen consumption product similar to
271 *rars-1(*RNAi*)* (Figure 4C)*.* Overall as for FRAP, the correlation of hypoxic survival with reduced
272 oxygen consumption did not reach statistical significance (Figure 4D). However with the
273 exclusion of *rars-1* and *eef-2*, the hypoxic survival to O_2 correlation for the rest of the RNAis was
274 highly significant (r=-0.93, p = 0.003).

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

293

294 **Hypoxia resistance and lifespan do not correlate.** Knockdown or mutation of a number of
295 translation factors has been found to increase longevity of *C. elegans* (Curran and Ruvkun,

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296 2007; Hansen et al., 2007; Hipkiss, 2007; Pan et al., 2007; Syntichaki et al., 2007; Rogers et al.,
297 2011). We have shown that long-lived *C. elegans* mutants of the insulin/IGF receptor homolog
298 *daf-2* are highly hypoxia resistant (Scott et al., 2002) and that *rars-1(gc47)* has a significantly
299 prolonged lifespan (Anderson et al., 2009). Thus, we hypothesized that lifespan and hypoxia
300 resistance would covary with translation factor knockdown. As previously shown for the *rars-*
301 *1(gc47)* mutation (Anderson et al., 2009), *rars-1*(RNAi) did result in a significant increase in
302 lifespan (Figure 6F,I). Likewise, *ifg-1* and *rps-15* RNAi animals were long lived (Figure 6C,H,I).
303 However, the five other RNAis did not significantly increase lifespan (Figure 6A,B,D,E,G,I). The
304 overall correlation of the lifespan and hypoxic survival phenotypes was not significant (Figure
305 6J). Lifespan also failed to correlate with FRAP or oxygen consumption (Figure 6K,L).
306
307 **Resistance to hypoxia and paraquat do not correlate.** Reactive oxygen species are generated
308 upon reoxygenation after hypoxia and can promote cell death (Allen and Bayraktutan, 2009;
309 Niizuma et al., 2009; Raedschelders et al., 2012). Resistance to reactive oxygen species in *C.*
310 *elegans* has been associated with translational suppression (Syntichaki et al., 2007; Wang et al.,
311 2010). Thus, we hypothesized that the hypoxia protective effect of translation factor
312 knockdown was secondary to resistance to oxidative damage. To test this hypothesis, we
313 measured the survival of RNAi-treated worms on agar plates containing the reactive oxygen
314 species generator paraquat. Six of the eight translation factor RNAis tested produced a
315 significant increase in survival on paraquat; *erfa-3* and *iftb-1* RNAis did not (Figure 7A). The
316 correlation of the paraquat and hypoxic survival phenotypes was not significant (Figure 7B).
317 However, the correlation of paraquat survival with lifespan was highly significant (Figure 7C).
318
319 **Resistance to hypoxia and protein misfolding stress strongly correlate.** Hypoxia produces a
320 significant stress on intracellular protein folding homeostasis and ultimately results in an
321 increase in misfolded proteins (Koumenis and Wouters, 2006; Ge et al., 2007; Rzymski and
322 Harris, 2007; Mao and Crowder, 2010). Misfolded proteins can directly or indirectly promote
323 cell death (Dobson, 2003; Powers et al., 2009). By measuring sensitivity to tunicamycin, which
324 increases the level of misfolded proteins (Merksamer et al., 2008), we tested whether

325 translation factor knockdown generally produces tunicamycin resistance and whether this
326 resistance covaries with hypoxia resistance. All but one translation factor RNAi conferred
327 significant resistance to tunicamycin (Figure 8A). As shown previously (Anderson et al., 2009),
328 *rars-1(gc47)* was strongly tunicamycin resistant. The correlation of resistance to tunicamycin
329 with that to hypoxia was highly significant (Figure 8B).
330

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331 **DISCUSSION**

332 The data presented herein supports four novel conclusions. First, in both mouse and
333 worm, knockdown of multiple translation factors can improve hypoxic survival. Second and
334 quite surprisingly, the hypoxia protection produced by knockdown of translation factors in *C.*
335 *elegans* is not explained entirely by the degree of reduction of translation rate. Third, reduction
336 in oxygen consumption during hypoxic exposure may be the primary mechanism of protection
337 for knockdown of some translation factors but is not necessary for the improved survival
338 produced by reduction of function of rars-1 and perhaps of others. Finally among the factors
339 tested, the survival advantage conferred by translation factor knockdown is most highly
340 correlated with resistance to the protein misfolding agent tunicamycin. We will discuss below
341 the implications and potential limitations of these conclusions.

342 Knockdown of translation factors mediating all three steps of translation, initiation,
343 elongation, and termination, as well as a knockdown of two ribosomal subunits were protective
344 against hypoxic death in *C. elegans*. After accounting for the effect on translation rate (Figure
345 4C), there was clearly a large range of efficiencies for hypoxic protection with knockdown of the
346 translation termination factor ERFA-3 having decidedly the weakest protective effect for the
347 level of translational suppression and IFTB-1 knockdown the strongest. IFTB-1 encodes eIF2β,
348 which is one of three subunits of the eIF2 initiation factors. Given the strong correlation of
349 resistances to misfolded protein stress and hypoxia, it is interesting to speculate that the highly
350 effective hypoxic protection derived from *iftb-1* knockdown is related to a reduction in the
351 levels of the eIF2:GTP:met-tRNAi ternary initiation complex. Reduction in this ternary complex,
352 not only reduces global translation rate, but can also increase initiation from non-AUG codons
353 (Marintchev and Wagner, 2004) in a manner similar to and perhaps synergistic with that
354 produced by activation of PERK, a component of the endoplasmic reticulum unfolded protein
355 response (UPR^{ER}). Upon activation by unfolded protein stress such as that produced by hypoxia,
356 PERK phosphorylates eIF2α and reduces global translation rate while paradoxically increasing
357 the translation of certain transcripts that help to ameliorate misfolded protein stress (Vattem
358 and Wek, 2004; Zhou et al., 2008). In mammalian cells, PERK minus cells are significantly
359 hypersensitive to hypoxia, and mutation of the PERK-phosphorylated serine on eIF2α also

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360 increases hypoxic sensitivity (Bi et al., 2005). In *C. elegans*, hypoxia does induce PERK-
361 dependent phosphorylation of eIF2α, but a PERK-1 loss-of-function mutant does not appear to
362 be hypersensitive to hypoxia, at least in a wild type background (Mao and Crowder, 2010). On
363 the other hand, a reasonable hypothesis for the weakly protective effect of the translation
364 termination factor ERFA-3 derives from the fact that eRF3 depletion should produce an increase
365 in nascent polypeptides still attached to ribosomes as well as novel polypeptides produced by
366 read through of stop codons (Chauvin et al., 2005; Dever and Green, 2012). This relative
367 increase in nascent polypeptides relative to mature protein as well as prolonged association
368 with the translation complex would be expected to increase the level of misfolded proteins
369 (Guerriero and Brodsky, 2012) and might mitigate the hypoxia protective effects from the
370 reduction in overall translation rates.

371 Prior to this work, the essentially universal and widely stated assumption was that the
372 improved survival from translational suppression derived from reduced oxygen consumption
373 (Hochachka et al., 1996; Liu and Simon, 2004; Fahling, 2009; Wheaton and Chandel, 2011).
374 While reasonable, this assumption was not experimentally tested. Our data demonstrate that
375 the oxygen consumption mechanism cannot explain the protection derived from knockdown of
376 at least one translation factor, *rars-1*, where knockdown does not significantly reduce oxygen
377 consumption. As we cannot independently manipulate oxygen consumption and translation
378 factor levels, it is unclear whether the oxygen consumption reductions produced by the
379 knockdown of the other translation factors are essential to their protective phenotype.
380 However, the strong correlation of hypoxic survival and oxygen consumption for knockdown of
381 all but *rars-1* and *eef-2* suggests that the oxygen consumption mechanistic hypothesis could be
382 correct for many translation factors. What is unique about the *rars-1* mechanism is unclear.
383 Whatever the mechanism, it is capable of improving survival when applied after hypoxic
384 survival.

385 A limitation of our results is the lack of anatomical expression or functional data for
386 most of the *C. elegans* translation machinery genes studied. Thus, one possibility for the lack of
387 correlation of oxygen consumption or FRAP with hypoxic survival is that the expression and,
388 more importantly, the function of some of the translation factor genes might not be ubiquitous.

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389 For example, if *rars-1* were functionally important in only a specific cell type that strongly
390 controls organismal hypoxic survival but that did not contribute greatly to organismal oxygen
391 consumption, then reduction of *rars-1* function could strongly improve hypoxic survival without
392 significantly altering oxygen consumption. In other words, *rars-1* might function at a cell
393 autonomous level to control translation rate and oxygen consumption and secondarily hypoxic
394 survival of that cell but at an organismal level *rars-1*-regulated hypoxic sensitivity might be cell
395 non-autonomous. Without detailed cell specific functional data for all three traits (hypoxic
396 sensitivity, oxygen consumption, translation rate) for *rars-1* and the other translation factor
397 genes, we cannot rule out cell specific functions for some of these genes as an explanation for
398 the lack of correlation of these traits at an organismal level.

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

411 An alternative hypothesis is that depletion of specific translation factors reduces
412 translation machinery aggregation produced by hypoxia. Components of the translation
413 machinery have been found to be aggregated after hypoxia and this aggregation may promote
414 or reduce cell death depending on the experimental model (Jamison et al., 2008; Buchan and
415 Parker, 2009) . Further, depletion of specific translation factors has differential effects on
416 translation machinery aggregation (Mokas et al., 2009). One reasonable hypothesis is that
417 reduction of some translation factors such as RARS-1 strongly alter the level of translation

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418 machinery aggregation produced by hypoxia in a way that improves recovery after hypoxia and
419 increases resistance to protein misfolding agents. This hypothesis remains to be tested.
420
421
422

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423

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426

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432

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551 **Figure Legends**

552 **Figure 1** Survival of mouse hippocampal neurons after hypoxia with translation factor
553 knockdown. **A.** Survival of mouse hippocampal neuron cultures after hypoxia when infected
554 with lentiviruses expressing shRNAs targeting translation factors. At least two distinct lentiviral
555 constructs (designated by an A, B, or C suffix) were effective for three of the four genes without
556 overt toxicity; only one Nars targeting virus was effective without toxicity. data are a mean ±
557 sem of a minimum of three independent trials * - $p < 0.01$ versus luciferase virus control, 1-way
558 ANOVA with Dunnett's multiple comparison correction. **B.** qRT-PCR shows > 4-fold knockdown
559 for all four translation factor transcripts. Gene names: Ei2b2 - eukaryotic translation initiation
560 factor 2B, subunit 2 beta, Gars - glycyl-tRNA synthetase, Nars - asparaginyl-tRNA synthetase,
561 Yars - tyrosyl-tRNA synthetase
562
563 **Figure 2** Organismal hypoxic survival of *C. elegans* with translation factor knockdown. **A.** *C.*
564 *elegans* translation machinery genes studied. **B.** *C. elegans* hypoxic survival after RNAi
565 knockdown of the various translation genes. Wild type *C. elegans* were grown from embryo to
566 adult on the particular RNAi bacteria, exposed to hypoxia at 26° for 20 hours, recovered for 24
567 hours, and scored for organismal survival. data are mean ± sem of a minimum of 3 independent
568 trials with at least 70 animals/trial. * - $p < 0.01$ versus vector control, 1-way ANOVA with
569 Dunnett's multiple comparison correction.
570

571 **Figure 3** Correlation of translational suppression with hypoxia resistance for *C. elegans*
572 translation factor knockdown. **A.** Adult animals were incubated on cycloheximide plates for 2
573 hours, at which time they were removed for photobleaching then returned to the same
574 cycloheximide plates for 24 hours before the fractional fluorescent recovery after
575 photobleaching (FRAP) was measured. **B.** FRAP normalized to L4440 vector control values for
576 young adult animals exposed to RNAi from embryo to adult. Data are mean ± sem of 6-22
577 animals. * - $p < 0.05$ versus vector control, 1-way ANOVA with Dunnett's multiple comparison
578 correction. **C.** Correlation of FRAP with hypoxic survival for the various RNA conditions is not
579 significant.

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580

581 **Figure 4** Correlation of oxygen consumption with hypoxia resistance for translation factor
582 knockdown/mutant animals. **A.** One day post L4 adult animals were incubated on
583 cycloheximide plates for 24 hours, at which time oxygen consumption was measured. Worms
584 were then lysed and protein concentration measured for normalization. Data are mean ± sem
585 of a minimum of three independent trials; error bars are smaller than the data points in most
586 cases. **B.** Oxygen consumption for young adult animals exposed to RNAi from embryo to adult.
587 Data are mean ± sem of a minimum of three independent trials. * - $p < 0.05$ versus vector
588 control, 1-way ANOVA with Dunnett's multiple comparison correction. **C.** Correlation of oxygen
589 consumption with hypoxic survival for the various RNAi conditions is not significant.
590

591 **Figure 5** Effect post-hypoxia RNAi knockdown of *rars*-1 and *iftb*-1 on survival. **A.** wild type N2
592 were grown under four RNAi conditions: 1) on L4440 empty vector from egg to adult and
593 before and after a 10 hour hypoxic exposure (L4440/L4440); 2) on *rars-1*(RNAi) bacteria from
594 egg to adult and before and after hypoxic exposure (*rars-1/rars-1*); 3) on *rars-1*(RNAi) from egg
595 to adult and before hypoxia with L4440 after hypoxia during recovery (*rars-1*/L4440); 4) on
596 L4440 from egg to adult and before hypoxia with *rars-1*(RNAi) after hypoxia during recovery
597 (L4440/*rars-1*(RNAi). Death was scored after each day of recovery. **B.** N2 incubated with *iftb-*
598 *1*(RNAi) before and after, only before, or only after a 10 hour hypoxic exposure. **C.** Percentage
599 dead after four days of recovery under the various conditions. Data are mean ± sem of at least
600 5 independent trials/condition. * $p < 0.05$ versus L4440/L4440 by two-tailed t-test. **D.** Short
601 exposure to *iftb-1* and *rars-1* RNAi prior to hypoxia. Animals were grown to adults on OP50
602 bacteria then exposed for 24 hours to empty vector (L4440), *iftb-1* RNAi, or *rars-1* RNAi then
603 washed free of bacteria, incubated for 20 hours in hypoxia, recovered for 24 hours on OP50
604 bacteria then scored. Data are mean ± sem of 3 independent trials/condition. * $p < 0.05$ versus
605 L4440 by two-tailed t-test.
606

607 **Figure 6** Correlations of lifespans of translation factor knockdown animals with oxygen
608 consumption, translation rate, and hypoxia resistance. **A-H.** Lifespan measured from egg

609 onward. 50 animals/condition were scored with RNAi applied from egg onward. **I.** Median
610 lifespans. * - $p < 0.01$ Log-rank test vs L4440 vector control. **J-L.** Lifespan does not correlate with
611 hypoxic survival, FRAP, or oxygen consumption.
612

613 **Figure 7** Correlation of paraquat resistance with lifespan and hypoxic survival with translation
614 factor knockdown. **A.** Kaplan-Meier curves were measured on 50 worms/condition to calculate
615 median duration of survival on 4 mM paraquat agar plates. Worms were transferred to plates
616 containing paraquat on day 7 after egg lay. * - $p < 0.01$ Log-rank text **B.** Survival duration on
617 paraquat does not correlate with hypoxic survival. **C.** Survival duration on paraquat correlates
618 significantly with lifespan.
619

620 **Figure 8** Correlation of tunicamycin resistance with hypoxic survival with translation factor
621 knockdown or mutation. **A.** Percentage of animals reaching adulthood four days post
622 embryonic stage on plates containing 1 µg ml^{-1} tunicamycin. Data are mean ± sem of six
623 independent trials. * - $p < 0.01$, Fisher's exact test. **B.** Tunicamycin resistance correlates
624 significantly with hypoxia resistance.
625

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A

*

Survival (%)

100
80
60
40
20
0

Luciferase
Eif2b2 A
Eif2b2 B
Gars A
Gars B
Gars C
Nars
Yars A
Yars B

B

Relative [mRNA] %

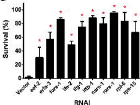
150
100
50
0

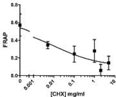
shRNA
Luciferase
Target

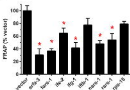
Eif2b2
Gars
Nars
Yars

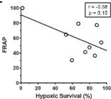
A

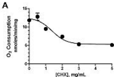
Gene name	Gene product
eef-2	EEF2, translation elongation factor
erfa-3	ERF3A translation termination factor
fars-1	Phenylalanyl-tRNA synthetase
ife-2	EIF4E cap binding protein
ifg-1	EIF4G cap binding protein
iftb-1	EIF2beta translation initiation factor
nars-1	Asparaginyl-tRNA synthetase
rars-1	Arginyl-tRNA synthetase
rpl-6	Large ribosomal subunit L6 protein
rps-15	Small ribosomal subunit S15 protein

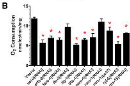


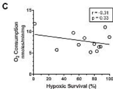

A
FRAP
[CHX] mg/ml

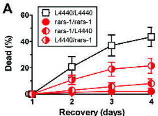

B
FRAP (% vector)
vector
erfa-3
fars-1
ife-2
iftg-1
iftb-1
nars-1
rars-1
rps-15

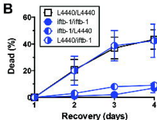

C
FRAP
Hypoxic Survival (%)
r = -0.58
p = 0.10

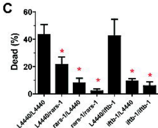

A
O2 Consumption
nmoles/min/mg
[CHX], mg/mL
0
1
2
3
4
5
15
10
5
0

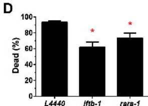

B
O2 Consumption
nmoles/min/mg
15
10
5
0
Vector

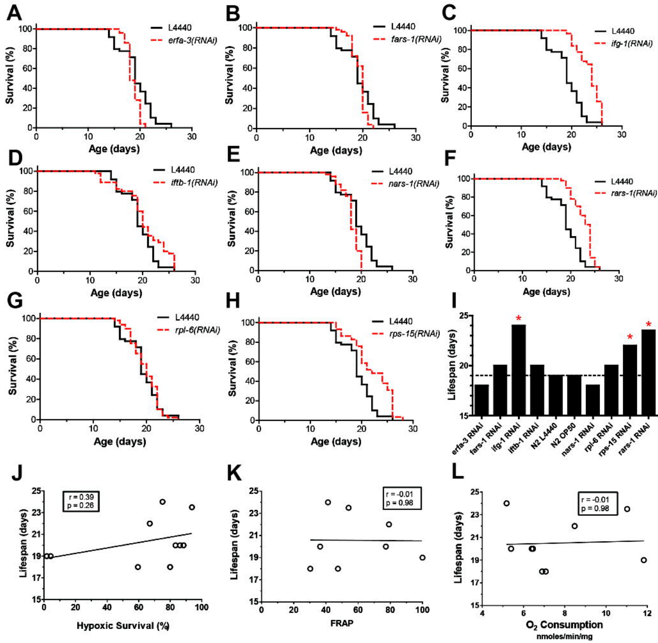

C
O2 Consumption
nmoles/min/mg
Hypoxic Survival (%)
r = -0.31
p = 0.33
15
10
5
0
0
20
40
60
80
100

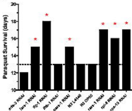

A
L4440/L4440
rars-1/rars-1
rars-1/L4440
L4440/rars-1
Dead (%)
Recovery (days)

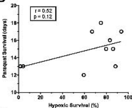

B
L4440/L4440
iftb-1/iftb-1
iftb-1/L4440
L4440/iftb-1
Dead (%)
Recovery (days)

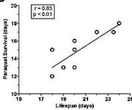

C
Dead (%)
L4440/L4440
L4440/rars-1
rars-1/L4440
rars-1/rars-1
L4440/iftb-1
iftb-1/L4440
iftb-1/iftb-1


D
Dead (%)
L4440
iftb-1
rars-1


A
B
C
D
E
F
G
H
I
J
K
L
L4440
erfa-3(RNAi)
fars-1(RNAi)
ifg-1(RNAi)
iftb-1(RNAi)
nars-1(RNAi)
rars-1(RNAi)
rpl-6(RNAi)
rps-15(RNAi)
Survival (%)
Age (days)
Lifespan (days)
erfa-3 RNAi
fars-1 RNAi
ifg-1 RNAi
iftb-1 RNAi
N2 L4440
N2 OP50
nars-1 RNAi
rpl-6 RNAi
rps-15 RNAi
rars-1 RNAi
r = 0.39
p = 0.26
Hypoxic Survival (%)
r = -0.01
p = 0.98
FRAP
r = -0.01
p = 0.98
O2 Consumption
nmoles/min/mg


A
Parasquat Survival (days)
20
18
16
14
12
10
iars-1 RNAi
ifg-1 RNAi
N2 L4440
N2 OP50
rpl-6 RNAi
rps-15 RNAi


B
r = 0.52
p = 0.12
Paraquat Survival (days)
Hypoxic Survival (%)
0
20
40
60
80
100


C
r = 0.83
p < 0.01
Paraquat Survival (days)
Lifespan (days)
15
17
19
21
23
25

A

% Adults

N2 L4440
eifa-3 RNAi
tars-1 RNAi
iftg-1 RNAi
ltd-1 RNAi
mars-1 RNAi
rpl-6 RNAi
rps-15 RNAi
cars-1 RNAi
rars-1(gc47)

B

r = 0.80
p < 0.01

% Adults

Hypoxic Survival (%)