Fasting prevents hypoxia-induced defects of proteostasis in *C. elegans*

Short title: Fasting prevents hypoxia-induced defects in proteostasis

Nicole N Iranon[1,2], Bailey E Jochim[2], and Dana L Miller[2]

[1] Graduate Program in Molecular and Cellular Biology, University of Washington School of Medicine, Seattle WA 98195-7350, USA
[2] Department of Biochemistry, University of Washington School of Medicine, Seattle WA 98195-7350, USA

Address correspondence to DLM: dlm16@uw.edu

**Abstract**

Low oxygen conditions (hypoxia) can impair essential physiological processes and cause cellular damage and death. We have shown that specific hypoxic conditions disrupt protein homeostasis in *C. elegans*, leading to protein aggregation and proteotoxicity. Here, we show that nutritional cues regulate this effect of hypoxia on proteostasis. Animals fasted prior to hypoxic exposure develop dramatically fewer protein aggregates compared to their fed counterparts, indicating that the effect of hypoxia is abrogated. Fasting is effective at protecting against hypoxia-induced proteostasis defects in multiple developmental stages, tissues, and in different models of misfolded or aggregation prone proteins. Our data also demonstrate that the effect of fasting is induced and reversed quite rapidly, suggesting that the nutritional environment experienced at the onset of hypoxia dictates at least some aspects of the physiological response to hypoxia. We further demonstrate that the insulin/IGF-like signaling pathway plays a role in mediating the protective effects of fasting in hypoxia. Animals with mutations in *daf-2*, the *C. elegans* insulin-like receptor, display wild-type levels of hypoxia-induced protein aggregation upon exposure to hypoxia when fed, but are not protected by fasting. However, we found that DAF-2 acts independently of the FOXO transcription factor, DAF-16, to mediate the protective effects of fasting. These results suggest a non-canonical role for the insulin/IGF-like signaling pathway in coordinating the effects of hypoxia and nutritional state on proteostasis.

**Author Summary**

When blood flow to various parts of the body becomes restricted, those tissues suffer from a lack of oxygen, a condition called hypoxia. Hypoxia can cause cellular damage and death, such as is observed as a result of stroke and cardiovascular disease. We have found that in the model organism *C. elegans* (a roundworm) specific concentrations of hypoxia cause aggregation of polyglutamine proteins – the same kind of proteins that are found in an aggregated state in the neurodegenerative disorder Huntington's disease. Here, we show that that worms can be protected from hypoxia-induced protein aggregation if they are fasted (removed from their food source) prior to experiencing hypoxia. Furthermore, we show that the insulin receptor is required for this protection. The insulin receptor is responsible for detecting insulin, a hormone that is released after feeding. Worms with a nonfunctional version of the insulin receptor displayed hypoxia-induced protein aggregation despite being fasted before the hypoxic exposure. Our results highlight a new role for the insulin signaling pathway in coordinating the effects of both hypoxia and nutritional state on protein aggregation.

32 **Introduction**

33 In order to survive in changing conditions, organisms need to successfully integrate a
34 number of environmental signals and respond appropriately in order to maintain homeostasis.
35 Aerobic heterotrophs must meet their requirements for food and oxygen by taking in these
36 resources from the environment. An inadequate response to low levels of oxygen (hypoxia) can
37 lead to cellular damage or death, an unsurprising outcome given oxygen's central role in
38 cellular metabolism. Like hypoxia, food deprivation presents an obstacle to homeostasis by
39 impinging on cellular metabolism and disturbing anabolic pathways. However, in many cases
40 food restriction can have beneficial effects, such as extending lifespan and delaying the onset of
41 neurodegenerative diseases and their associated pathologies [1]. In a mouse model of
42 Alzheimer's disease, 12 weeks of caloric restriction reduces Aβ plaque burden [2], and mice
43 expressing human mutant huntingtin maintained on an alternate-day-feeding diet have
44 reduced brain atrophy and decreased huntingtin aggregate formation [3]. Similarly, depriving *C.*
45 *elegans* of their bacterial food source reduces damage associated with expressing
46 polyglutamine proteins [4].

47 The protective effect of fasting is not limited to symptoms of neurodegeneration – there
48 are many studies that show fasting can protect against damage associated with hypoxia in
49 mammals. For example, mice on an alternate-day feeding regimen have higher survival rates
50 after myocardial ischemia induced via coronary occlusion [5]. Similar results have been
51 obtained with ischemic damage to the liver. Mice on a calorically restricted diet have reduced
52 infarct damage compared to ad-libitum fed controls [6], and mice that have been fasted for 3
53 days display reduced hepatocellular apoptosis and damage [7]. Calorie restriction can also

54 improve outcomes after cerebral ischemic injury by protecting cortical and striatal neurons [8],
55 and reducing neurological deficits and infarct volume [9]. These observations suggest that
56 understanding the mechanistic basis underlying the protective effects of fasting in hypoxia
57 could provide novel insight into therapeutic strategies to treat pathological conditions
58 associated with ischemia and reperfusion injury.

59 We have previously shown that in *C. elegans* the cellular response to specific hypoxic
60 conditions involves a disruption of proteostasis – the coordination of protein synthesis, folding,
61 degradation, and quality control required to maintain a functional proteome [10]. Here we
62 show that fasting prevents the hypoxia-induced disruption of proteostasis. Our data indicate
63 that the nutritional context of an animal at the onset of hypoxia has the power to alter
64 hypoxia's effect on proteostasis and that the insulin-like signaling (IIS) pathway plays a role in
65 fasting's ability to protect against proteostasis decline independently of the canonical
66 downstream transcription factor DAF-16/FOXO.

67

68 **Results**

69 In order to investigate the effect of nutritional status on proteostasis in hypoxia, we first
70 used transgenic *C. elegans* that express yellow fluorescent protein (YFP) fused to a
71 polyglutamine tract in the body wall muscles [11]. We refer to these animals as QX::YFP, where
72 X refers to the number of glutamine residues fused to YFP, such that Q35::YFP animals express
73 YFP with 35 glutamine residues. In these animals, the number of YFP foci, which correspond to
74 large protein aggregates, can be used as an *in vivo* measure of cellular proteostasis [12].

75 Exposing animals to 0.1% oxygen for 24 hours while fed resulted in an increase in the
76 number of YFP foci (Fig. 1B-1D), consistent with a decrease in proteostasis as has been
77 demonstrated previously [10]. However, we found that the number of YFP foci that formed in
78 hypoxia was dramatically reduced if the animals were removed from food for six hours before
79 the hypoxic exposure and remained off of food for the duration of hypoxia (Fig. 1A). Hypoxia-
80 induced protein aggregation (HIPA) was prevented by fasting in fourth-stage larvae (L4)
81 Q35::YFP animals (Fig. 1C) as well as in first-stage larvae (L1) Q40::YFP (Fig. 1D). We conclude
82 that fasting prevents HIPA and that this effect persists across development.
83

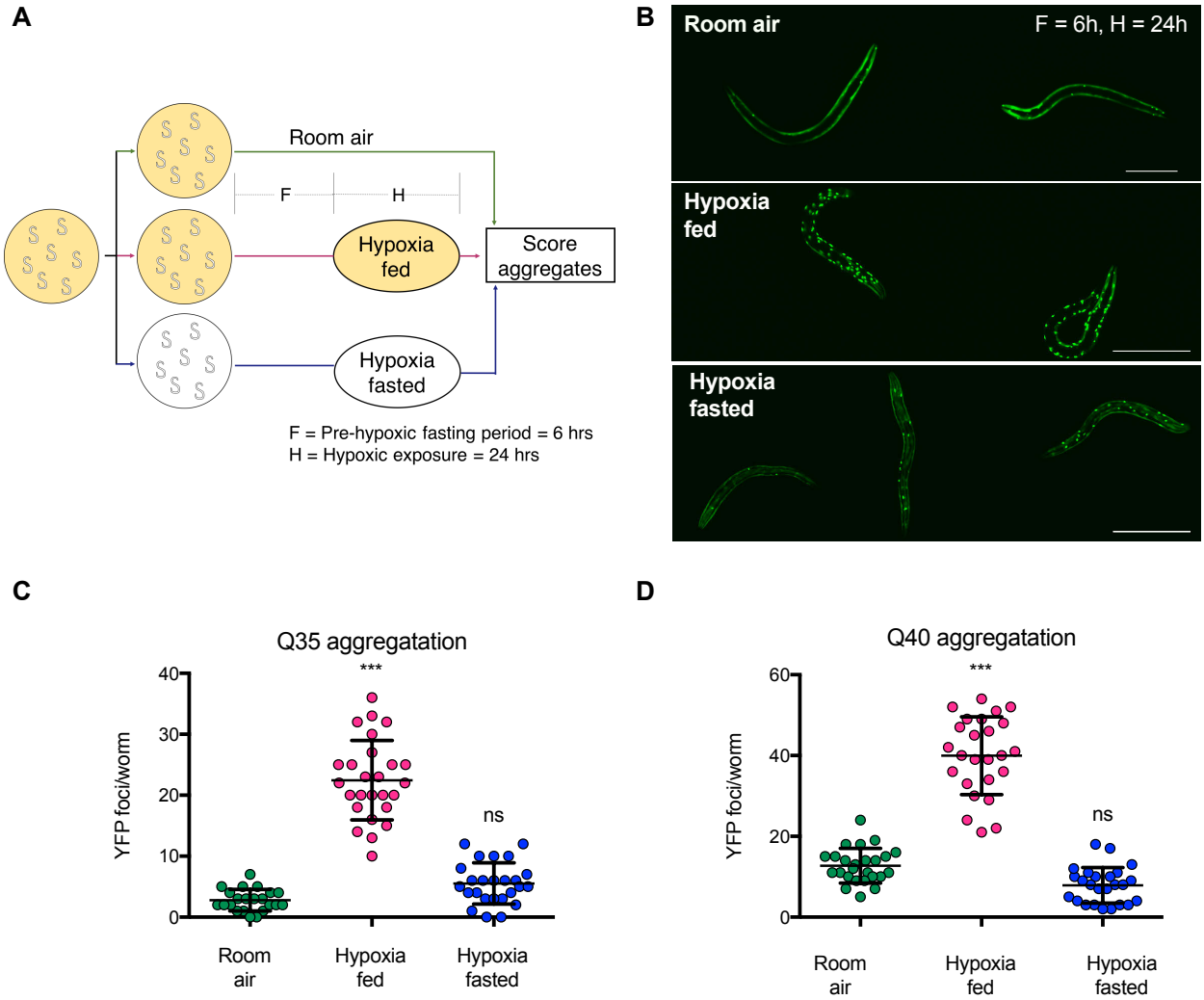



**Figure 1. Fasting protects against hypoxia-induced protein aggregation. A.** Experimental Schematic. Cohorts of age-synchronized animals were split into three groups: the first was maintained on food in room air, the second was maintained on food before and during exposure to hypoxia, and the third was removed from food before exposure to hypoxia. Fasting is indicated by white plates, yellow plates indicate animals on food. F= the duration of fasting (h) before hypoxia; H = duration of hypoxia (h). Unless otherwise noted, aggregates were counted immediately upon removal from hypoxia. **B.** Representative images of Q40::YFP animals from cohorts of animals maintained in room air, exposed to hypoxia on food (hypoxia fed), or exposed to hypoxia while fasted (hypoxia fasted). F=6h, H=24h. Scale bars = 100µm**. C-D.** Aggregation measurements for L4 Q35::YFP (**C**) and L1 Q40::YFP (**D**) animals exposed to hypoxia on food (fed, magenta) or after removal from food (fasted, blue). Controls remained in room air (green). Data from one representative experiment is shown. Each experiment was repeated at least 3 times. Each circle is the number of YFP foci in a single animal, the mean is indicated by the line, and error bars are the standard deviation. Statistical comparisons were made between animals exposed to hypoxia and controls maintained in room air. Significance: *** $p < 0.001$; ns, not significant.

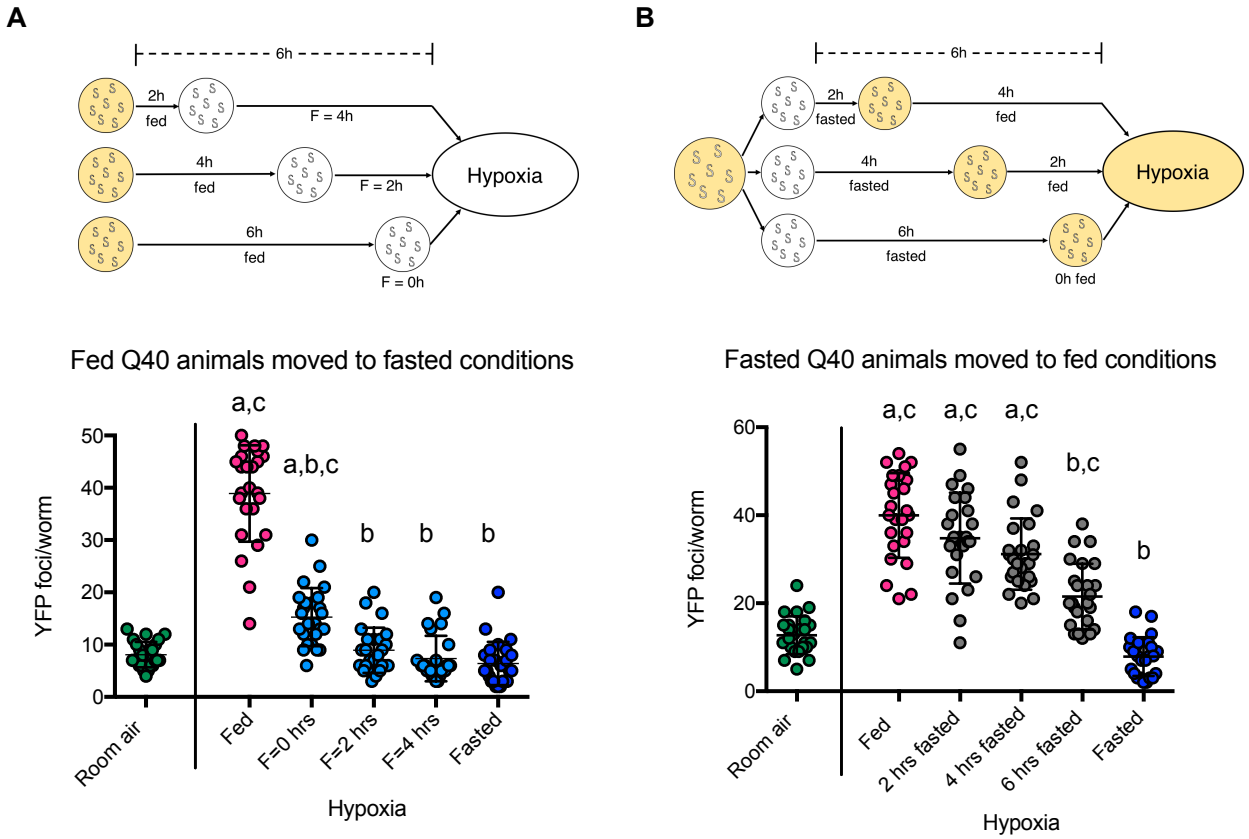


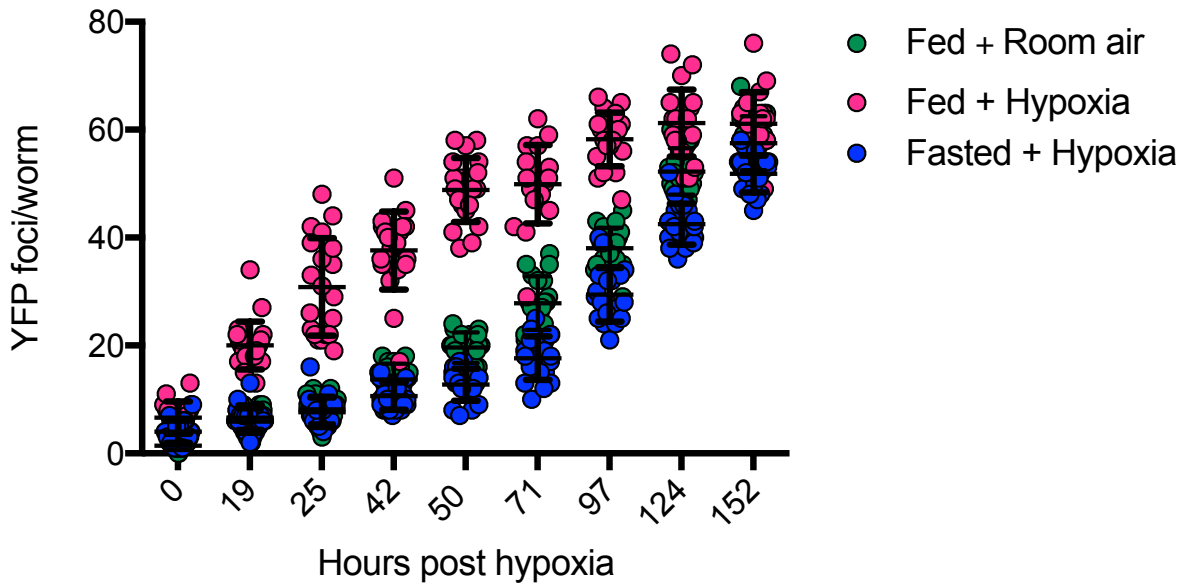

**Figure 2: Fasting protection against HIPA is quickly induced and reversed. A.** Effect of fasting occurs rapidly in hypoxic conditions. Cohorts of L1 Q40::YFP animals were removed from food before exposure to hypoxia (F = 0, 2, or 4 h; H=24 h). All animals were off of food when exposed to hypoxia and the number of foci was scored immediately upon removal from hypoxia (cyan). Controls remained in room air (green), were continuously on food (fed, magenta), or were fasted for a full 6 h before hypoxia (fasted, blue). Data from one representative experiment is shown. Each experiment was repeated at least 3 times. Each circle is the number of YFP foci in a single animal, the mean is indicated by the line, error bars are the standard deviation. Significance was calculated using a Kruskal-Wallis test and Dunn's multiple comparisons post hoc analysis. Significant differences ($p < 0.05$) in aggregation between conditions are indicated by letters above each group as follows: a - significantly different from room air controls; b - significantly different from fed hypoxic controls; c - significantly different from fasted hypoxic controls. **B.** Fasting before exposure to hypoxia improves proteostasis. As shown in the schematic above the graph, cohorts of L1 Q40::YFP animals were removed from food 6h before exposure to hypoxia, and fasted for 2, 4, or 6 h before being returned to food. All cohorts were on food when exposed to hypoxia (H=24 h). The number of foci was scored immediately upon return to room air (gray). Controls remained in room air (green), were continuously on food and exposed to hypoxia (fed, magenta), or were not returned to food before hypoxia (fasted, blue). Data from one representative experiment is shown. Each experiment was repeated at least 3 times. Each circle is the number of YFP foci in a single animal, the mean is indicated by the line,

120 error bars are the standard deviation. Statistical comparisons were made between animals
121 fasted for the indicated amount of time and controls maintained in room air, fed controls
122 exposed to hypoxia after being continuously on food, and fasted controls that were not
123 returned to food before hypoxia. Significance was calculated using a Kruskal-Wallis test and
124 Dunn's multiple comparisons post hoc analysis. Significant differences ($p < 0.05$) in aggregation
125 between conditions are indicated by letters above each group as follows: a - significantly
126 different from room air controls; b - significantly different from fed hypoxic controls; c -
127 significantly different from fasted hypoxic controls.

128 We originally chose to fast animals for 6h before exposure to hypoxia to allow animals
129 time to alter gene expression [13], and this period of time off of food is sufficient to deplete
130 stored glycogen as measured by iodine staining (DLM unpublished). However, there is no
131 evidence to suggest that the protective effects of fasting in hypoxia requires changes in gene
132 expression or glycogen stores. Therefore we next measured how long of a fasting period was
133 required to mitigate the effects of hypoxia on aggregation of polyglutamine proteins.

134 To determine the pre-hypoxia fasting duration required to protect against HIPA, we
135 removed Q35::YFP animals from food for varying lengths of time before being exposed to
136 hypoxia (as diagrammed in Fig. 2A). We found that animals removed from food immediately
137 before exposure to hypoxia developed significantly fewer YFP foci in hypoxia as compared to
138 controls that remained on food in hypoxia (Fig. 2A, 6h fed compared to fed). We conclude that
139 extended fasting before exposure to hypoxia is not required to prevent HIPA. Instead, our data
140 show that the protective effects of fasting occur very rapidly. In fact, the full protection against
141 HIPA is realized with only 2h fasting before exposure to hypoxia (Fig. 2A). These results suggest
142 that at least some of the protective effects of fasting are due to the absence of food directly,
143 rather than metabolic changes or alterations in gene expression that occur during fasting prior
144 to the hypoxic insult.

145 Work in other systems has shown that fasting can have a protective effect that persists
146 even after animals are returned to food (Robertson and Mitchell 2014). To further explore the
147 requirements for fasting to protect against HIPA we next asked whether the protective effects
148 of fasting against HIPA could be reversed. In these experiments (Fig. 2B), we began fasting
149 animals 6h before exposure to hypoxia but then returned the animals to food prior to initiation
150 of hypoxia. We observed that animals fasted for a full 6h and then returned to food
151 immediately before exposure to hypoxia (Fig. 2B, 6h fasted) developed significantly more YFP
152 foci than animals that were fasted for 6h and then exposed to hypoxia in the absence of food
153 (Fig 2B, fasted), suggesting that the nutritional context of an animal as it experiences hypoxia is
154 able to mediate the effect of hypoxia on proteostasis. Furthermore, we found no protection
155 from HIPA if animals were fasted for 4h, but then fed for 2 h before exposure to hypoxia (Fig.
156 2B, 4h fasted), even though 4h of fasting was sufficient for complete protection against HIPA in
157 the absence of food (Fig. 2A, 2h fed). This result indicates that the protective effects of fasting
158 are fully reversed within 2h of return to food. We conclude that the protective effects of fasting
159 in hypoxia are rapidly reversed.
160




161

162 **Figure 3: Fasting protects against long-term effects of hypoxia on proteostasis.**
163 Cohorts of L4 Q35::YFP animals were exposed to hypoxia (H=10 h) on food (magenta) or fasted
164 (blue, F=6h). Controls remained in room air on food (green). The number of YFP foci was scored
165 after return to room air as indicated. Data from one representative experiment is shown. The
166 experiment was repeated at least 3 times. Each cohort included at least 20 animals per time
167 point.
168

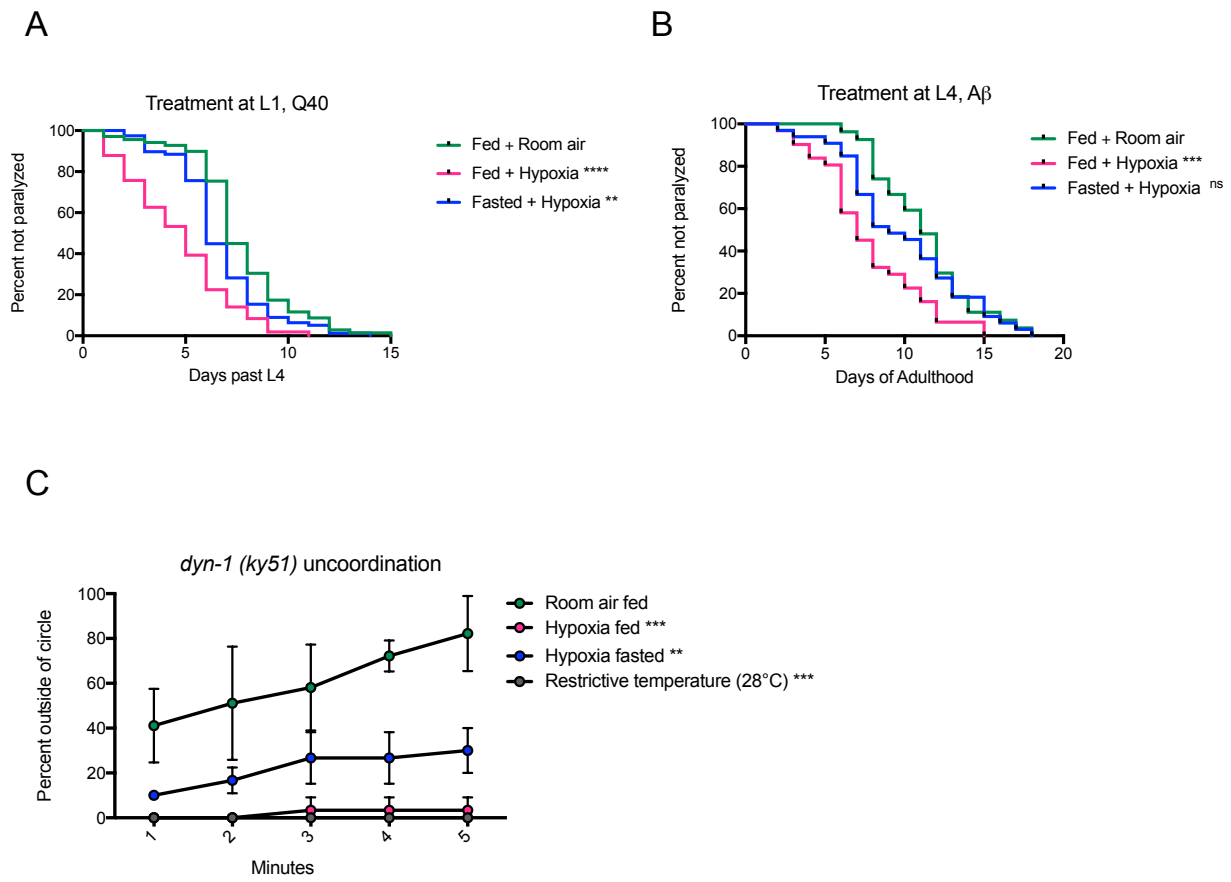
169 Shorter exposures to hypoxia that do not immediately increase the number of
170 polyglutamine protein aggregates still disrupt long-term proteostasis, as evidenced by the
171 increased rate of age-associated protein aggregation after return to room air [10]. We
172 therefore asked whether fasting could protect against these long-term proteostasis deficits in
173 addition to HIPA. We exposed Q35::YFP L4 animals to hypoxia for only 10h either in the fed
174 state or after fasting for 6h (F = 6 hours, H = 10 hours as per Fig. 1A). Control animals remained
175 on food in room air. Immediately after this short hypoxic exposure, there was no observed
176 increase in the number of YFP foci in animals exposed to hypoxia regardless of whether food
177 was present (Fig. 3, 0 hours post-hypoxia). As expected, the animals exposed to hypoxia in the
178 fed state accumulate aggregates faster than control animals. In contrast, animals exposed to

179 hypoxia while fasted accumulate YFP foci at the same rate as control animals. These data

180 indicate that fasting both prevents HIPA and protects against the long-term effects on

181 proteostasis induced by a short exposure to hypoxia.

182




183

184 **Figure 4. Fasting has general protective effects against hypoxia-induced defects in**
185 **proteostasis. A.** Fasting protects against toxicity of Q40::YFP. Cohorts of L1 animals expressing
186 Q40::YFP were exposed to hypoxia on food (magenta), or fasted (blue) before exposure to
187 hypoxia (F=6h, H=24 h). Paralysis was scored after return to room air, beginning the first day of
188 adulthood. Controls remained on food in room air (green). Data from one representative
189 experiment is shown, each cohort included at least 70 animals. Each experiment was repeated
190 at least 3 times. Significance was calculated using a Log-rank (Mantel-Cox) test with a
191 Bonferroni correction for multiple comparisons. Statistical comparisons were made between
192 animals exposed to hypoxia and animals maintained in room air. **** $p < 0.0001$; ** $p < 0.01$.
193 **B.** Fasting protects against toxicity of $A\beta_{1-42}$. Cohorts of L4 animals expressing $A\beta_{1-42}$ were
194 exposed to hypoxia on food (magenta) or fasted (blue) before exposure to hypoxia (F=6h,
195 H=24h). Paralysis was scored after return to room air, beginning at the first day of adulthood.

196 Controls remained on food in room air (green). Data from one representative experiment is
197 shown, each cohort included at least 70 animals. Each experiment was repeated at least 3
198 times. Significance was calculated using a Log-rank (Mantel-Cox) test with a Bonferroni
199 correction for multiple comparisons. Statistical comparisons were made between animals
200 exposed to hypoxia and animals maintained in room air. *** $p < 0.001$. **C.** Fasting protects
201 against hypoxia effects on metastable DYN-1. Temperature-sensitive *dyn-1(ky51)* mutant
202 animals were exposed to hypoxia at the permissive temperature on food (magenta), or after
203 fasting (blue). Controls remained on food in room air at the permissive temperature (green) or
204 on food at the non-permissive temperature (28°C, gray). Paralysis was scored 1h after return to
205 room air. Average data from 3 independent experiments is shown, each cohort included 10
206 animals. Significance was calculated using a repeated measures two-way ANOVA and Dunnett's
207 multiple comparisons test. Statistical comparisons were made between animals exposed to
208 hypoxia or animals maintained at the restricted temperature and animals maintained in room
209 air. Significance: *** $p < 0.001$; ** $p < 0.01$
210

211 The cellular role of protein aggregates is controversial, with some reports finding a
212 protective role and others suggesting a cytotoxic effect [14]. We have previously shown that
213 aggregates induced by hypoxia are cytotoxic, resulting in accelerated paralysis after animals are
214 returned to room air [10]. We therefore next asked if fasting would protect against increased
215 proteotoxicity in addition to HIPA. To address this, we exposed cohorts of L1 Q40::YFP animals
216 to hypoxia for 24 hours while fed or fasted, then returned the animals to room air and
217 measured the onset of paralysis in each cohort. We found that fasting slowed the rate at which
218 paralysis developed relative to animals exposed to hypoxia while fed (Fig. 4A). This result
219 indicates that fasting protects against hypoxic effects of increased protein aggregation and
220 proteotoxicity.

221 We next sought to determine whether fasting's protective effects on proteostasis
222 extend to other models of proteotoxicity. Human amyloid β $(A\beta)_{1\text{-}42}$ peptide expressed in the
223 body wall muscles of *C. elegans* results in cytoplasmic plaque formation, with a subsequent
224 phenotype of progressive paralysis [15]. *C. elegans* expressing $A\beta_{1\text{-}42}$ in their body wall muscles

225 become paralyzed more quickly when they are exposed to hypoxia [10]. We found that this
226 effect of hypoxia was reversed by fasting, as the rate that paralysis develops is slowed if animals
227 expressing $A\beta_{1-42}$ are exposed to hypoxia while fasting (Fig. 4B). Because $A\beta_{1-42}$ and Q40::YFP are
228 both expressed in body wall muscles, we also evaluated if fasting protected animals expressing
229 a metastable version of the neuronal dynamin protein DYN-1 from the effects of hypoxia. The
230 *dyn-1(ky51)* mutant contains a temperature-sensitive (ts) mutation, such that the DYN-1
231 protein is functional and *dyn-1(ky51)* mutant animals exhibit wild-type motility at the
232 permissive temperature (20°C), but become uncoordinated at the restrictive temperature
233 (28°C) due to improper folding of the DYN-1 protein [16]. Genetic and environmental factors
234 that disrupt proteostasis, including hypoxia, prevent the proper folding of the DYN-1 protein at
235 the permissive temperature, thereby rendering the *dyn-1(ky51)* animals uncoordinated [17,
236 10]. Similar to our experiments with Q40::YFP and $A\beta_{1-42}$, we found that fasting *dyn-1(ky51)*
237 mutant animals before exposure to hypoxia results in a partial rescue of hypoxia-induced
238 uncoordination at the permissive temperature (Fig. 4C). Together, our results suggest that
239 fasting has a general protective effect against proteostasis defects induced by hypoxia, and that
240 this protective effect is not specific to a particular tissue, developmental stage, or
241 misfolded/aggregation prone model.
242 Dysregulation of insulin-like signaling (IIS) has been tied to protein aggregation and
243 neurodegeneration in a number of model organisms [18]. As the IIS pathway links food
244 availability to growth, development, stress resistance, and aging, we hypothesized that changes
245 in IIS could explain how fasting modulates the effect of hypoxia on proteostasis. The IIS

246 pathway is widely conserved in metazoans [19]. We therefore explored the hypothesis that IIS
247 would mediate the effects of fasting to prevent HIPA.
248 We first looked at the localization of DAF-16::GFP in animals exposed to hypoxia to
249 determine if IIS is active in hypoxia. DAF-16 is the *C. elegans* orthologue of the FOXO
250 transcription factor. When active, the insulin/IGF-like receptor DAF-2 initiates a
251 phosphorylation cascade that results in the phosphorylation and nuclear exclusion of DAF-16
252 protein [20, 21]. Conversely, when nutrients are scarce, DAF-16 remains unphosphorylated by
253 upstream kinases and is able to enter the nucleus and bind to its target genes [20, 22]. We
254 found that DAF-16::GFP remained diffuse and cytoplasmic in control worms maintained in room
255 air on food (Fig 5B, 5C), but accumulated in the nucleus of animals that were removed from
256 food in room air (Fig. 5B, 5C) or were exposed to hypoxia on food (Fig. 5B, 5C). These results
257 suggest that IIS activity is reduced by fasting and hypoxia, consistent with previous reports [23,
258 24]. Surprisingly, DAF-16::GFP did not accumulate in the nuclei of animals exposed to hypoxia
259 after fasting (Fig 5B, 5C), despite hypoxia and fasting both individually resulting in nuclear
260 accumulation.
261 These DAF-16::GFP localization patterns led us to interrogate requirements for DAF-16
262 and the upstream IIS receptor DAF-2 in mediating fasted and fed responses to hypoxia. To this
263 end, we crossed the Q35::YFP transgene into *daf-2(e1370)* and *daf-16(mu86)* backgrounds. The
264 fact that DAF-16::GFP is localized to the nucleus in fed animals exposed to hypoxia suggests the
265 possibility that DAF-16 facilitates HIPA. We found that *Q35::YFP; daf-16(mu86)* mutant animals
266 exhibit robust HIPA on food (Fig 5D), indicating that DAF-16 is not required for HIPA despite its
267 nuclear accumulation in fed hypoxic animals. We also asked if there was a genetic requirement

268 for the IIS receptor DAF-2. Our data indicate that IIS does not mediate the effects of hypoxia on
269 proteostasis in fed animals, as *Q35::YFP; daf-2(e1370)* mutant animals exhibit robust HIPA
270 when fed (Fig. 5D). Thus, neither DAF-16 nor DAF-2 activities are required for HIPA in fed
271 animals.

272 Given the IIS-independent nature of HIPA in fed animals, we next investigated whether
273 fasting protection requires IIS. We discovered that DAF-2, but not DAF-16 is required for fasting
274 protection against HIPA. Fasting protects the *Q35::YFP; daf-16(mu86)* similar to wild-type (Fig
275 5D); however, we observe significant HIPA when *Q35; daf-2(e1370)* and *Q35; daf-2(e1368)*
276 mutant animals are exposed to hypoxia when fasted (Fig 5D and Supplemental Fig. 1). These
277 results show that protective effects of fasting in hypoxia require DAF-2, but not DAF-16. This is
278 consistent with our observation that DAF-16::GFP is not localized to the nucleus in fasted
279 animals exposed to hypoxia (Fig 5B, 5C).

280 We found that the insulin/IGF-like receptor DAF-2 mediates the protective effects of
281 fasting on HIPA, while the the FOXO transcription factor DAF-16 is not required for protection.
282 Given this finding, we also checked the DAF-16::GFP localization pattern in worms with a *daf-*
283 *2(e1370)* mutation. These mutants have constitutively nuclear DAF-16 in the fed state due to
284 decreased signaling through the IIS pathway [20]. Since DAF-16::GFP is not localized to the
285 nucleus in fasting-protected wild-type animals exposed to hypoxia, we sought to investigate
286 whether the nuclear localization of DAF-16 in *daf-2(e1370)* mutants, which are not protected by
287 fasting, would be altered by hypoxia. We found that DAF-16::GFP is fully nuclear in all
288 conditions, including fasted hypoxia, in these animals (Supplemental Fig. 2).

289 In *C. elegans*, DAF-16 mediates the effects of decreased signaling through DAF-2.

290 Mutations in *daf-16* suppress most *daf-2* mutant phenotypes including increased lifespan,

291 enhanced dauer formation, increased fat storage, reproductive delays, and increased resistance

292 to heat and oxidative stress. [25, 26]. This coupled with the nuclear localization of DAF-16::GFP

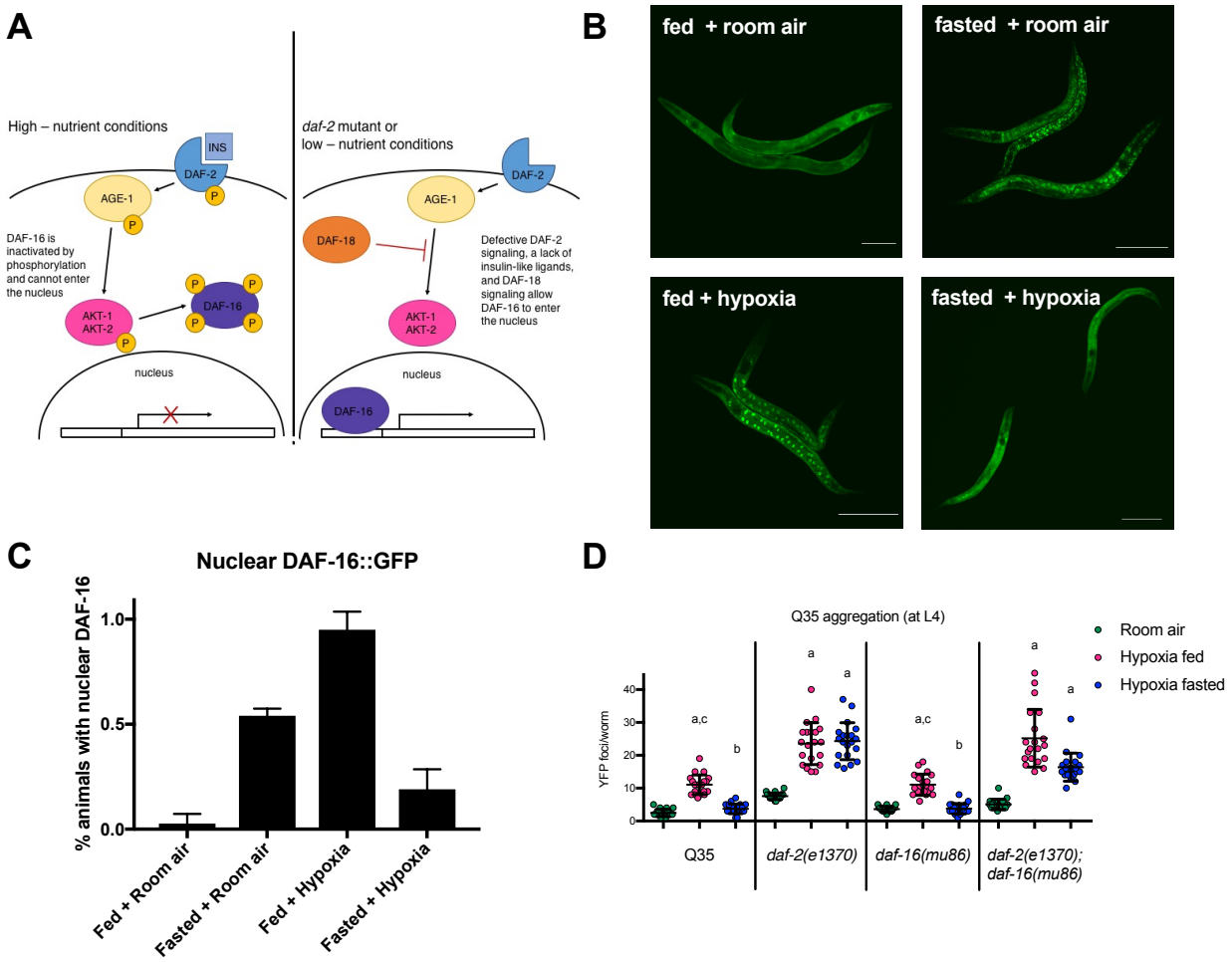
293 in *daf-2* mutants led us to hypothesize that *daf-16* would be required for the HIPA in fasted

294 *Q35; daf-2(e1370)* mutant animals. While *Q35; daf-16(mu86)* mutant animals were protected

295 from HIPA by fasting similar to wild-type controls, *Q35; daf-2(e1370);daf-16(mu86)* animals still

296 exhibit significant HIPA when fasted (Fig. 5D). These results indicate that DAF-2 mediates the

297 effects of fasting to prevent HIPA at least partly independently of DAF-16.




298

299 **Figure 5. The insulin-like signaling pathway is required for fasting protection. A** Schematic of
300 key insulin-signaling pathway members in *C. elegans*. Under nutrient-rich conditions, insulin-like
301 peptides bind to the insulin receptor DAF-2, initiating a phosphorylation cascades that
302 ultimately leads to the phosphorylation of the FoxO transcription factor DAF-16, excluding it
303 from the nucleus. Conversely, when nutrients are scarce, DAF-16 remains unphosphorylated
304 and is able to enter the nucleus and bind to its target genes. **B.** DAF-16 is not localized to the
305 nucleus in fasted animals exposed to hypoxia. Cohorts of 20 DAF-16::GFP animals were
306 maintained in room air on food for 24 hrs (fed + room air), fasted in room air for 24 hrs (fasted
307 + room air), exposed to hypoxia for 24 hrs on food (fed + hypoxia), or exposed to hypoxia after
308 fasting (fasted + hypoxia; F=6h, H=24hr). Scale bars = 100μm. **C** Quantification of DAF-16::GFP
309 nuclear accumulation. The percent of animals with nuclear GFP was scored immediately post
310 hypoxia. Average data from 3 independent experiments is shown. The bar height indicates the
311 mean. Error bars are the standard deviation. **D** Fasting does not protect *daf-2* mutants against
312 HIPA. Aggregation measurements (F=6h, H=24h) for L4 Q35::YFP animals with mutations in *daf-*
313 *2(e1370)*, *daf-16(mu86)*, and the *daf-2(e1370); daf-16(mu86)* double mutant. Animals were
314 maintained on food in room air (room air, green), were exposed to hypoxia on food (fed,
315 magenta), or were exposed to hypoxia after removal from food (hypoxia fasted, blue). Each
316 circle is the number of YFP foci in a single animal, the mean is indicated by the line, error bars
317 are the standard deviation. Data from one representative experiment is shown. Each cohort
318 included at least 20 animals, and each experiment was repeated at least 3 times. Significance
319 was calculated using a Kruskal-Wallis test and Dunn's multiple comparisons post hoc analysis.
320 Significant differences ($p < 0.05$) in aggregation for a given strain between conditions are
321 indicated by letters above each group as follows: a - significantly different from room air
322 controls; b - significantly different from fed hypoxic controls; c - significantly different from
323 fasted hypoxic controls.
324

325

326 **Discussion**

327 This study illustrates the power of fasting to ameliorate the deleterious effects of
328 hypoxia on proteostasis. These findings are consistent with phenomena that have been
329 observed in mammals – fasting mice for a single day increases survival after kidney ischemia
330 and also reduces ischemic damage to the liver [27]. Our results suggest that the nutritional
331 milieu present at the onset of hypoxia can dictate the effect of hypoxia on proteostasis, as
332 fasting protection against hypoxia can be induced quite quickly. Animals that are removed from
333 food immediately before hypoxia are protected against HIPA to a significant degree, even after

334 being maintained on food for the entire pre-hypoxic period. This implies that worms are
335 integrating information about their environment, including nutrient availability, right as they
336 sense hypoxia. The importance of the nutritional environment of the animal as it experiences
337 hypoxia is further supported by the fact that we also see a rapid reversal of fasting protection.
338 Worms fasted for six hours but that are moved onto food immediately preceding hypoxia are
339 not as protected against HIPA compared to worms that were fasted and remained off of food
340 for the duration of hypoxia. The speed with which fasting protection can be induced and
341 reversed indicates that protection cannot be explained solely by changes in gene expression
342 resulting in a hypoxia-resistant pre-adapted state. Furthermore, the rapidity with which fasting
343 protection can be reversed suggests that altered gene expression or metabolism resulting from
344 the fasting period is alone insufficient to protect against HIPA. Although *C. elegans* enter a
345 reproductive and developmental diapause in 0.1% oxygen [28], the protection conferred by
346 fasting does not represent a simple delay in the onset of proteostasis decline due to the time
347 spent in hypoxia. Rather, fasting provides long-term protection against the accrual of protein
348 aggregates and toxicity even after the return to room air.

349 We found that IIS mediates fasting protection against HIPA. Notably, IIS is not required
350 for the fed response to hypoxia, as fed IIS mutants show increased aggregate levels comparable
351 to wild-type animals. In worms and flies, mutations in the insulin receptor are generally
352 considered protective against hypoxia. In *C. elegans, daf-2* mutants have a hypoxia-resistant
353 phenotype, displaying reduced muscle and neuronal cell death following hypoxia [29, 30], while
354 flies with defective insulin signaling (mutations in the insulin receptor *InR*, or *Chico*, the insulin
355 receptor substrate) are protected against anoxia/reoxygenation injury [31]. The *daf-2*

356 phenotype uncovered here is therefore distinct in that these mutants are sensitive to hypoxia in
357 the fasted state, with fasted *daf-2* mutant animals exhibiting increased HIPA compared to wild-
358 type controls. These results contradict the *a priori* expectation that *daf-2* mutants might be
359 resistant to hypoxia even in the fed state due to their inability to detect insulin-like peptides.
360 Mammalian systems offer precedents of insulin receptor mutations causing sensitivity
361 to hypoxic stress. Knockdown of neuronal insulin-like growth factor 1 receptor (IGF-1R)
362 exacerbates hypoxic injury and increases mortality in mice [32], and IGF-1R is required in order
363 for IGF-1 to protect myocardial cell exposed to ischemia [33]. However, data on the role of
364 mammalian IIS in response to hypoxia are mixed, and are complicated by the fact that different
365 types of insulin receptors mediate distinct cellular functions [34]. As such, the simplified *C.*
366 *elegans* IIS system may be useful for understanding contextual inputs that alter IIS outputs.
367 DAF-16 is believed to be the main nexus of IIS [20, 35-37], which makes the DAF-2-
368 dependent, but DAF-16-independent nature of the protective effect of fasting described here
369 unusual in *C. elegans*. Decreased DAF-2 activity results in phenotypes such as increased
370 lifespan, reproductive delays, and increased resistance to heat and oxidative stress, all of which
371 require DAF-16 [26]. However, a few other examples exist in the literature of DAF-2 dependent,
372 DAF-16 independent phenomena: dauer formation at 27°, meiotic progression of oocytes, salt
373 chemotaxis learning, and regulation of the dao-3 and hsp-90 genes [38- 42]. In chemotaxis
374 learning, DAf-2 acts on learning through phosphatidylinositol 3,4,5-triphosphate ($PIP_3$), but not
375 DAF-16. Similar to these studies, fasting-mediated protection against HIPA supports the
376 existence of downstream targets of DAF-2 separate from DAF-16 that are capable of influencing
377 stress responses and proteostasis.

378 **Materials and methods**

379 *C. elegans strains and methods*

380 Animals were maintained on nematode growth media (NGM) with OP50 *E. coli* at 20°C
381 (Brenner, 1974). See Supplementary Table S6 for worm strains. Strains were obtained from the
382 *Caenhorabditis* Genetics Center at the University of Minnesota. Double and triple mutants were
383 generated using standard genetic techniques, and genotypes were verified using PCR.

384

385 *Construction of hypoxic chambers*

386 Hypoxic conditions were maintained using continuous flow chambers, as described in
387 Fawcett et al. 2012. Compressed gas tanks (1000 ppm $O_2$ balanced with $N_2$) were Certified
388 Standard (within 2% of target concentration) from Airgas (Seattle, WA). Oxygen flow was
389 regulating using Aalborg rotameters (Aalborg Intruments and Controls, Inc., Orangeburg, NY,
390 USA). Hypoxic chambers (and room air controls?) were maintained in a 20°C incubator for the
391 duration of the experiments.

392

393 *YFP::polyQ aggregation assays*

394 Synchronous cohorts of L1 YFP::polyQ$_{40}$ animals were generated by either bleaching
395 first-day adult animals in a 20% alkaline hypochlorite solution or allowing first-day adult animals
396 to lay eggs for 1-2 hrs on seeded NGM plates. The adults were then removed, and the plates
397 were incubated at 20°C. The next morning, cohorts of hatched L1 larvae were suspended in M9
398 and mouth-pipetted to new NGM plates for hypoxic exposure. Synchronous cohorts of L4

399 YFP::polyQ$_{35}$ animals were generated by picking L4 animals from well-fed, logarithmically
400 growing populations.
401 Cohorts of 25-35 YFP::polyQ animals were exposed to hypoxia for approximately 24 h at
402 20°C on unseeded 3 cm NGM plates with 40mg/mL carbenicillin or NGM plates seeded with live
403 OP50 food. Plates were ringed with palmitic acid (10mg/mL in ethanol), creating a physical
404 barrier around the edge of each plate to discourage animals from leaving the surface of the
405 agar.
406 To quantify the number of YFP foci, worms were mounted a 2% agar pad in a drop of
407 50mM sodium azide as anesthetic. Control experiments showed that azide did not affect the
408 aggregation of YFP::polyQ$_{35}$ or YFP::polyQ4$_{0}$ (Moronetti Mazzeo et al. 2012). YFP foci were
409 identified and quantified as described in Morley et al. (2002) and Silva et al. (2011). A Nikon 90i
410 fluorescence microscope with the YFP filter and 10x objective (Nikon Instruments Inc., Melville,
411 NY, USA) was used to visualize and quantify aggregates. In all experiments, the number of
412 aggregates was counted blind to treatment and genotype. Statistical significance was evaluated
413 by calculating P-values between conditions using a Kruskal-Wallis test and Dunn's multiple
414 comparisons post hoc analysis in GraphPad Prism version 7.0c for Mac OSX (GraphPad Softare,
415 San Diego, California, USA) In all cases, $P < 0.05$ was considered statistically significant.
416
417 *Paralysis and uncoordination assays of proteotoxicity*
418 Animals expressing A$\beta_{1\text{-}42}$ or YFP::polyQ$_{40}$ were exposed to 1000 ppm $O_2$ for 24 at 20°C
419 as L4 or L1, respectively. For both, animals were grown on seeded NGM plates until 6 hrs before
420 hypoxic exposure, at which point fasted animals were transferred to unseeded NGM plates,

421 where they remained until the end of the hypoxic exposure. Fed animals were transferred to
422 new seeded NGM plates. After hypoxic exposure, all animals were returned to food and
423 normoxia, and incubated at 20°C. Paralysis was scored daily. Worms were considered paralyzed
424 if they failed to respond, other than with movement of the nose or pharyngeal pumping, when
425 tapped with a platinum wire pick 3 consecutive times. Dead or bagged worms were censored
426 from the experiment on the day of death/bagging. Paralyzed worms were removed from the
427 plate on the day of paralysis. Live worms that were not paralyzed were moved to a new plate
428 each day until all worms were scored as either paralyzed or dead. Statistical significance was
429 calculated using Kaplan-Meier log-rank (Mantel-Cox) tests and a Bonferroni correction for
430 multiple comparisons using GraphPad Prism version 7.0c for Mac OSX (GraphPad Softare, San
431 Diego, California, USA).
432
433 *DAF-16::GFP localization*
434 Synchronous cohorts of L2 animals expressing DAF-16::GFP were exposed to hypoxia for
435 24 h at 20°C on unseeded unseeded 3 cm NGM plates with 40mg/mL carbenicillin or NGM
436 plates seeded with live OP50 food. Plates were ringed with palmitic acid (10mg/mL in ethanol),
437 creating a physical barrier around the edge of each plate to discourage animals from leaving the
438 surface of the agar. To visualize the localization of DAF-16::GFP, worms were mounted a 2%
439 agar pad in a drop of 10mM levamisole as anesthetic. A Nikon 90i fluorescence microscope with
440 the GFP filter and 10x objective (Nikon Instruments Inc., Melville, NY, USA) was used to visualize
441 DAF-16::GFP. For quantification, percent of animals with nuclear GFP was scored immediately
442 after removal from hypoxia. In all experiments, the GFP localization was scored blind to
443 treatment and genotype. Statistical significance was evaluated by calculating P-values between

conditions using a Kruskal-Wallis test and Dunn's multiple comparisons post hoc analysis in GraphPad Prism version 7.0c for Mac OSX (GraphPad Softare, San Diego, California, USA). $P < 0.05$ was considered statistically significant.

**Acknowledgements**

We

**References:**


1. Contestabile A. Benefits of caloric restriction on brain aging and related pathological states: understanding mechanisms to devise novel therapies. Curr Med Chem. 2009;16:350-361.
2. Patel NV, Gordon MN, Connor KE et al. Caloric restriction attenuates Abeta-deposition in Alzheimer transgenic models. Neurobiol Aging. 2005;26:995-1000.
3. Duan W, Guo Z, Jiang H, Ware M, Li XJ, Mattson MP. Dietary restriction normalizes glucose metabolism and BDNF levels, slows disease progression, and increases survival in huntingtin mutant mice. Proc Natl Acad Sci U S A. 2003;100:2911-2916.
4. Steinkraus KA, Smith ED, Davis C et al. Dietary restriction suppresses proteotoxicity and enhances longevity by an hsf-1-dependent mechanism in Caenorhabditis elegans. Aging Cell. 2008;7:394-404.
5. Katare RG, Kakinuma Y, Arikawa M, Yamasaki F, Sato T. Chronic intermittent fasting improves the survival following large myocardial ischemia by activation of BDNF/VEGF/PI3K signaling pathway. J Mol Cell Cardiol. 2009;46:405-412.
6. Menezes-Filho SL, Amigo I, Prado FM et al. Caloric restriction protects livers from ischemia/reperfusion damage by preventing $Ca^{2+}$-induced mitochondrial permeability transition. Free Radic Biol Med. 2017;110:219-227.
7. Qin J, Zhou J, Dai X et al. Short-term starvation attenuates liver ischemia-reperfusion injury (IRI) by Sirt1-autophagy signaling in mice. Am J Transl Res. 2016;8:3364-3375.
8. Duan W, Guo Z, Mattson MP. Brain-derived neurotrophic factor mediates an excitoprotective effect of dietary restriction in mice. J Neurochem. 2001;76:619-626.
9. Ran M, Li Z, Yang L, Tong L, Zhang L, Dong H. Calorie restriction attenuates cerebral ischemic injury via increasing SIRT1 synthesis in the rat. Brain Res. 2015;1610:61-68.
10. Fawcett EM, Hoyt JM, Johnson JK, Miller DL. Hypoxia disrupts proteostasis in Caenorhabditis elegans. Aging Cell. 2015;14:92-101.
11. Morley JF, Brignull HR, Weyers JJ, Morimoto RI. The threshold for polyglutamine-expansion protein aggregation and cellular toxicity is dynamic and influenced by aging in Caenorhabditis elegans. Proc Natl Acad Sci U S A. 2002;99:10417-10422.
12. Satyal SH, Schmidt E, Kitagawa K et al. Polyglutamine aggregates alter protein folding homeostasis in Caenorhabditis elegans. Proc Natl Acad Sci U S A. 2000;97:5750-5755.
13. Van Gilst MR, Hadjivassiliou H, Yamamoto KR. A Caenorhabditis elegans nutrient response system partially dependent on nuclear receptor NHR-49. Proc Natl Acad Sci U S A. 2005;102:13496-13501.
14. Soto C. Unfolding the role of protein misfolding in neurodegenerative diseases. Nat Rev Neurosci. 2003;4:49-60.
15. Link CD. Expression of human beta-amyloid peptide in transgenic Caenorhabditis elegans. Proc Natl Acad Sci USA. 1995;92:9368.

16. Clark SG, Shurland DL, Meyerowitz EM, Bargmann CI, van der Bliek AM. A dynamin GTPase mutation causes a rapid and reversible temperature-inducible locomotion defect in C. elegans. Proc Natl Acad Sci U S A. 1997;94:10438-10443.

17. Ben-Zvi A, Miller EA, Morimoto RI. Collapse of proteostasis represents an early molecular event in Caenorhabditis elegans aging. Proc Natl Acad Sci U S A. 2009;106:14914-14919.

18. Cohen E, Bieschke J, Perciavalle RM, Kelly JW, Dillin A. Opposing activities protect against age-onset proteotoxicity. Science. 2006;313:1604-1610.

19. Piñero González J, Carrillo Farnés O, Vasconcelos AT, González Pérez A. Conservation of key members in the course of the evolution of the insulin signaling pathway. Biosystems. 2009;95:7-16.

20. Lin K, Hsin H, Libina N, Kenyon C. Regulation of the Caenorhabditis elegans longevity protein DAF-16 by insulin/IGF-1 and germline signaling. Nat Genet. 2001;28:139-145.

21. Henderson ST, Johnson TE. daf-16 integrates developmental and environmental inputs to mediate aging in the nematode Caenorhabditis elegans. Curr Biol. 2001;11:1975-1980.

22. Murphy CT, McCarroll SA, Bargmann CI et al. Genes that act downstream of DAF-16 to influence the lifespan of Caenorhabditis elegans. Nature. 2003;424:277-283.

23. Honjoh S, Yamamoto T, Uno M, Nishida E. Signalling through RHEB-1 mediates intermittent fasting-induced longevity in C. elegans. Nature. 2009;457:726-730.

24. Leiser SF, Fletcher M, Begun A, Kaeberlein M. Life-span extension from hypoxia in Caenorhabditis elegans requires both HIF-1 and DAF-16 and is antagonized by SKN-1. J Gerontol A Biol Sci Med Sci. 2013;68:1135-1144.

25. Ogg S, Paradis S, Gottlieb S et al. The Fork head transcription factor DAF-16 transduces insulin-like metabolic and longevity signals in C. elegans. Nature. 1997;389:994-999.

26. Zhou KI, Pincus Z, Slack FJ. Longevity and stress in Caenorhabditis elegans. Aging (Albany NY). 2011;3:733-753.

27. Mitchell JR, Verweij M, Brand K et al. Short-term dietary restriction and fasting precondition against ischemia reperfusion injury in mice. Aging Cell. 2010;9:40-53.

28. Miller DL, Roth MB. C. elegans are protected from lethal hypoxia by an embryonic diapause. Curr Biol. 2009;19:1233-1237.

29. Scott BA, Avidan MS, Crowder CM. Regulation of hypoxic death in C. elegans by the insulin/IGF receptor homolog DAF-2. Science. 2002;296:2388-2391.

30. Mabon ME, Scott BA, Crowder CM. Divergent mechanisms controlling hypoxic sensitivity and lifespan by the DAF-2/insulin/IGF-receptor pathway. PLoS One. 2009;4:e7937.

31. Vigne P, Tauc M, Frelin C. Strong dietary restrictions protect Drosophila against anoxia/reoxygenation injuries. PLoS One. 2009;4:e5422.

32. Liu W, D'Ercole JA, Ye P. Blunting type 1 insulin-like growth factor receptor expression exacerbates neuronal apoptosis following hypoxic/ischemic injury. BMC Neurosci. 2011;12:64.

33. Liu A, Zhang X, Gu H, Li P, Yu T. Insulin-like growth factor-1 protects ischemic myocardial cells via PI3K/AKT pathway. Int J Clin Exp Pathol. 2016;9:12574-12580.

34. Cai W, Sakaguchi M, Kleinridders A et al. Domain-dependent effects of insulin and IGF-1 receptors on signalling and gene expression. Nat Commun. 2017;8:14892.

35. Dillin A, Crawford DK, Kenyon C. Timing requirements for insulin/IGF-1 signaling in C. elegans. Science. 2002;298:830-834.

36. Hsu AL, Murphy CT, Kenyon C. Regulation of aging and age-related disease by DAF-16 and heat-shock factor. Science. 2003;300:1142-1145.

37. Honda Y, Honda S. The daf-2 gene network for longevity regulates oxidative stress resistance and Mn-superoxide dismutase gene expression in Caenorhabditis elegans. FASEB J. 1999;13:1385-1393.

38. Ailion M, Thomas JH. Dauer formation induced by high temperatures in Caenorhabditis elegans. Genetics. 2000;156:1047-1067.

39. Lopez AL, Chen J, Joo HJ et al. DAF-2 and ERK couple nutrient availability to meiotic progression during Caenorhabditis elegans oogenesis. Dev Cell. 2013;27:227-240.

40. Tomioka M, Adachi T, Suzuki H, Kunitomo H, Schafer WR, Iino Y. The insulin/PI 3-kinase pathway regulates salt chemotaxis learning in Caenorhabditis elegans. Neuron. 2006;51:613-625.

41. Vellai T, McCulloch D, Gems D, Kovács AL. Effects of sex and insulin/insulin-like growth factor-1 signaling on performance in an associative learning paradigm in Caenorhabditis elegans. Genetics. 2006;174:309-316.

42. Yu H, Larsen PL. DAF-16-dependent and independent expression targets of DAF-2 insulin receptor-like pathway in Caenorhabditis elegans include FKBPs. J Mol Biol. 2001;314:1017-1028.