Hypoxia disrupts proteostasis in *Caenorhabditis elegans*

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Summary

Oxygen is fundamentally important for cell metabolism, and as a consequence, O_2 deprivation (hypoxia) can impair many essential physiological processes. Here, we show that an active response to hypoxia disrupts cellular proteostasis – the coordination of protein synthesis, quality control, and degradation that maintains the functionality of the proteome. We have discovered that specific hypoxic conditions enhance the aggregation and toxicity of aggregation-prone proteins that are associated with neurodegenerative diseases. Our data indicate this is an active response to hypoxia, rather than a passive consequence of energy limitation. This response to hypoxia is partially antagonized by the conserved hypoxia-inducible transcription factor, *hif-1*. We further demonstrate that exposure to hydrogen sulfide (H_2S) protects animals from hypoxia-induced disruption of proteostasis. H_2S has been shown to protect against hypoxic damage in mammals and extends lifespan in nematodes. Remarkably, our data also show that H_2S can reverse detrimental effects of hypoxia on proteostasis. Our data indicate that the protective effects of H_2S in hypoxia are mechanistically distinct from the effect of H_2S to increase lifespan and thermotolerance, suggesting that control of proteostasis and aging can be dissociated. Together, our studies reveal a novel effect of the hypoxia response in animals and provide a foundation to understand how the integrated proteostasis network is integrated with this stress response pathway.

Key words: *C. elegans*; hypoxia; H_2S; oxygen; Polyglutamine; protein aggregation; proteostasis.

Introduction

Fluctuations in O_2 availability are common in nature. Effective O_2 concentration declines with altitude, and steep concentration gradients of O_2 occur in marine environments and wet soil because O_2 is poorly soluble in water and diffuses slowly in aqueous solution. Animals have evolved a variety of physiological and behavioral responses to low O_2 (hypoxia). Nevertheless, hypoxia can be quite damaging, as O_2 availability contributes to cellular damage and death in human

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pathological conditions where blood flow is interrupted such as severe blood loss, stroke, and cardiovascular disease. Cellular damage from hypoxia can be mitigated by a preconditioning exposure, in which a nonlethal hypoxic event precedes the damaging insult (reviewed in Semenza, 2011). This suggests that there are endogenous cellular mechanisms that can protect against damaging effects of hypoxia when appropriately activated.

It has been suggested that cellular damage occurs when arterial blood O_2 concentration drops below 5000 ppm O_2 (Carreau *et al.*, 2011). However, the O_2 available to different tissues is not uniform, and the sensitivity of different cell types to withstand hypoxia can vary dramatically. Tumor cells are particularly resistant to hypoxia, likely as an adaptation to poor O_2 delivery in tumors. In fact, tumor hypoxia is strongly associated with poor prognosis and resistance to therapy (reviewed in Brown, 2007). There is great need to understand the diversity and integration of cellular responses to hypoxia. It is technically quite difficult to precisely measure or experimentally control cellular O_2 concentrations in living mammals. We therefore have used *Caenorhabditis elegans* to investigate responses to specific hypoxic conditions. In this animal, all cells are directly exposed to the gaseous environment (Shen & Powell-Coffman, 2003). This allows for precise control of cellular O_2 availability in a genetically tractable model, without the confounding effects of compensatory responses that increase blood flow to hypoxic tissues, which are common in larger animals.

The physiological response to hypoxia depends greatly on the amount of O_2 that is available, as has been well demonstrated in the nematode *C. elegans*. *C. elegans* is broadly tolerant to hypoxia and can continue development and reproduction in as little as 5000 ppm O_2 (Nystul *et al.*, 2003; Miller & Roth, 2009). In anoxia (operationally defined here as < 10 ppm O_2), *C. elegans* enters into a reversible state of suspended animation, in which all observable biological processes arrest (Padilla *et al.*, 2002; Nystul *et al.*, 2003). Upon return to normoxia (which we define as room air, 210 000 ppm O_2), animals reanimate and resume normal biological activity without apparent consequence.

Curiously, there are a range of O_2 conditions in which *C. elegans* can neither induce suspended animation nor continue development. In 1000 ppm O_2, isolated embryos continue to develop and die with gross morphological and developmental defects (Nystul *et al.*, 2003). Exposure to 1000 ppm O_2 is not lethal after embryogenesis, but instead induces diapause in which development and reproduction – but not movement and other biological activities – reversibly arrest (Miller & Roth, 2009). These observations support the idea that there are multiple distinct but highly coordinated responses to hypoxia.

Hypoxia extends lifespan in both *C. elegans* and *Drosophila* (Rascon & Harrison, 2010; Leiser *et al.*, 2013). These observations suggest that hypoxia responses integrate mechanistically with longevity-associated cellular processes. Many studies suggest that proteostasis is essential to prevent cellular decline associated with aging (reviewed in Morley *et al.*, 2002; Taylor & Dillin, 2011). Proteostasis is the coordination of protein translation, folding, quality control, trafficking, and degradation that is essential to maintain the proteome in a functional state. Hypoxia can impact many, if not all, of the cellular processes involved in proteostasis. In flies, turtles, and mammalian cell culture, protein translation arrests in hypoxia (Teodoro & O'Farrell, 2003; Liu & Simon, 2004; Liu *et al.*, 2006). Chaperones, heat shock proteins, and the

unfolded protein response are activated by hypoxia in mammalian cells as well as *C. elegans* (Koumenis *et al.*, 2002; Wouters & Koritzinsky, 2008; Mao & Crowder, 2010; Powell-Coffman, 2010). Decreasing translation and upregulating heat shock proteins might be predicted to maintain proteostasis. However, O_2 is required for correct disulfide bond formation in protein folding and in *Drosophila*, protein turnover arrests upon O_2 deprivation (Teodoro & O'Farrell, 2003), which could impair cellular proteostasis. Thus, the effects on global proteostasis are not easily predicted.

The gas hydrogen sulfide (H_2S) has been shown to improve outcome in several models of hypoxic and ischemic damage in mammals (reviewed in Nicholson & Calvert, 2010). Mice exposed to H_2S survive otherwise lethal hypoxia (Blackstone & Roth, 2007; Elrod *et al.*, 2007). In preclinical mammalian models, treatment with H_2S improves outcome in myocardial infarct and cerebral ischemic injury (Predmore & Lefer, 2011; Liu *et al.*, 2012). One possibility is that H_2S signaling impinges on pathways similar to those that mediate the protective effects of hypoxic preconditioning. Consistent with this view, H_2S stabilizes and activates HIF-1 in both mice and *C. elegans* (Budde & Roth, 2010), and HIF is important for hypoxic preconditioning in myocardial infarct (Liu *et al.*, 2010; Sarkar *et al.*, 2012). Curiously, in *C. elegans*, different genes are regulated by *hif-1* in H_2S and hypoxia (Miller *et al.*, 2011), suggesting that the protective effects of H_2S are not simply a result of activating the HIF-mediated response to hypoxia.

In this study, we measured the functional effect of hypoxia on proteostasis in living animals using the nematode *C. elegans*. Our results indicate that in specific hypoxic conditions, there is an active cellular response that perturbs proteostasis. The perturbation of proteostasis persists even when O_2 is restored. The *hif-1* transcription factor is not required for this aspect of the hypoxia response. Instead, we show *hif-1* partially suppresses the effect of hypoxia on proteostasis in some conditions. We also demonstrate that treatment with H_2S can both prevent and reverse detrimental effects of hypoxia on proteostasis. Our observation that H_2S protects against hypoxia is reminiscent of the situation in mammals, suggesting that the functional integration of hypoxia and H_2S responses is conserved.

Results

In *C. elegans*, cells acquire O_2 directly from the environment, rather than by active transport through a vascular system. Therefore, in contrast to larger animals, *C. elegans* do not respond to hypoxia with adaptations that improve delivery of O_2 to cells, such as increased respiration or heart rate. We took advantage of this feature of *C. elegans* biology and exposed animals to constructed environments with defined concentrations of O_2 to precisely control cellular O_2 (Nystul & Roth, 2004; Fawcett *et al.*, 2012). To evaluate the effects of hypoxia on proteostasis *in vivo*, we utilized a well-established polyglutamine protein model. In these animals, the yellow fluorescent protein (YFP) is fused to a series of glutamine residues and expressed in the body wall muscle. We refer to this transgene as YFP::polyQ$_x$ (the subscript indicates the number of glutamine residues fused to YFP). YFP::polyQ$_x$ is soluble and diffuse throughout the muscle cells when first expressed, but aggregates as proteostasis mechanisms fail, forming bright fluorescent foci (Fig. 1C, for example). Thus, the localization of YFP::polyQ$_x$ is a read-out of proteostasis efficiency *in vivo*. This model has been validated in studies that have determined the effects of aging, genetic disruption of quality control machinery, and osmotic stress on the proteostasis network (Morley *et al.*, 2002; Gidalevitz *et al.*, 2006; Moronetti Mazzeo *et al.*, 2012).
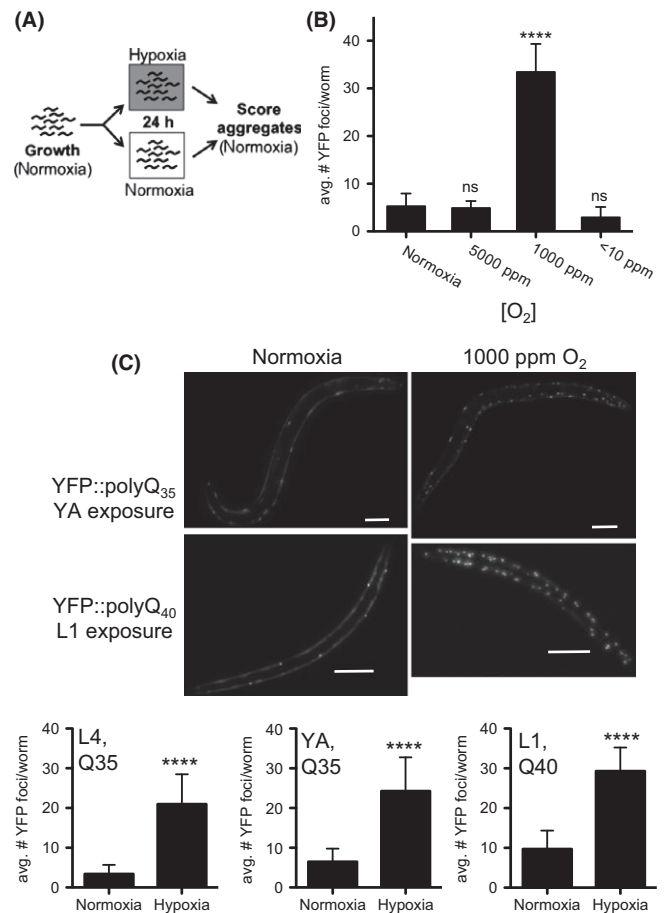

Fig. 1 Hypoxia induces polyglutamine protein aggregation. (A) Experimental design. Synchronized populations of YFP::polyQ$_{35}$ animals were grown in normoxia, then the population was divided and half were exposed to hypoxia. The number of YFP foci was scored immediately upon removal from hypoxia. (B) Specific hypoxic O_2 concentrations induce polyglutamine protein aggregation. Cohorts of young adult YFP::polyQ$_{35}$ were exposed to atmosphere containing the indicated concentration of O_2. YFP foci were counted immediately upon removal. (C) Polyglutamine protein aggregation is induced by exposure to 1000 ppm O_2. Fluorescence microscopy images show YFP::polyQ$_{35}$ (top) and YFP::polyQ$_{40}$ (bottom) in live *Caenorhabditis elegans* after 24 h in either normoxia (left) or 1000 ppm O_2 (right). Bar graphs (below) show the mean number of YFP::polyQ foci after 24 h in hypoxia (1000 ppm O_2) or normoxia (RA = 210 000 ppm O_2). Animals were exposed as either as fourth-stage larvae (L4, left), young adult (YA, middle), or first-stage larvae (L1, right). Q35 = YFP::polyQ$_{35}$; Q40 = YFP::polyQ$_{40}$. In all panels, graphs show mean ± SD. Each cohort included at least 30 animals. Statistical comparisons were between groups exposed to hypoxia and normoxic controls: ****P-value < 0.0001; ns, not significant. Summary of data from replicate experiments is included in Table S1 (Supporting information).

Specific hypoxic condition induces aggregation of polyglutamine proteins

We exposed animals expressing YFP::polyQ$_{35}$ to hypoxia as first-day adults, before the onset of age-associated protein aggregation, to determine the effect of hypoxia on proteostasis (schematized in Fig. 1A). *C. elegans* survive exposure to all O_2 conditions tested in our experiments. We observed that animals exposed to environments with as little as 5000 ppm O_2 (a 40-fold reduction in O_2 from room air, 210 000 ppm O_2) showed no difference in the number of YFP foci compared to controls that remained in room air (Fig. 1B). This suggests that

proteostasis is effectively maintained in this condition, even though the decrease in O_2 causes a severe decrease in metabolic and developmental rate (Miller & Roth, 2009; Van Voorhies, 2009).

In contrast to the situation in 5000 ppm O_2, the number of YFP foci increased dramatically in animals exposed to 1000 ppm O_2 (Fig. 1B), suggesting that proteostasis has been disrupted. YFP foci did not form in 1000 ppm O_2 in YFP::polyQ$_0$ control animals that express YFP without a polyglutamine tract indicating that the effect of hypoxia depends on the polyglutamine tract. Animals also developed increased YFP::polyQ$_{35}$ foci when they were exposed to 1000 ppm O_2 as fourth-stage larvae (L4) (Fig. 1C), indicating that this response was robust across developmental stages. Animals exposed to 1000 ppm O_2 enter into a developmental and reproductive diapause (Miller & Roth, 2009) and are therefore developmentally younger than controls that remain in room air.

We did not observe increased formation of YFP foci in animals exposed to anoxia (Fig. 1B). These results suggest that the disruption of proteostasis in 1000 ppm O_2 is an active consequence of the response to hypoxia, and not simply a passive consequence of decreased aerobic energy production. In anoxia, *C. elegans* enter into a state of suspended animation, which is poorly understood mechanistically. We cannot exclude the possibility that suspended animation itself protects the proteostasis network. Another possible interpretation of this result is that the process of aggregation itself is an active process, requiring a functioning cellular metabolic state. These specific effects on proteostasis in distinct O_2 concentrations are yet another piece of evidence that there are distinct physiological responses to different hypoxic conditions.

Aggregation of YFP::polyQ$_x$ occurs in a polyglutamine tract length- and age-dependent manner in normoxic conditions (Morley *et al.*, 2002). We therefore considered the possibility that hypoxia could have an age-dependent effect on proteostasis. To test this possibility, we compared the effects of hypoxia on strains expressing either YFP::polyQ$_{35}$ or YFP::polyQ$_{40}$. In normoxia, YFP::polyQ$_{35}$ does not begin to aggregate until the animals are adults, whereas YFP::polyQ$_{40}$ protein is more aggregation-prone and forms foci starting at L1/L2. We found that exposure to 1000 ppm O_2 did not induce aggregation of YFP::polyQ$_{35}$ in first-stage larvae (L1), in contrast to our previous results showing increased aggregation in both L4 and adults (Fig. 1C and data not shown). However, we did observe an increase in the number of fluorescent foci when YFP::polyQ$_{40}$ animals were exposed as L1 (Fig. 1C). We conclude that the effect of hypoxia on proteostasis is similar at all developmental stages. The difference between the effect of hypoxia on L1 animals expressing YFP::polyQ$_{35}$ and YFP::polyQ$_{40}$ suggests that these proteins are differentially vulnerable to how the proteostasis network is perturbed in hypoxia. For example, one possibility is that hypoxia disrupts proteostasis in a manner that promotes the growth of aggregates but not the formation of new aggregate seeds. The fact that protein aggregation is induced by hypoxia throughout life indicates that hypoxia has age-independent effects on proteostasis.

Hypoxia enhances proteotoxicity of neurodegeneration disease models in *C. elegans*

It has been proposed that aggregation of proteins involved in neurodegeneration is a cytoprotective response to sequester more toxic, smaller aggregates. One possibility is that protein aggregation in hypoxia might similarly be a protective mechanism to reduce toxic effects of unfolded or damaged proteins. We evaluated this hypothesis by measuring the proteotoxicity of YFP::polyQ$_x$ after hypoxia. In room air, YFP::polyQ$_x$ toxicity leads to age-associated disruption of muscle cell function and

paralysis. We reasoned that if increased protein aggregation is a cytoprotective response to hypoxia, then animals exposed to hypoxia would maintain muscle function as long as, or longer than, controls. To assess this, we measured the onset of paralysis in animals exposed to 1000 ppm O_2. We observed that both YFP::polyQ$_{40}$ and YFP::polyQ$_{35}$ animals become paralyzed sooner when exposed to 1000 ppm O_2, but not 5000 ppm O_2 (Fig. 2A,B, Fig. S1, and data not shown). Importantly, hypoxia does not induce paralysis in wild-type (N2) or YFP::polyQ$_0$ animals, suggesting that the proteotoxicity we observe in YFP::polyQ$_x$ animals is due to cytotoxicity associated with polyQ$_x$ (Fig. 2C,D). We conclude that proteotoxicity of polyglutamine proteins is enhanced by hypoxia.

To further establish that hypoxia causes a general defect in proteostasis, we examined whether other aggregation-prone proteins were also affected by exposure to hypoxia. We first measured the effect of hypoxia on animals expressing $A\beta_{1-42}$ in body wall muscle (Link, 1995). In *C. elegans*, $A\beta_{1-42}$ forms aggregates similar to amyloid plaques associated with Alzheimer's disease in humans, and leads to age-associated muscle dysfunction and paralysis. We found that, similar to the YFP::polyQ$_x$ model, animals expressing $A\beta_{1-42}$ became paralyzed sooner when exposed to 1000 ppm O_2 (Fig. 3A). Based on this finding, we conclude that hypoxia-induced disruption in proteostasis is not specific to YFP::polyQ$_x$.

Both the YFP::polyQ$_x$ and $A\beta_{1-42}$ transgenes are expressed in the body wall muscle. This raises the possibility that hypoxia-induced disruption of proteostasis is specific to this tissue. To address this possibility, we measured the effect of hypoxia in neurons of animals that express the human tau(V337M) protein variant from the *aex-3*
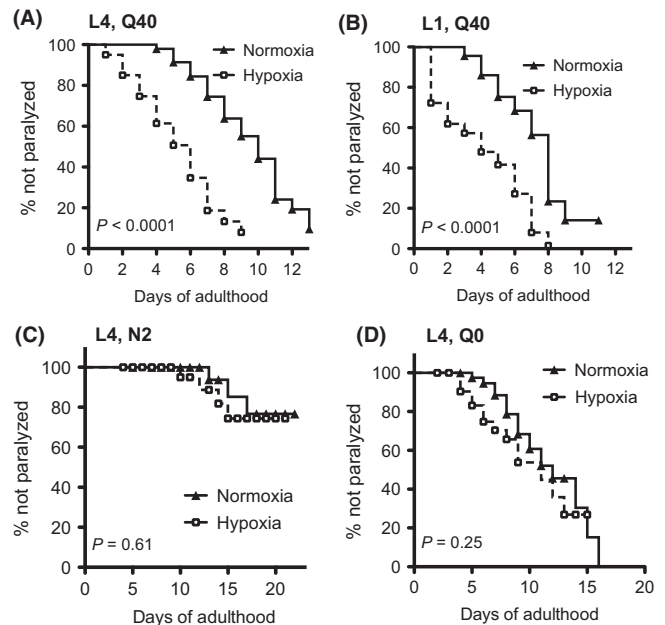

Fig. 2 Hypoxia accelerates paralysis associated with expression of polyglutamine proteins. YFP::polyQ$_x$ animals exposed to hypoxia become paralyzed sooner than normoxic controls. Age-matched animals were exposed to 1000 ppm O_2 for 24 h and then returned to normoxia. Paralysis was scored daily. (A) Paralysis of YFP::polyQ$_{40}$ animals exposed as L4. (B) Paralysis of YFP::polyQ$_{40}$ animals exposed as L1. (C) Paralysis of N2 (wild-type) animals exposed as L4. (D) Paralysis of YFP::polyQ$_0$ animals exposed as L4. Each cohort included 30–50 animals. *P*-values on each graph compare hypoxia to normoxia using Kaplan–Meyer statistics. Summary of data from replicate experiments is included in Table S2 (Supporting information).
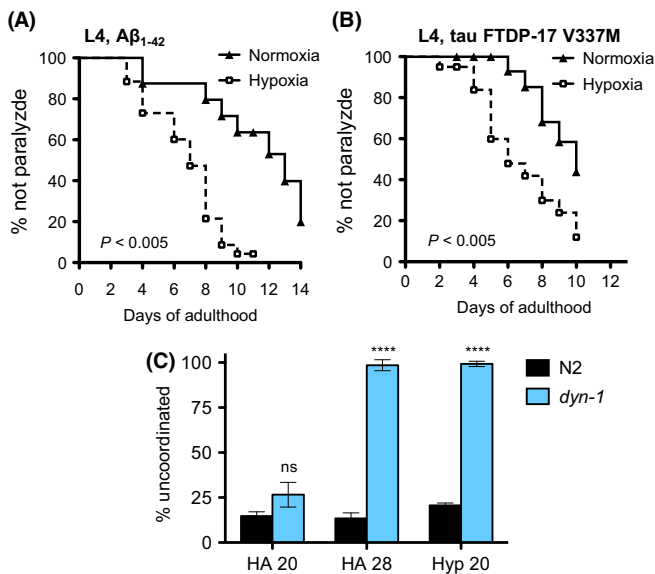
Fig. 3 Hypoxia accelerates paralysis associated with aggregation-prone and metastable proteins. (A) Wild-type animals expressing human $A\beta_{1-42}$ in body wall muscle become paralyzed more rapidly when exposed to 1000 ppm O_2 as L4. (B) *Caenorhabditis elegans* expressing the human V337M tau variant associated with FTDP-17 in neurons become paralyzed more rapidly when exposed to 1000 ppm O_2 as L4. (C) Metastable proteins are less functional after animals are exposed to 1000 ppm O_2. Percentage of uncoordinated *dyn-1(ky51)* and WT animals at the permissive (20°C) or nonpermissive (28°C) temperature exposed as L4s to hypoxia (1000 ppm O_2) for 24 h or maintained in normoxia. ****$P < 0.0001$ when compared to wild-type; ns, not significant. Each cohort included 30–50 animals. Summary of data from replicate experiments is included in Table S3 (Supporting information).

pan-neuronal promoter. This mutation causes a progressive neurodegenerative disease in humans (frontotemporal dementia with parkinsonism linked to chromosome 17). The tau(V337M) variant reduces binding affinity of tau to microtubules, accelerates tau aggregation, and leads to age-associated paralysis (Kraemer *et al.*, 2003). We found that animals expressing tau(V337M) in the nervous system became paralyzed more rapidly after exposure to 1000 ppm O_2 (Fig. 3B). We conclude that hypoxia disrupts global proteostasis in both neurons and muscle, supporting the idea that exposure to hypoxia results in an organism-wide disruption of proteostasis.

Thus far, the models we have investigated rely on transgenic expression of exogenous aggregate-prone proteins. To further test our model that hypoxia responses lead to a general disruption of proteostasis, we utilized animals with temperature sensitive (ts) mutations in the neuronal dynamin protein DYN-1. The *dyn-1(ky51)* ts mutant allele encodes a metastable DYN-1 protein. *dyn-1(ts)* mutant animals are uncoordinated at the restrictive temperature (28°C), but have normal motility at the permissive temperature (20°C). Conditions that disrupt proteostasis prevent the proper folding of the metastable DYN-1, causing *dyn-1(ts)* animals to become paralyzed even at the permissive temperature (Clark *et al.*, 1997; Gidalevitz *et al.*, 2006). We predicted that *dyn-1(ts)* animals exposed to 1000 ppm O_2 would become paralyzed at the permissive temperature as a result of the hypoxia-induced disruption of proteostasis.

We monitored the motility of *dyn-1(ts)* mutant animals at 20°C to assess the effect of hypoxia on proteostasis. Consistent with our hypothesis that hypoxia disrupts proteostasis, we found that animals exposed to hypoxia for 24 h at the permissive temperature displayed a severe impairment of motility (Fig. 3C). The same hypoxic conditions had no effect on wild-type (N2) animals. We conclude that the response to hypoxia disrupts the cellular folding environment and impairs the ability of the DYN-1 protein to function. Taken together with our experiments using YFP::polyQ$_x$, $A\beta_{1-42}$, and Tau, our results support a model in which exposure to hypoxia results in a widespread loss of proteostasis.

hif-1 is necessary but not sufficient to protect against hypoxia-induced protein aggregation

In studies of age-induced changes to proteostasis, changes in expression of proteasome subunits, autophagy, and chaperones are commonly noted (Lapierre *et al.*, 2011; Vilchez *et al.*, 2012; Taylor & Dillin, 2013). Insofar as we observed a functional effect on proteostasis, we hypothesized that exposure to hypoxia may also change the expression of key components of the proteostasis network. To address this possibility, we measured the abundance of transcripts of genes that are modified in other conditions that alter proteostasis using qRT–PCR. We did not observe any changes of transcript abundance after hypoxia for genes encoding several core proteasome subunits, or those genes critically involved in autophagy, TOR signaling, or the unfolded protein response (Fig. S2). This finding is in agreement with previously published microarray data of animals exposed to hypoxia, in which few changes to genes involved in proteostasis were observed (Shen *et al.*, 2005).

Although we did not observe a change in transcription of core proteostasis factors, we predicted that there would be genetic factors that mediate the effect of hypoxia on proteostasis if it was a result of the active response to hypoxia. We considered HIF-1 as a candidate for such a factor. HIF-1 is a well-studied and highly conserved bHLH transcription factor that coordinates the transcriptional response to hypoxia in metazoans, including *C. elegans* (reviewed in Semenza, 2009; Powell-Coffman, 2010). HIF-1 protein stability and transcriptional activity are directly regulated by O_2 availability (schematized in Fig. 4A). Earlier studies demonstrate that constitutive activation of HIF-1 reduces the toxicity of the aggregation-prone protein $A\beta_{1-42}$ in normoxia (Mehta *et al.*, 2009).

We crossed YFP::polyQ$_x$ transgenes into *hif-1(ia04)* mutant animals to determine whether *hif-1* is required for the effect of hypoxia on proteostasis. The *ia04* allele is a deletion of exons 2-4 and predicted molecular null (Jiang *et al.*, 2001). In 5000 ppm O_2, *hif-1(ia04)* mutant embryos die, while larvae and adults precociously enter hypoxia-induced diapause (Nystul & Roth, 2004; Miller & Roth, 2009). We found the number of YFP::polyQ$_{35}$ foci increased in *hif-1(ia04)* animals exposed to 5000 ppm O_2 (Fig. 4B), although there was no change in the number of foci when wild-type animals were exposed to the same conditions (see also Fig. 1B). Importantly, we did not observe any difference in the extent of age-associated aggregation of YFP::polyQ$_{35}$ in *hif–1(ia04)* mutant animals compared to wild-type controls (Fig. 4B). These experiments indicate that HIF-1 activity is necessary to stop the perturbation of proteostasis in wild-type animals exposed to 5000 ppm O_2.

Our observation that wild-type animals, with fully functional HIF-1, cannot maintain proteostasis in 1000 ppm O_2 (Fig. 1B) suggests that *hif-1* is not sufficient to prevent the disruption of proteostasis in these conditions, in contrast to the situation in 5000 ppm O_2 (Fig. 4B). This observation is consistent with earlier studies showing that HIF-1 mediates physiological responses to hypoxic O_2 concentrations ≥ 5000 ppm O_2, but not in more severe hypoxia. For example, *hif-1* is required for survival of embryos exposed directly to 5000 ppm O_2 but has no effect on viability of embryos exposed to 1000 ppm O_2 or anoxia (Nystul *et al.*, 2003; Miller & Roth, 2009). Similarly, *hif-1* is required for continued
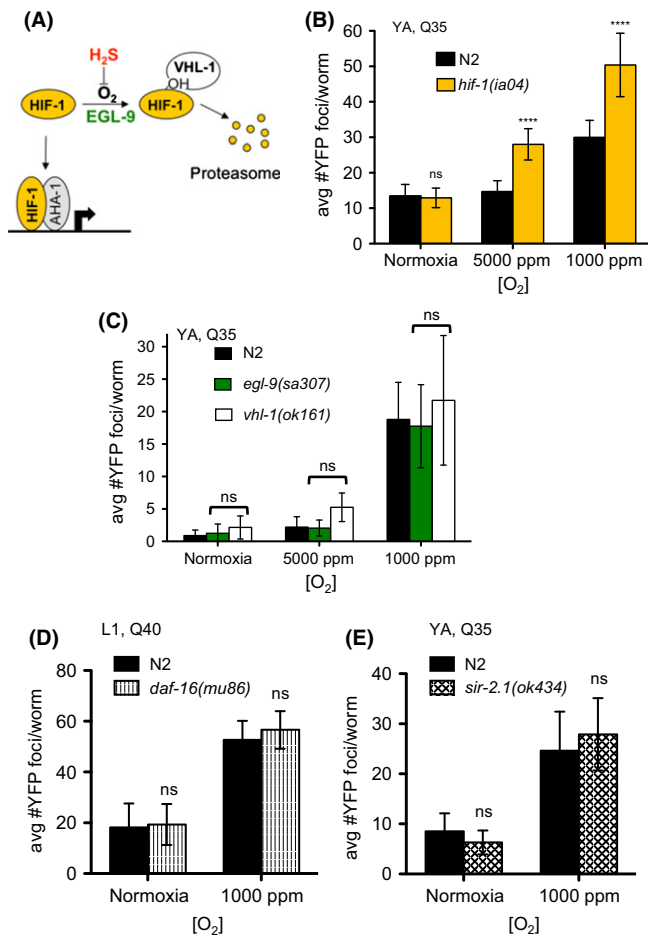
Fig. 4 HIF-1 is necessary but not sufficient to protect against hypoxia-induced protein aggregation. (A) HIF-1 protein levels are regulated by O_2 and H_2S (reviewed in Semenza, 2011). In the presence of O_2, the transcription factor HIF-1 is hydroxylated by the prolyl hydroxylase EGL-9. Hydroxylated HIF-1 is recognized by the E3 ubiquitin ligase von Hippel–Lindau protein 1 (VHL-1) and targeted for degradation by the proteasome. In hypoxia, the hydroxylation reaction is inefficient, resulting in accumulation of HIF-1 protein, which enters the nucleus and induces transcription. HIF-1 protein also accumulates in animals exposed to H_2S, even when O_2 is abundant (Budde & Roth, 2010). (B) HIF-1 is necessary to protect against protein aggregation in hypoxia. The number of YFP foci is greater in *hif-1(ia04); YFP::polyQ35* mutant animals than wild-type controls in both 5000 ppm O_2 and 1000 ppm O_2 but not normoxia. (C) HIF-1 is not sufficient to protect against hypoxia-induced protein aggregation. Mutations in negative regulators *egl-9* and *vhl-1* cause constitutive stabilization of HIF-1, even in normoxia. The number of YFP foci is not different from wild-type in *egl-9(sa307); YFP::polyQ35* or *vhl-1(ok161);YFP::polyQ35* mutant animals exposed to each hypoxic O_2 concentration. (D) Hypoxia-induced protein aggregation is independent of the insulin-like/IGF signaling pathway. The number of YFP foci in *daf-16(mu86); YFP::polyQ40* mutant animals is not different from wild-type after exposure to 1000 ppm O_2. (E) Sirtuin activity does not regulate hypoxia-induced protein aggregation. The number of YFP foci in *sir-2.1(ok434); YFP::polyQ35* mutant animals is not different from wild-type after exposure to 1000 ppm O_2. In all panels, graphs show mean ± SD error, each cohort contained at least 30 animals. Statistical comparisons were to wild-type controls in the same condition: ****$P < 0.0001$; ns, not significant. Summary of data from replicate experiments is included in Tables S4 and S6 (Supporting information).

postembryonic development and reproduction in 5000 ppm O_2 (Miller & Roth, 2009).

The fact that *hif-1* is necessary to prevent hypoxia-induced protein aggregation in 5000 ppm O_2 but not able to protect proteostasis in

wild-type animals exposed to 1000 ppm O_2 could indicate that the perturbation of proteostasis is mechanistically different in these two conditions. In this scenario, we expect that disrupting *hif-1* would not affect protein homeostasis in 1000 ppm O_2. To test this, we counted the number of fluorescent foci in *hif-1(ia04)* mutant animals expressing YFP::polyQ$_{35}$ after exposure to 1000 ppm O_2. We observed more aggregates in *hif-1(ia04)* mutant animals exposed to 1000 ppm O_2 than in wild-type controls (Fig. 4B). These data suggest that activation of HIF-1 antagonizes the disruption of proteostasis, at least partially, in 1000 ppm O_2 as well as preventing a perturbation in proteostasis in 5000 ppm O_2.

Increased HIF-1 activity delays age-induced proteotoxicity of both YFP::polyQ$_{35}$ and Aβ_{1-42} (Mehta *et al.*, 2009). Our data show that HIF-1 activity can reduce protein aggregation after exposure to hypoxia. However, we observed no difference between the rate of hypoxia-induced paralysis in *hif-1(ia04)* mutant animals and wild-type controls that express YFP::polyQ$_{35}$ (Fig. S3). One possibility is that mutations in *hif-1* separate the effects of proteostasis on protein aggregation and proteotoxicity in hypoxia. However, technical differences in the assays used to measure protein aggregation and toxicity complicate this interpretation. The number of fluorescent foci is scored immediately after the exposure to hypoxia, whereas YFP::polyQ$_x$-associated paralysis must be measured days after the exposure to hypoxia. Another important feature of the assays is that paralysis is a binary measurement – animals are either paralyzed or they are not. In contrast, the number of fluorescent foci in YFP::polyQ$_x$ is quantitative, so there are more than two possible outcomes. We cannot rule out the possibility that, unlike the aggregation assay, the paralysis assay is simply not sensitive enough to detect partial changes in proteostasis.

The fact that *hif-1* has only a partial effect to prevent protein aggregation in 1000 ppm O_2 could indicate that there are additional protective mechanisms needed to protect proteostasis in this condition. Alternatively, it is possible that *hif-1* could induce the necessary factors but that in these severe conditions *hif-1* is simply overwhelmed. In this scenario, we would expect that increasing the activity of HIF-1 would reduce the number of protein aggregates after exposure to hypoxia.

Mutations in either *egl-9* or *vhl-1* lead to the constitutive stabilization and increased transcriptional activity of *hif-1*, even in normoxia (schematized in Fig. 4) (Epstein *et al.*, 2001; Shen *et al.*, 2006; Budde & Roth, 2010). The level of HIF-1 stabilization in these mutants is sufficient to reduce the toxicity of Aβ_{1-42} proteins in normoxia (Mehta *et al.*, 2009) and dramatically increases expression of common *hif-1* reporters (Shen *et al.*, 2006). We found that both *vhl-1(ok161)* and *egl-9(sa304)* mutant animals expressing YFP::polyQ$_{35}$ accumulate the same number of foci as wild-type controls when exposed to 1000 ppm O_2 (Fig. 4C). This result argues that factors other than HIF-1 are required to protect against effects of 1000 ppm O_2 on proteostasis.

The conserved transcription factor DAF-16 and the SIRT1 homolog SIR-2.1 are attractive candidates for factors that could be working with HIF-1 in hypoxia. DAF-16 interacts with HIF-1 and has been shown to regulate proteostasis and lifespan (Murphy *et al.*, 2003; Shen *et al.*, 2005; Leiser *et al.*, 2013). Similarly, SIR-2.1 has been shown to regulate lifespan and plays a role in coordinating the maintenance of proteostasis under stress conditions (Kaeberlein *et al.*, 1999; Parker *et al.*, 2005; Raynes *et al.*, 2012). To determine whether DAF-16 or SIR-2.1 contributes to hypoxia-induced protein aggregation, we crossed the YFP::polyQ$_x$ transgenes into *daf-16(mu86)* and *sir–2.1(ok434)* mutant animals. The *daf–16(mu86)* allele, an 11 kb genomic deletion that removes nearly all of the winged-helix domain, is a presumed molecular null. The *sir–2.1(ok434)* allele contains a 1 kb deletion and an insertion

resulting in a frameshift. When exposed to hypoxia, we observed that both daf-$16(mu86)$;YFP::polyQ$_{40}$ and sir-$2.1(ok434)$;YFP::polyQ$_{40}$ mutant animals developed as many aggregates as wild-type controls (Fig. 4D,E). We therefore conclude that the effect of hypoxia on proteostasis is independent of the insulin/IGF like signaling pathway and sir-2.1.

The response to hypoxia has long-lasting effects on proteostasis

We next investigated whether the perturbation of proteostasis in hypoxia was reversible, or if the damage had a lasting effect upon the return to normoxia. We reasoned that if proteostasis recovered after the hypoxic insult, the rate of protein aggregation would be the same in animals regardless of whether they had been exposed to hypoxia. One complicating factor is that the rate of aggregation depends partly on how many aggregates had already formed. To separate the effects of hypoxia from the effect of increased aggregate number, we exposed animals to 1000 ppm O_2 for only 3 h (schematized in Fig. 5A). There is no increase in the number of YFP::polyQ$_{35}$ foci immediately after this short exposure to hypoxia. However, we found that the appearance of aggregates was accelerated in animals exposed to hypoxia (Fig. 5B). The number of aggregates in control animals that remain in room air did not increase, confirming that there were no age-associated defects in proteostasis over the course of this experiment.

Our result suggests that response(s) to hypoxia (or the transition between hypoxia and normoxia) induces long-lasting defects in proteostasis that cannot be corrected in room air. Consistent with this hypothesis, animals expressing YFP::polyQ$_{40}$ became paralyzed more rapidly after return to room air whether exposure was during L1 or L4

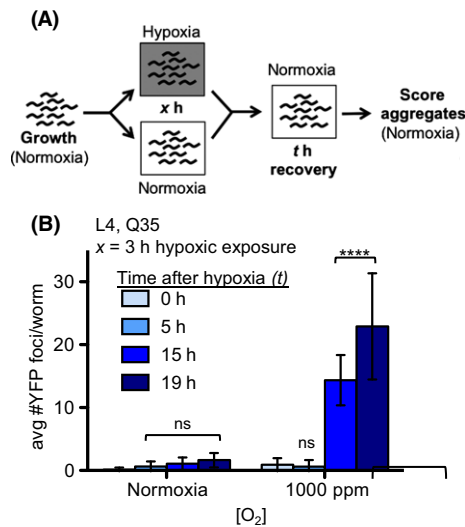**(A)**

(B) L4, Q35
x = 3 h hypoxic exposure

Fig. 5 Exposure to hypoxia has long-lasting effects on proteostasis. (A) Experimental design. YFP::polyQ$_{35}$ animals were grown in normoxia then transiently exposed to hypoxia for 3 h as L4. The number of YFP foci was scored after recovery in normoxia at each designated time (t) during recovery. (B) Short exposure to 1000 ppm O_2 disrupts proteostasis after return to normoxia. No aggregates were observed immediately after YFP::polyQ$_{35}$ animals were exposed to hypoxia, but the number of aggregates increased significantly more rapidly in the hypoxia-exposed cohort than controls during the recovery. Foci number was statistically compared to control animals at t = 0 h: ****P < 0.0001; ns, not significant. Each cohort had at least 30 animals. Graphs show mean ± SD. Summary of data from replicate experiments is included in Table S5 (Supporting information).
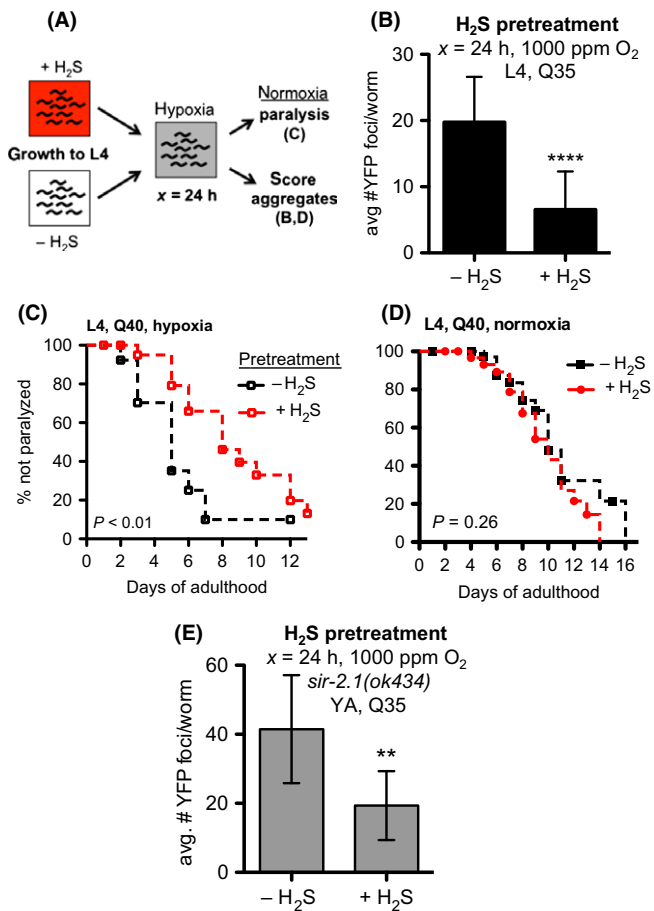
(Fig. 2A,B). In both situations, increased protein aggregation was observed at the time of the hypoxic exposure, but animals became paralyzed at adulthood. Thus, although the duration of hypoxic insults and transitions between hypoxia and normoxia were the same for the L1 and L4 cohorts, the time between protein aggregation and toxicity was longer in the L1 cohort than for those animals exposed as L4. This result supports the idea that aggregation that occurs during exposure to hypoxia does not alone explain the tissue damage that leads to eventual paralysis.

Adaptation to H$_2$S protects against hypoxia-induced disruption of proteostasis

Many studies suggest an intimate relationship between proteostasis and aging. Accumulating evidence shows that H$_2$S can effectively reduce cellular damage and death resulting from ischemia/reperfusion (I/R) injury in mammals (reviewed in Nicholson & Calvert, 2010). Moreover, H$_2$S increases lifespan and thermotolerance in *C. elegans* (Miller & Roth, 2007).

We considered the hypothesis that H$_2$S would protect against the hypoxia-induced defect in proteostasis. For these experiments, we grew YFP::polyQ$_{35}$ animals in 50 ppm H$_2$S before exposure to hypoxia (schematized in Fig. 6A). This concentration of H$_2$S activates HIF-1 and extends lifespan in *C. elegans* (Miller & Roth, 2007; Budde & Roth, 2010). We observed significantly fewer YFP::polyQ$_{35}$ foci in animals exposed to 1000 ppm O_2 that were grown in H$_2$S (Fig. 6B). The improvement in proteostasis is functionally important, as we also measured a significant delay in paralysis after exposure to 1000 ppm O_2 in YFP::polyQ$_{40}$ animals raised in H$_2$S relative to untreated controls (Fig. 6C). We conclude that pretreatment with H$_2$S enhances the ability to maintain proteostasis when challenged with hypoxia. More generally, these data indicate that, as in mammals, adaptation to H$_2$S can protect against the effects of hypoxia in *C. elegans*.

One curious aspect of our results is the effect of H$_2$S to increase lifespan appears to be distinct from its modulation of proteostasis in hypoxia. We noticed continuous exposure to H$_2$S is not required for the effects on proteostasis, although it is for increased lifespan [Fig. 6B,C (Miller & Roth, 2007)]. We also observed that treatment with H$_2$S for 48 h starting at adulthood is insufficient to protect against age-induced paralysis in YFP::polyQ$_{40}$ animals (Fig. 6D). Moreover, SIR-2.1, the *C. elegans* homolog of the sirtuin SIRT1 that is required for the effects of H$_2$S on lifespan and thermotolerance (Miller & Roth, 2007), is not required for H$_2$S to protect proteostasis in hypoxia (Fig. 6E). Just as in wild-type animals, sir–$2.1(ok434)$ mutant animals grown in H$_2$S develop significantly fewer YFP::polyQ$_{35}$ foci in hypoxia than controls grown in the absence of H$_2$S. Taken together, these results suggest that the effects of H$_2$S on proteostasis and lifespan are genetically distinct.

H$_2$S has been shown to improve outcome in mammalian preclinical models of severe blood loss and myocardial infarct even when administered after the ischemic event (Predmore & Lefer, 2011; Luan et al., 2012). This led us to consider the possibility that H$_2$S treatment would be sufficient to reverse this effect of hypoxia on proteostasis. To test this, we grew YFP::polyQ$_{35}$ animals in room air (normoxia, without H$_2$S), exposed them to 1000 ppm O_2, and then allowed to recover in the presence or absence of 50 ppm H$_2$S (schematized in Fig. 7A). Remarkably, animals treated with H$_2$S after exposure to hypoxia developed significantly fewer YFP::polyQ$_{35}$ foci during the recovery period than controls that were not exposed to H$_2$S (Fig. 7B). Posttreatment with H$_2$S also delayed the onset of hypoxia-induced paralysis in both YFP::polyQ$_{40}$ and Aβ_{1-42} animals (Fig. 7C,D). These data further support our

Fig. 6 Adaptation to H₂S protects against hypoxia-induced effects on proteostasis. (A) YFP::polyQₓ animals were grown in normoxia in the presence or absence of 50 ppm H₂S and then transiently exposed to 1000 ppm O₂ for 24 h. (B) Animals grown in H₂S develop fewer aggregates in 1000 ppm O₂. Animals were grown to L4 in H₂S then exposed to hypoxia. Aggregates were counted immediately after exposure to hypoxia. (C) H₂S pretreatment delays polyglutamine-associated paralysis after exposure to 1000 ppm O₂. Animals were grown to L4 in H₂S and then exposed to hypoxia. After return to normoxia (room air), paralysis was scored daily. (D) H₂S does not alter age-associated paralysis induced by YFP::polyQ₄₀. Animals were exposed to H₂S for first 48 h of adulthood, and then paralysis was monitored in room air. (E) The effect of H₂S on proteostasis in hypoxia is independent of sir-2.1. The number of YFP foci in sir-2.1 (ok434);YFP::polyQ₃₅ mutant animals after exposure to 1000 ppm O₂ was decreased by pretreatment similar to wild-type. For all panels, graph shows mean number of foci with SD error bars, each cohort consisting of 30–40 animals. Statistical comparisons were to matched normoxic controls: ****$P < 0.0001$; **$P < 0.005$; ns, not significant. Summary statistics from replicate experiments are provided in Table S6 (Supporting information).

assertions that hypoxia responses induce defects in proteostasis that persist after the hypoxic insult, but also imply that the detrimental effects of hypoxia on proteostasis are reversible.

Discussion

Disruption of proteostasis contributes to pathologies associated with aging, neurodegenerative diseases, and cancer. There are well-known responses to hypoxia that might be expected to improve proteostasis, such as reduced global translation and induction of protein chaperones. However, our data reveal that *in vivo* the response to specific hypoxic conditions actively disrupts the integrated proteostasis network. Our

results are consistent with observations in mammalian systems that ubiquitinated proteins and transgenically expressed proteotoxic proteins aggregate in neurons after ischemia/reperfusion injury *in vivo* (Hu et al., 2000; Unal-Cevik et al., 2011). Clinically, stroke is often associated with neurodegenerative sequelae and many studies have found an association between stroke and increased risk of Alzheimer's disease (reviewed in Kelleher & Soiza, 2013).

Our studies provide a unique and powerful model to begin to understand how metabolic and physiological adjustments to hypoxia could have long-lasting cellular consequences with important medical implications. For example, in mouse models of Alzheimer's disease, tau protein continues to aggregate even three months after ischemic injury (Koike et al., 2011). Similarly, acute ischemia/reperfusion injury in rat models of kidney transplants results in increased fibrosis and kidney dysfunction that are consistent with long-term physiological and cellular changes (Gueler et al., 2004). We showed that it is possible to prevent the hypoxia-induced disruption of proteostasis with H₂S. H₂S activates HIF-1 by inhibiting the EGL-9 prolyl hydroxylase (Budde & Roth, 2010; Ma et al., 2012). However, it is unlikely that H₂S acts solely through *hif-1* to improve proteostasis, especially as constitutive activation of HIF-1 does not improve proteostasis in hypoxia. H₂S exposure also results in the transcriptional upregulation of F-box proteins, which are adaptors for SCF ubiquitin ligases. Thus, it may be that H₂S protects the proteostasis network against hypoxia-induced protein aggregation by modulating the ubiquitin proteasome system. Nevertheless, our results show that the beneficial effects of H₂S are conserved from mammals to nematodes, which suggests a fundamental integration of H₂S signaling and cellular responses to hypoxia.

Our results indicate that hypoxia-induced disruptions of proteostasis are reversible, as treatment with H₂S after the hypoxic insult is sufficient to reduce both protein aggregation and cytotoxicity. In mammals, postconditioning with H₂S protects against ischemic injury and severe blood loss (King & Lefer, 2011). Proteostasis decreases with age and contributes to a variety of devastating neurodegenerative diseases. It has been proposed that proteostasis failure is a key driver of the aging process (Morley et al., 2002; Douglas & Dillin, 2010; Taylor & Dillin, 2011; O'Neill et al., 2012). However, we found that protective effects of H₂S are independent of *sir-2.1*, which is required for increased lifespan in H₂S (Miller & Roth, 2007). Moreover, short treatments with H₂S that do not increase lifespan are sufficient to protect against hypoxia-induced proteostasis. While the maintenance of proteostasis and lifespan has been shown to be tightly linked, our work adds to a growing collection of evidence that these two processes can be decoupled (Christie et al., 2014; El-Ami et al., 2014). We propose that proteostasis and aging are decoupled by H₂S and suggest the exciting possibility that other defects in proteostasis may be reversible even in aged organisms.

Materials and methods

C. elegans strains and methods

Animals were maintained on nematode growth media (NGM) with OP50 *E. coli* at 20°C (Brenner, 1974). For worm strains, see Table S7 (Supporting information).

Constructing hypoxic and H₂S-containing environments

Hypoxic and H₂S conditions were maintained using continuous flow chambers, as previously described (Padilla et al., 2002; Fawcett et al.,
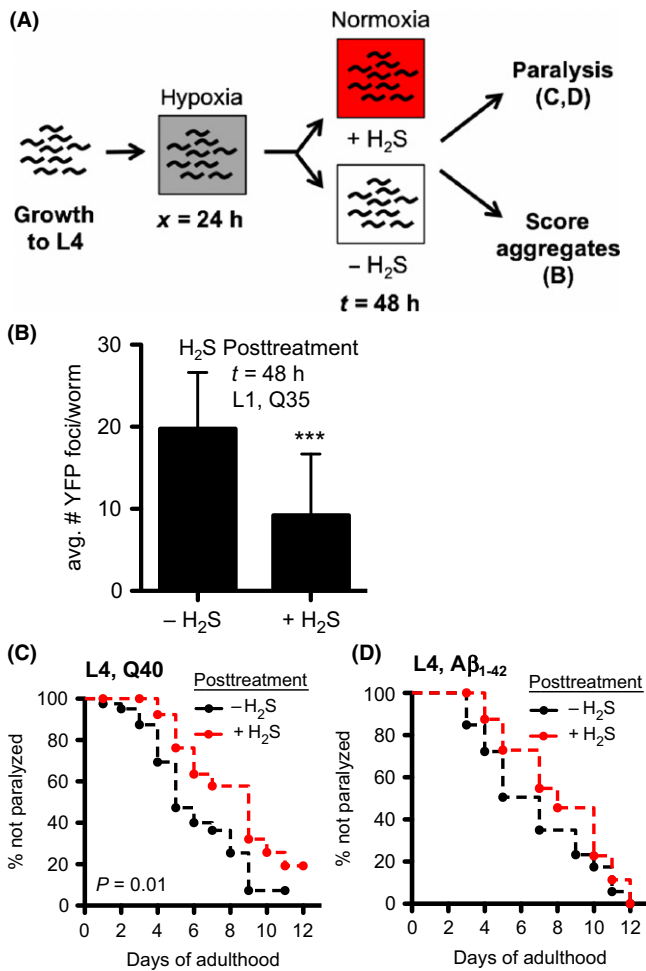
Fig. 7 Posttreatment with H_2S reverses effects of hypoxia on YFP::polyQ$_x$ aggregation and toxicity. (A) YFP::polyQ$_x$ animals were grown in normoxia (without H_2S) and then exposed to 1000 ppm O_2 (x = 24 h). The animals were returned to normoxia to recover ± 50 mM H_2S (t = 48 h). (B) Recovery in H_2S slows polyglutamine protein aggregation after return to normoxia. Statistical comparison between H_2S-treated and untreated controls: ***P < 0.0005. (C,D) H_2S posttreatment delays hypoxia-induced proteotoxicity. Paralysis is delayed by exposure to H_2S after removal from hypoxia in both YFP::polyQ$_{40}$ (C) and Aβ_{1-42} (D) animals. For all panels, each cohort consists of 30–50 animals. Summary of data from replicate experiments is included in Table S7 (Supporting information).

2012). Compressed gas tanks were purchased from Airgas (Seattle, WA) and were certified standard to within 2% of the indicated O_2 concentration (balanced with N_2). Hypoxic chambers were maintained in a 20°C incubator for the duration of the experiments. H_2S was diluted to 50 ppm with house air from a 5000 ppm stock tank (balance N_2) as previously described (Fawcett et al., 2012). H_2S environments were maintained in a fume hood at room temperature, with matched house-air (without H_2S) environments. Cultures were maintained continuously in H_2S for pretreatment experiments.

YFP::polyQ$_x$ aggregation assay

Synchronized cohorts of 50–75 YFP::polyQ$_x$ animals were exposed to hypoxic environments for approximately 24 h at 20°C on NGM plates seeded with live OP50 food. Palmitic acid (10 mg mL^{-1} in ethanol) was used to form a physical barrier around the edge of each plate to

encourage the animals to remain on the surface of the plate when in hypoxia. To visualize the localization of the YFP, worms were mounted on an agar pad in a drop of 20 mM sodium azide as anesthetic. Control experiments showed that azide did not affect the aggregation of YFP::polyQ$_{35}$ or YFP::polyQ$_{40}$, as observed by Moronetti Mazzeo et al. (2012). To evaluate protein aggregation in hypoxia, YFP foci were counted immediately after the hypoxic exposure. YFP foci were identified and quantified as described in Morley et al. (2002) and Silva et al. (2011). Aggregates were visualized and counted using a Nikon 90i fluorescence microscope with the GFP filter and oil-immersion 20× objective (Nikon Instruments Inc., Melville, NY, USA).

Synchronous YFP::polyQ$_{40}$ populations were generated by allowing first-day adult animals lay eggs for 1 h, after which time the adults were removed and the plates were incubated at 20°C overnight. Cohorts of 50–75 larvae were suspended in M9 and mouth-pipetted to NGM plates for hypoxic exposure. L4 animals were picked from well-fed, logarithmically growing populations and either exposed to hypoxia or allowed to develop to young adults overnight at 20°C.

In all experiments, the number of aggregates was counted blind to treatment. Statistical significance was evaluated by calculating P-values using Mann–Whitney nonparametric tests in GraphPad Prism version 5.0d for Mac OS X, GraphPad Software, San Diego California USA, www.graphpad.com. In experiments containing more than 2 experimental conditions or strains, a Kruskal–Wallis test and Dunn's multiple comparisons post hoc analysis were performed to calculate the P-values between conditions. In experiments with time courses, a two-way paired ANOVA was performed to calculate the P-value between time points. In all cases, P < 0.05 was considered to be statistically significant. Summary data from replicate experiments are included in Tables S1–S6 (Supporting information).

Paralysis assays of proteotoxicity

Animals expressing Aβ_{1-42}, tau(V337M), or YFP::polyQ$_x$ were exposed to 1000 ppm O_2 for 24 h at 20°C either as L4 or L1. After hypoxic exposure, animals were returned to normoxia and incubated at 20°C. Paralysis was scored daily. Worms were considered paralyzed if they exhibited movement of the nose or tail or pharynx pumping, but remained immobile after tapping with a platinum wire pick 3 consecutive times. Animals that did not move or pump were scored as dead. Dead and bagged worms were censored from the experiment on the day of death/bagging. Paralyzed worms were removed from the plate on the day of paralysis. Live worms that were not paralyzed were moved to a new plate each day until all worms were either scored as paralyzed or dead. Kaplan–Meier log-rank (Mantel–Cox) tests using GraphPad Prism were used to evaluate statistical significance.

Uncoordination in *dyn-1(ky51)* ts mutants was measured as described in Gidalevitz et al. (2006). Experiments shown were performed using unseeded NGM plates.

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Author contributions

EF, JH, and DM conceived and designed the experiments. EF, JJ, JH, and DM performed experiments and analyzed data. EF and DM wrote the manuscript.

Conflict of interest

None declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Fig. S1 5000 ppm O_2 does not accelerate paralysis associated with expression of polyglutamine proteins.

Fig. S2 Exposure to hypoxia does not result in upregulation of genes involved in maintenance of proteostasis.

Fig. S3 Mutation of *hif-1* in YFP::polyQ35 animals does not exacerbate hypoxiainduced proteotoxicity.

Table S1 Summary of data from replicate experiments as in Fig. 1.

Table S2 Summary of data from replicate experiments as in Fig. 2.

Table S3 Summary of data from replicate experiments as in Fig. 3.

Table S4 Summary of data from replicate experiments as in Fig. 4.

Table S5 Summary of data from replicate experiments as in Fig. 5.

Table S6 Summary of data from replicate experiments as in Figs 4 and 6.

Table S7 Summary of data from replicate experiments as in Fig. 7.

Table S8 List of worm strains.

Table S9 Summary of data from replicate experiments as in Figs 1, 2, and 3 (Supporting information).