Fasting protects against proteostasis defects induced by hypoxia

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Abstract

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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 impair essential physiological processes and cause cellular damage and death, such as is observed as a result of stroke and cardiovascular disease. We have found that specific concentrations of hypoxia cause a disruption of protein homeostasis in *C. elegans*. However, the genetic signaling pathways involved in hypoxia-induced proteostasis defects remain poorly defined. Furthermore, although animals must respond appropriately to hypoxia in order to survive, a lack of oxygen may not be the only environmental stress with which an animal needs to contend. Yet the ways in which organisms integrate responses to the presence of multiple

environmental stresses is also not well understood. In my dissertation research, I utilized the nematode *C. elegans* to study the response to hypoxia in conjunction with nutrient deprivation.

I show that nutritional cues regulate the effect of hypoxia on proteostasis and that both the insulin/IGF-1 signaling (IIS) pathway and AMP-activated protein kinase (AMPK) play roles in mediating the effects of hypoxia and nutrient deprivation on protein aggregation. Animals that are fasted prior to hypoxic exposure develop dramatically fewer protein aggregates compared to their fed counterparts. I discovered that IIS is required for fasting protection, as animals with mutations in *daf-2*, the *C. elegans* insulin/IGF-1-receptor, display wild-type levels of hypoxia-induced protein aggregation upon exposure to hypoxia when fed, but are not protected by fasting. However, this requirement for IIS is independent of the downstream transcription factor DAF-16/FOXO.

Furthermore, I found a role for AMPK in regulating the response to hypoxia that depends on the nutritional status of the animal. In fed conditions AMPK promotes protein aggregation, but without food AMPK is required for fasting-induced protection against aggregation. Taken together, my results outline a non-canonical role for the IIS pathway in coordinating the effects of both hypoxia and nutritional state on proteostasis, and also underscore AMPK's role in modulating cellular pathways that maintain proteostasis in response to a complex interaction of environmental cues.

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CHAPTER 1. HISTORY AND INTRODUCTION

1.1 OXYGEN HOMEOSTASIS

AUTHORS NOTE: This section is adapted from the following published paper:
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All organisms must maintain homeostasis to survive. Walter Cannon defined the modern concept of homeostasis as "the coordinated physiological reactions which maintain most of the steady states in the body. . .." (Cannon 1929). At the cellular level, maintaining homeostasis requires the coordination of metabolic reactions and cellular processes with environmental conditions. Homeostatic mechanisms are also centrally important for regulating longevity assurance. One consequence of the physiological decline associated with aging is degradation of the ability to maintain homeostasis, which narrows the range of conditions that can be tolerated. At least partly as a result of this defect in homeostasis, the likelihood of death from injury, infection, and disease increases. Oxygen (O_2) is an essential environmental resource for all metazoans, with only one known exception (Danovaro *et al.* 2010). The ability to sense and respond to changes in O_2 likely arose early in evolution (O'Farrell 2001). Nevertheless, even short exposure to decreased O_2 availability (hypoxia) leads to irreversible cellular damage and death in most metazoans.

There is great diversity in sensitivity to hypoxia between different animals and even between cell types in the same animal. For example, hibernating mammals have decreased respiration, with up to 30 min between breaths, and can survive in hypoxic conditions that

are damaging to related euthermic non-hibernators (Drew *et al.* 2004). In global cerebral ischemia, CA1 pyramidal neurons in the hippocampus begin to die before other neurons when blood flow is disrupted (Lipton 1999). This variation suggests there are mechanisms that promote homeostasis in hypoxia, but that they are only employed in specific physiological contexts. It is important also to consider the precise hypoxic conditions experienced by the cells and organism. The physiological consequences of hypoxia depend greatly on the duration and severity of the hypoxic insult.

Hypoxia, where O_2 levels are "less than normal" or low enough to disrupt normal function, includes a wide range of conditions. The ambient concentration of O_2 at sea level (1 atm atmospheric pressure) is 210,000 ppm (21%) O_2. At high altitude, though the concentration of O_2 remains the same, the lower atmospheric pressure results in decreased effective ambient O_2 tension. O_2 is poorly soluble in aqueous solutions and diffuses slowly. Therefore, steep O_2 gradients can exist in poorly mixed water environments and waterlogged soil. It can take >3 h for a 100 mm tissue culture dish to equilibrate with ambient O_2 levels (Chapman *et al.* 1970). In large animals, O_2 is delivered to cells by a complex circulatory system. The concentration of O_2 at the tissue level is lower than ambient, varies between tissue types, and depends both on O_2 delivery and tissue metabolic activity (Montgomery 1957; Dyson and Singer 2011). Fluctuations in ambient O_2 supply or tissue metabolic demand stimulate compensatory responses to increase blood flow and O_2 delivery, including vasodilation, increased respiratory rate, and production of red blood cells. This makes it difficult to experimentally control the hypoxic exposure of cells in an intact animal in order to investigate different cellular responses to hypoxia. It is important also to consider that it is experimentally difficult or impossible to separate damage that occurs in hypoxia or ischemia from effects that occur as a result of reoxygenation.

In contrast, *C. elegans* does not have a circulatory system, relying instead on diffusion for O_2 delivery to cells. This allows for precise experimental control of both genotype and cellular environment (Shen and Powell-Coffman 2003; Fawcett *et al.* 2012). Because it is an attractive model for hypoxia research, we have built a framework of hypoxia responses as a function of O_2 tension using *C. elegans*, drawing connections with other systems when possible. There have been several excellent reviews recently about signaling pathways that coordinate cellular responses to hypoxia (Gorr *et al.* 2006; Powell-Coffman 2010; Hand *et al.* 2011; Padilla and Ladage 2012; Semenza 2011). In this chapter I compare how strategies to respond to hypoxia vary with O_2 concentration and focus on how response mechanisms could integrate with other signaling pathways to influence organism physiology and lifespan. An overview of C. elegans respnsonses to various concentrations of hypoxia can be seen in Fig 1.1.

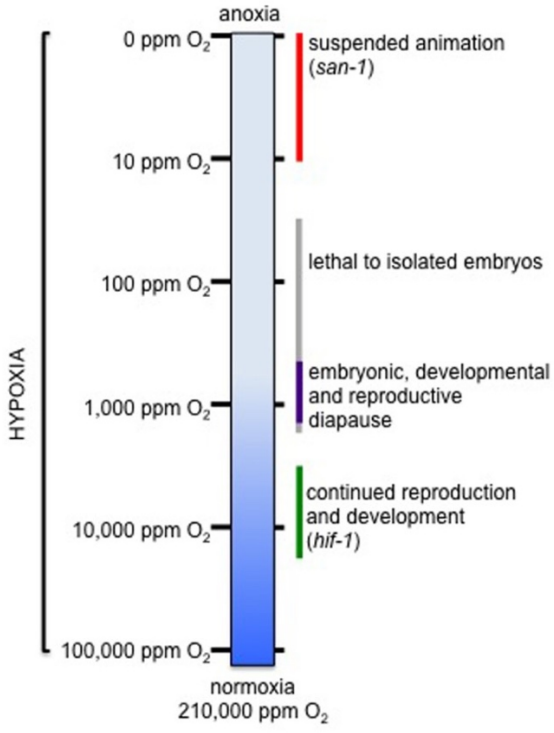


FIGURE 1.1. HYPOXIA RESPONSES AT DIFFERENCE CONCENTRATIONS OF O_2. The bar represents decreasing O2 levels, with normoxia at the bottom and anoxia at the top. For the purposes of this review, normoxia is considered to be room air, which is 210,000 ppm (21%) O2. Hypoxia includes all concentrations of O2 that are less than this. On the right,

the physiological response of *C. elegans* to different O_2 concentrations is noted, as described in the main text.

Adaptations to Anoxia

In the laboratory, *C. elegans*, *Drosophila melanogaster*, and *Danio rerio* all survive without O_2 (anoxia; operationally defined as <10 ppm O_2) by entering into a state of suspended animation (Foe and Alberts 1985; DiGregorio *et al.* 2001; Padilla and Roth 2001; Padilla *et al.* 2002). In suspended animation, all microscopically observable activity reversibly arrests, including embryonic cell divisions, post-embryonic development, movement, and reproduction. Upon reoxygenation, developmental processes resume and animals grow to healthy, fertile adults. Suspended animation can be successfully maintained for several days in *C. elegans*, weeks in *Drosophila* embryos, and years in the brine shrimp *Artemia franciscana* (Foe and Alberts 1985; Clegg 1997; Padilla *et al.* 2002). Mechanisms that underlie the ability to survive severe hypometabolic and quiescent states may be widely conserved. Metabolism is dramatically reduced in dogs that survive for several hours after total exsanguination with cold saline flush, for example (Behringer *et al.* 2003).

One common feature of suspended animation is the reversible arrest of cell divisions. The point at which cell cycle arrest occurs differs between organisms. *C. elegans* embryonic blastomeres arrest in interphase, prophase, and metaphase, but the transition to anaphase will not occur in anoxia (Padilla *et al.* 2002; Nystul *et al.* 2003; Hajeri *et al.* 2005). The spindle assembly checkpoint is activated by anoxia, and stopping the cell cycle is important to prevent lethal chromosome segregation defects. Embryos that have been depleted of *san-1*, a component of the spindle assembly checkpoint, die when exposed to anoxia and exhibit chromosome segregation defects (Nystul *et al*. 2003). In cells that arrest in interphase or prophase, the chromatin condenses and chromosomes align near the nuclear envelope, whereas metaphase blastomeres display reduced spindle and astral microtubule

density. The prophase arrest is characterized by inactivation of *cdk-1* and requires the *npp-16* nucleoporin (Hajeri *et al.* 2005). These results indicate that there are at least two distinct cell cycle checkpoints activated to arrest embryonic cell divisions in anoxia-induced suspended animation in *C. elegans*.

The spindle assembly checkpoint is not required for suspended animation in adults, possibly because somatic cells are all post-mitotic. However, germline stem cell divisions arrest in adults in suspended animation without any apparent decrease in full reproductive potential (Padilla *et al*. 2002; our unpublished observation). Thus, there may be other mechanisms that contribute to anoxia-induced suspension of cell division post-embryonically. The mechanisms by which anoxia signaling integrates with the spindle checkpoint are not well understood, though the effect is conserved. *Drosophila* embryos exposed to anoxia also arrest during interphase, prophase, and metaphase, and the arrest is characterized by chromatin localization near the nuclear membrane (Foe and Alberts 1985; Douglas *et al.* 2001). Similarly, *Danio rerio* embryos suspend cell division in anoxia, though arrest is exclusively during interphase (Padilla and Roth 2001).

In anoxia metabolic networks must be substantially rearranged, with important phenotypic consequences. O_2 is essential for both mitochondrial respiration and fatty acid oxidation. A major consequence of O_2 deprivation is that cellular energy metabolism is disrupted. The survival of both embryos and adult *C. elegans* in anoxia is correlated with available glycogen stores, which serve as a source for glycolytic energy production (Frazier and Roth 2009; LaRue and Padilla 2011). Glycogen decreases progressively as embryos are exposed to anoxia (Frazier and Roth 2009). Mutations in genes that have little in common, other than decreased glycogen content, all show an anoxia-sensitive phenotype during embryogenesis (Frazier and Roth 2009). Similarly, hyperosmotic shock, an environmental

perturbation that increases glycerol production at the expense of glycogen, reduces the viability of embryos in anoxia (Frazier and Roth 2009).

In contrast, in adults hypomorphic loss-of-function mutations in the insulin/IGF receptor homolog *daf-2* increase glycogen content and survival in anoxia (Scott *et al.* 2002; Mendenhall *et al.* 2006; Frazier and Roth, 2009; LaRue and Padilla 2011). Diet-induced increases in glycogen are also associated with increased survival in anoxia in *Drosophila* (Vigne *et al.* 2009). Depletion of the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase (*gpd-2/3*) by RNAi decreases survival of adult *daf-2* mutant animals in anoxia (Mendenhall *et al.* 2006). The significance of this result is not clear, insofar as *gpd-2/3(RNAi)* does not reduce survival of wild-type animals in anoxia (Mendenhall *et al.* 2006). One possibility is that the difference between wild-type and *daf-2* mutant animals reflects a difference in metabolic state. Both gene expression, oxygen consumption measurements, and physiological studies suggest that the *daf-2* mutant animals have a metabolic architecture that is very different from wild-type (Van Voorhies and Ward 1999; Lee *et al.* 2003; Murphy *et al.* 2003; Houthoofd *et al.* 2005). Moreover, RNAi directed against other glycolytic enzymes does not alter survival in anoxia (Mendenhall *et al.* 2006). This may suggest that simply decreasing glycolysis does not explain the effect on anoxia survival. However, it is difficult to assess whether the RNAi treatment sufficiently decreased the activity of the glycolytic enzymes in these experiments, and no direct measurements of effects on glycogen were reported.

In anoxia, fatty acid oxidation is not possible. Instead, increased fatty acid synthesis may be important for anabolic activity and to regenerate reducing equivalents for continued glycolytic activity. Fatty acid synthesis is a hallmark of hypoxic tumor cells (Romero-Garcia *et al.* 2011), and in *C. elegans* the SREBP homolog *sbp-1* is required for fatty acid

accumulation after anoxia (Taghibiglou *et al.* 2009). This result suggests that changes in lipid metabolism are essential parts of the response to hypoxia. However, it is also possible that lipid signaling plays an important role during O_2 deprivation. Consistent with this view, mutations that are predicted to disrupt ceramide synthesis modulate survival in anoxia. Survival was decreased by loss-of-function of *hyl-2*, whereas similar mutations in the related *hyl-1* increase survival in anoxia (Menuz *et al.* 2009). In mammalian models, altered ceramide signaling has been associated with hypoxia-induced changes in tumors and may contribute to cell death in neurological disorders including cerebral ischemia (Jana *et al.* 2009; Yin *et al.* 2010). *Hyl-1* and *hyl-2* are functional homologs of LAG1 (longevity assurance gene 1), which was reported to increase replicative lifespan in *Saccharomyces cerevisiae* (D'Mello *et al.* 1994). However, RNAi knockdown of neither *hyl-1* nor *hyl-2* increase lifespan in *C. elegans* (Menuz *et al.* 2009). Lipid metabolism and signaling are increasingly recognized as playing an important role in the regulation of aging and lifespan (Lapierre and Hansen 2012). Considering the important role that aberrant lipid signaling plays in the progression of cancer cells, elucidating the role that these processes play in adaptations to hypoxia is likely to be a productive direction for future research.

There is surprising overlap between genes and pathways that increase survival in anoxia and those that modulate lifespan, though the mechanistic basis of this correlation is not understood. In a screen for genes that increased survival in anoxia when depleted by RNAi, 11 of 198 hits (5.6%) had previously been identified to increase lifespan in *C. elegans* (Mabon *et al.* 2009). In contrast, the frequency of finding genes that increase lifespan from RNAi screens that use longevity as the primary phenotype ranged from 0.1 to 0.5% (Hamilton *et al.* 2005; Hansen *et al.* 2005). Thus, the genes identified by enhanced anoxia survival are enriched for longevity genes. In addition to a variety of metabolic genes identified in this screen, anoxia survival also requires autophagy, which may serve as an

important source for catabolic energy production. Disruption of genes important for autophagy by RNAi or mutation reduces survival in anoxia (Samokhvalov *et al.* 2008). In mammalian systems, autophagy is regulated by hypoxia, particularly in cancer cells (Rouschop and Wouters 2009; Eskelinen 2011). Moreover, autophagy is important for increased lifespan by both *daf-2* loss-of-function mutations and dietary restriction (DR) in *C. elegans* (Meléndez *et al.* 2003; Hansen *et al.* 2008). Overexpression of autophagy gene LC3/Atg8 in the nervous system increases lifespan in *Drosophila* (Simonsen *et al.* 2008).

The insulin/IGF1 signaling (IIS) pathway is another conserved pathway that is involved both in longevity assurance and the response to hypoxia. In. *C. elegans*, the IIS receptor homolog *daf-2* increases lifespan as well as survival in anoxia (Kenyon *et al.* 1993; Scott *et al.* 2002; Mendenhall *et al.* 2006). Increased stress resistance is a well-known feature of *daf-2* mutant animals, suggesting that increased survival in anoxia is a consequence of a correlation between increased stress resistance and lifespan (Lithgow *et al*. 1995; Honda and Honda 1999; Mendenhall *et al.* 2006; Scott *et al.* 2002). However, five of six *daf-2* regulated gene products depleted by RNAi increased resistance to anoxia but had no effect on lifespan (Mabon *et al.* 2009). Moreover, mutations that increase resistance to osmotic stress, including loss-of-function alleles of *dpy-10* and *osm-7*, decrease survival in anoxia (Wheeler and Thomas 2006; Frazier and Roth 2009). Thus, a general increase in stress resistance does not explain the relationship between lifespan and anoxia resistance.

Protein metabolism is another central aspect of cellular physiology affected by hypoxia. Protein synthesis and the chaperones that help to maintain cellular proteins in the correctly folded state are energetically expensive. The coordination of protein synthesis, quality control, and degradation, referred to as proteostasis, is essential to maintain cellular function (Hartl *et al.* 2011; Taylor and Dillin 2011). Reduced protein translation is

associated with increased lifespan in *C. elegans* (Hansen *et al.* 2007; Pan *et al.* 2007). Many genes that increase survival in anoxia when depleted by RNAi are involved in protein translation. Protein translation is inhibited in low O_2 (Hochachka *et al.* 1996; Teodoro and O'Farrell, 2003; Storey and Storey, 2004; Wouters *et al.* 2005; Liu *et al.* 2006), making it somewhat surprising that genetic manipulations that decrease translation would increase anoxia survival. It may be that indirect consequences of, or adaptations to, decreased translation confer the protective effect in anoxia. For instance, decreased energy utilization for protein translation could increase energy stores available in anoxia. Another possibility is that reduced translation rates improve proteostasis networks and improve the capacity to deal with unfolded protein stress in anoxia.

In the endoplasmic reticulum, the ERO1 enzyme uses O_2 to catalyze oxidative protein folding (Tu and Weissman 2002), which would be inhibited in anoxia. In *C. elegans*, the ER unfolded protein response (UPR) is activated in anoxia, and UPR genes *xbp-1* and *ire-1* are required for survival (Mao and Crowder 2010). This suggests that anoxia increases the burden of misfolded proteins in the secretory path. Decreasing translation by knock-down of aminoacyl tRNA synthase genes reduces expression of UPR mediators, and increases survival in anoxia (Anderson *et al.* 2009). UPR activity is increased by decreased O_2 in pancreatic β-cells and liver (but not cardiomyocytes), suggesting that it plays a conserved role in the cellular response to hypoxia (Tagliavacca *et al.* 2012; Zheng *et al.* 2012). Understanding general mechanisms that integrate stress homeostasis pathways with the proteostasis network could reveal new strategies to manipulate proteostasis. This would have broad significance, particularly as defects in proteostasis have been associated with the aging process (Haigis and Yankner 2010; Gidalevitz *et al.* 2011).

Responses to Hypoxia

A common strategy to survive hypoxia is to avoid conditions with insufficient O_2. Indeed, animals have evolved sophisticated behavioral strategies to avoid hypoxic conditions. In a gradient of O_2, blue crabs, New Zealand snapper, and *C. elegans* will all avoid low O_2 and show preference for an optimal O_2 environment (Dusenbery 1980; Bell *et al.* 2009; Gray *et al*. 2004; Cook and Herbert 2012). Interestingly, other environmental conditions can modulate what is perceived as the optimal O_2 concentration. Hypoxia avoidance in *C. elegans* decreases as animals are starved (Dusenbery 1980). Both alligators and cold-submerged frogs prefer lower ambient temperature in hypoxia (Branco *et al.* 1993; Tattersall and Boutilier 1997). This may reflect a physiological interaction between temperature and O_2. Consistent with this idea, *C. elegans* survive much longer in anoxia at low temperature than at higher temperature (Padilla *et al.* 2002; Scott *et al.* 2002; Mendenhall *et al.* 2006). It is not clear if the mechanisms that regulate survival are identical in these conditions, though the insulin/IGF receptor ortholog *daf-2* can increase survival at both temperatures (Scott *et al.* 2002; Mendenhall *et al.* 2006). The interaction between temperature and hypoxia may also have clinical relevance, as therapeutic hypothermia can reduce neurodevelopmental disability in infants surviving hypoxic ischemic encephalopathy from perinatal asphyxiation, and is used in adults clinically to improve outcome after pelvic surgery, cardiac arrest, and brain ischemia (Selway 2010; Finley 2011; Sunde and Søreide 2011; Yenari and Han 2012).

In moderate hypoxia (5,000–20,000 ppm O_2) *C. elegans* embryos complete development and grow to gravid adults, albeit more slowly than in room air (Jiang *et al.* 2001; Nystul and Roth 2004; Miller and Roth 2009). This indicates that the response to these hypoxic conditions is physiologically distinct from anoxia, in which animals enter suspended

animation. Consistent with this, embryos do not require *san-1*, the spindle assembly checkpoint protein essential for suspended animation (Nystul and Roth 2004), to survive exposure to hypoxia. Instead, HIF-1, the single worm homolog of the hypoxia-inducible factor (HIF) is required for embryo survival in 5,000–20,000 ppm O_2 (Jiang *et al.* 2001; Nystul and Roth 2004). HIF is a highly conserved bHLH-PAS domain transcription factor that helps maintain O_2 homeostasis by coordinating the transcriptional response to hypoxia in metazoans. There are many excellent reviews of HIF function and its role in development and disease (e.g., Semenza 2009; Semenza 2010; Semenza 2011; Semenza 2012; Majmundar *et al.* 2010; Powell-Coffman 2010). HIF was first identified biochemically as the factor that bound the erythropoietin promoter in hypoxia (Wang and Semenza 1993).

HIF is directly regulated by O_2 levels. HIF is hydroxylated at the conserved proline in the LxxLAP motif by a 2-oxoglutarate-dependent prolyl hydroxylase of the EGLN family, named after *egl-9* in *C. elegans* (Epstein *et al.* 2001). Hydroxylated HIF is then recognized by an E3-ubiquitin ligase, the Von Hippel–Lindau factor VHL-1, and degraded by the proteasome (Kaelin 2008). In hypoxia the hydroxylation is inefficient and HIF accumulates, dimerizes with the aryl hydrocarbon nuclear translocator (ARNT; *aha-1*), and induces expression of target genes that facilitate adaptation to hypoxia. In mammals, HIF is essential for early developmental events, and both HIF1α and HIF2α mutant mice die early in embryogenesis (Iyer *et al.* 1998; Compernolle *et al.* 2002). HIF homologs are also important for tracheal branching in *Drosophila* and neuronal patterning in *C. elegans*, highlighting the conserved role for HIF in development (Keith and Simon 2007; Centanin *et al.* 2008; Pocock and Hobert 2008). Constitutive stabilization of HIF has been implicated in tumor progression and mutations in VHL, a negative regulator of HIF, are associated with Von Hippel–Lindau syndrome, which is characterized by renal clear cell carcinoma (Kim and Kaelin 2004; Shen

and Kaelin 2012). Importantly, HIF-1 is not required for embryos to survive suspended animation in *C. elegans*, demonstrating that these two physiological responses to low O_2 are genetically distinct (Padilla *et al.* 2002). Although HIF has been the focus of most studies into transcriptional responses to hypoxia, there is also evidence that other factors are involved. HIF-independent transcriptional responses to hypoxia have been observed in *C. elegans* and mammals (Dong *et al.* 2001; Shen *et al.* 2005; Piret *et al.* 2006; Ndubuizu *et al.* 2010). The factors that mediate these effects are not well understood.

Despite the fact there are at least two separate adaptive responses to low O_2 – suspended animation in anoxia or continued development in moderate hypoxia – there are hypoxic conditions that are lethal during embryogenesis. Isolated embryos die when exposed to O_2 concentrations between 100 and 1,000 ppm O_2 (Nystul and Roth 2004). In these conditions, continued developmental progression is associated with increased lethality. Embryos exposed to 1,000 ppm O_2 undergo more cell divisions and experience a higher rate of lethality than those exposed to 100 ppm O_2, for 24 h (Nystul and Roth 2004). Although the cellular mechanisms that underlie these defects are not well understood, it has been demonstrated that inducing suspended animation in isolated embryos using carbon monoxide rescues embryo survival in hypoxia (Nystul and Roth 2004). Anoxia-induced suspended animation also protects *C. elegans* embryos against otherwise lethal cold exposure (Chan *et al.* 2010). These results suggest that arresting cell division and development facilitates coordination between cellular events and prevents irrevocable errors. Although embryos cannot autonomously engage suspended animation in these hypoxic conditions, embryos exposed to 1,000 ppm O_2 *in utero* arrest development and survive (Miller and Roth 2009). Embryo survival *in utero* requires *san-1*, suggesting that the embryos are in a state genetically related to anoxia-induced suspended animation (Miller and Roth 2009). We refer to this as a hypoxia-induced diapause, because it is reminiscent

of mammalian embryonic diapause, in which the adults remain active but arrest development of embryos *in utero* (Renfree and Shaw 2000). This embryonic diapause is coordinated by as-yet uncharacterized maternal factors that alter the uterine environment to impinge on embryonic development. Many facets of suspended animation and the mechanisms by which suspended animation can be non-autonomously controlled in the presence of O_2 remain a mystery and are likely to be a fruitful area of future research.

Developmental context also influences the response to hypoxia, with greater flexibility after embryogenesis. Newly hatched larvae survive in hypoxic conditions that are lethal to embryos (1,000 ppm O_2), and survival is associated with a reversible arrest of postembryonic development (Miller and Roth 2009). This suggests that there are mechanisms that can arrest cell division in 1,000 ppm O_2, but that embryos cannot enact this response. The arrest of post-embryonic cell divisions is genetically distinct from suspended animation, in that *san-1* is not required to arrest cell division of germline stem cells (Miller and Roth 2009). One caveat to this interpretation is that it has not been demonstrated that *san-1* is required for successful suspension of germline stem cell divisions in adults exposed to anoxia, and it is possible that suspended animation in adults employs different strategies to arrest cell division. Further delineation of the mechanisms used to arrest cell division in these conditions is required to evaluate this possibility. In addition to this developmental arrest, adults exposed to 1,000 ppm O_2 enter a reproductive diapause (Miller and Roth 2009). Gravid adults cease laying eggs, arrest the development and fertilization of oocytes, and halt embryonic development *in utero*. The arrest of progeny production ensures that embryos are not produced into conditions where they cannot survive. Moreover, energy shunted away from reproductive activity can be used instead for locomotion to search for a new environment. Therefore, by delaying progeny production animals can find a time and place more suited to successful reproduction. In this way,

hypoxia-induced reproductive diapause is similar to diapause in insects and mammals that ensures progeny production is synchronized with seasonal and nutritional conditions that maximize fitness (Renfree and Shaw 2000; Tatar *et al.* 2001; Allen 2007; Guidetti *et al.* 2008; Tachibana and Watanabe 2008).

HIF-1 is not required for hypoxia-induced diapause, as animals with a null allele of *hif-1* arrest post-embryonic development and reproduction in 1,000 ppm O_2 as efficiently wild-type animals (Miller and Roth 2009). Unlike the situation in embryos, *hif-1* mutant larvae and adults exposed to 5,000 ppm O_2 survive 24 h with >90% viability to adult upon reoxygenation (Nystul and Roth 2004; Miller and Roth 2009). Nevertheless, HIF-1 is necessary for the normal response to 5,000 ppm O_2. Whereas wild-type animals continue development in these conditions, *hif-1* mutant animals precociously enter into hypoxia-induced developmental and reproductive diapause (Miller and Roth 2009). This observation supports the idea that responses to hypoxia are specific to the concentration of O_2 that is available, and that HIF-1 does not play a major role in the response to 1,000 ppm O_2. In fact, even constitutive activation of HIF-1, by loss-of-function mutations in negative regulator *vhl-1* or *egl-9*, does not prevent diapause in 1,000 ppm O_2. This result further suggests that HIF-1 promotes continued developmental activity in both larvae and embryos, though it may have different targets in each developmental context. In contrast, early stage *hif-1* mutant embryos die in 5,000 ppm O_2, suggesting that HIF-1 acts autonomously during embryogenesis, when the nervous system is not fully developed, to protect against hypoxia (Jiang *et al.* 2001; Nystul and Roth 2004). The neuronal circuits and neuroendocrine factors that coordinate the systemic response to hypoxia have not been delineated, though it has been shown that hypoxia-induced diapause does not require the same neurons that mediate hyperoxia avoidance behavior (Gray et al. 2004; Miller and Roth 2009).

The AMP-activated protein kinase (AMPK) is also involved in regulating hypoxia-induced diapause in 5,000 ppm O_2. In 1,000 ppm O_2, *aak-2* mutant animals lacking a functional catalytic subunit are fully capable of entering into and surviving diapause. However, *aak-2* mutant animals precociously enter diapause in 5,000 ppm O_2 (Miller and Roth 2009). Thus, like HIF-1, AAK-2 acts to oppose diapause in hypoxia and support continued developmental activity. AAK-2 is not required for embryonic or larval survival in either 1,000 or 5,000 ppm O_2 (Miller and Roth 2009), though it is required for long-term survival in anoxia (LaRue and Padilla 2011). The source of this discrepancy could be either the duration or severity of O_2 deprivation. Another possibility is that AMPK has different functions in different hypoxic conditions. This could result if different AMPK complexes are active in each O_2 concentration. In addition to *aak-2*, *aakb-1/2 and aakg-2* contribute to long-term survival in anoxia (LaRue and Padilla 2011). It is not known which subunits other than *aak-2* are involved in coordinating hypoxia-induced diapause. Another possibility is that different AMPK substrates mediate these different physiological effects, depending on context. Recent proteomic studies have revealed that AMPK directly phosphorylates many components of the cell cycle machinery (Banko *et al.* 2011). These studies suggest a preliminary model in which HIF-1 acts upstream or in parallel to AMPK, which regulates cell division in hypoxia. Working out the mechanistic details that govern this effect is likely to provide unique insight into how AMPK coordinates cellular activities in response to metabolic stress.

1.2 Hypoxia Induces Proteostasis Defects

Hypoxia and Ischemia-Reperfusion Induce Proteostasis Defects

As described in the previous section, hypoxia is a condition characterized by insufficient oxygen availability. Cells and tissues in the body can become hypoxic as the result of ischemia, a lack of blood supply (and therefore oxygen) to tissues or organs. Ischemia and subsequent reperfusion of oxygen into the tissue can cause cellular and tissue damage and/or death. These types of injuries are often termed ischemia-reperfusion (I/R) injuries due to the inextricable link between blood flow disruption and reperfusion as well as the fact the reperfusion itself may have a causative role in exacerbating the damage incurred during the ischemic bout (Kalogeris *et al.* 2012). Although the extent of cellular damage is highly dependent on the severity and duration of the ischemic insult, specific tissues and organs have varying susceptibilities to I/R injury, with the brain, heart, and kidneys being the three most sensitive organs (Ordy *et al.* 1993, Boersma *et al.* 1996, Humphreys *et al.* 2009, Kalogeris *et al.* 2012).

A connection between hypoxia and protein aggregation has been uncovered in a number of systems. As mentioned above, the brain is the organ most sensitive to restrictions in blood supply. However, not all regions of the brain are equally susceptible to injury. Hippocampal neurons, particularly in the CA1 region, are especially vulnerable to hypoxic damage and death (Schmidt-Kastner and Freund 1991; Schmidt-Kastner 2015). The earliest studies on ischemia-induced proteostasis defects focused on proteostasis machinery expression profiles following cerebral ischemia. These early studies found a loss of ubiquitin staining and HSP70 expression following ischemia in rats and gerbils respectively, with a gradual return of immunoreactivity in all brain areas except the CA1 region (Vass *et al.* 1988; Magnusson and

Wieloch 1989). However, it was later shown that loss of free ubiquitin, rather than ubiquitin conjugation, may be behind the loss in ubiquitin immunoreactivity observed after ischemia (Morimoto *et al.* 1996). Subsequently, Hayashi and colleagues found that ischemia caused an increase in insoluble ubiquitin-protein conjugates (Hayashi *et al.* 1992a; Hayashi *et al.* 1992b; Hayashi *et al.* 1993), which finally provided a link between ubiquitin-related changes and biochemical data indicative of protein aggregates.

It wasn't until 2000 that ischemic protein aggregates were actually visualized using electron microscopy in a rat model of cerebral ischemia (Hu *et al.* 2000). Interestingly, the full extent of neuronal injury and death following ischemia does not become obvious until 2-4 days after the ischemic episode, a phenomenon known as 'delayed neuronal death' (Kirino 2000, Pulsinelli *et al.* 1982, Kirino 1982). Although protein synthesis is transiently suppressed in response to stressors such as ischemia, CA1 neurons that are destined to undergo delayed neuronal death post-ischemia display an irreversible translation arrest (Hossmann 1993; DeGracia and Hu 2007). Ubiquitin-marked aggregates are enriched in these sensitive CA1 neurons, and it was later found that at least some proportion of ischemia-induced aggregates contain translational machinery, including ribosomal subunits, co-translational chaperones, and eukaryotic initiation factors, suggesting that persistent translational arrest via translational machinery aggregation might be one mechanism for delayed neuronal death (Zhang *et al.* 2006; Liu *et al.* 2005).

Although less extensively studied, non-neuronal protein aggregation has also been reported. Polyglutamine proteins expressed in the body wall muscles of *C. elegans* aggregate following exposure to specific concentrations of hypoxia (Fawcett *et al.* 2015). *C. elegans* also exhibit intra-mitochondrial protein aggregation after exposure to hypoxia in addition to widespread mitochondrial dysfunction, including depolarization and abnormal pathology (Kaufman and Crowder 2015). This finding is in accordance with earlier findings from

Hayashi *et al.*, as their post-ischemic insoluble ubiquitin-conjugates were enriched in the mitochondrial fraction (Hayashi *et al.* 1992; Hayashi *et al.* 1993). Neonatal rat cardiomyocytes expressing tracts of polyglutamine display aggregation phenotypes after hypoxia (Ma *et al.* 2012) Finally, hypoxia increases amyloid-β in exosomes isolated from human neuroblastoma cells expressing human wild-type amyloid precursor protein (Xie *et al.* 2018).

Although it seems clear that ischemia, at least under some circumstances, can lead to protein aggregation, the relationship between the proteins that aggregate in hypoxia and those that aggregate with age or those that are found in neurodegenerative disorders has only recently begun to be investigated. In *C. elegans*, widespread protein aggregation is intrinsic to the aging process; furthermore, aggregates known to be present in human neurodegeneration are enriched in the set of proteins that that become more insoluble with age in worms (David *et al.* 2010). RNA-binding proteins, heat shock proteins, ubiquitin, and SUMO aggregate in mice exposed to cerebral ischemia. The aggregated RNA-binding proteins include TDP43, FUS, hnRNPA1, PSF/SFPQ, and p54/NONO, all of which have been linked to neurodegenerative diseases (Kahl *et al.* 2018).

Potential Collapse of the Ubiquitin Proteasome System and Autophagy in Hypoxia and I/R

The ubiquitin proteasome system (UPS) and autophagy are the two major protein degradation mechanisms employed by cells to deal with misfolded and aggregated proteins. The UPS is the more selective of the two and involves tagging substrates with polyubiquitin chains, thereby targeting them for degradation by the proteasome. In contrast, autophagy is a bulk degradative process, in which proteins and other cellular components are degraded via hydrolytic enzymes in the lysosome (Ji and Kwon 2017). Given the importance of these pathways in clearing misfolded and aggregated proteins it is no surprise that one hypothesis

for the phenomenon of hypoxia-induced protein aggregation is that a lack of oxygen results in a failure in one or both of these systems, thus causing the protein aggregation phenotypes observed following hypoxia.

Measuring changes in autophagy in response to a stress can be challenging because of its stepwise nature. As shown in Fig 1.2, the process of autophagy involves the formation of double-membrane-bound autophagosomes (APs) containing the cargo destined to be degraded. APs fuse with lysosomes to create autolysosomes (ALs), wherein hydrolases degrade the cellular material (Klionsky *et al.* 2012). Therefore, measuring the number of autophagic elements such as APs present at a given stage of autophagy does not necessarily inform researchers about the amount of flux through the pathway. In fact, although increased numbers of APs were often historically interpreted to represent an upregulation of autophagy, accumulation of APs is often due to a blockage of the AP to AL transition, and therefore represent a failure of autophagy rather than an increase (Klionsky *et al.* 2012).

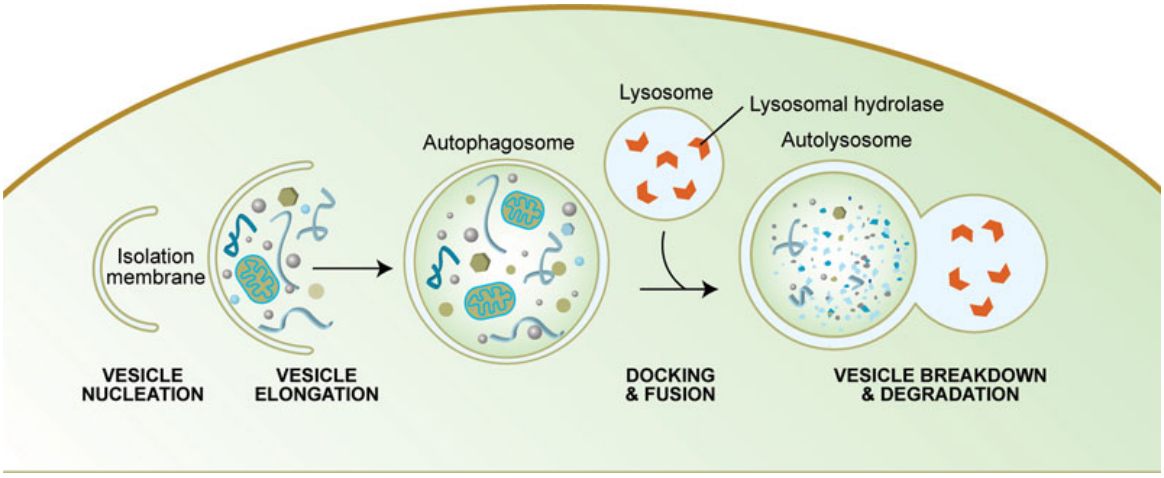


FIGURE 1.2 SCHEMATIC DIAGRAM OF THE STEPS OF AUTOPHAGY. Autophagy begins with the formation of the phagophore or isolation membrane (vesicle nucleation step). The concerted action of the autophagy core machinery proteins at the phagophore assembly site (PAS) is thought to lead to the expansion of the phagophore into an autophagosome (vesicle elongation). The autophagosome can engulf bulk cytoplasm nonspecifically, including entire organelles, or target cargos specifically. When the outer membrane of the autophagosome fuses with an endosome (forming an amphisome before fusing with the

lysosome) or directly with a lysosome (docking and fusion steps), it forms an autophagolysosome. Finally, the sequestered material is degraded inside the autophagolyosome (vesicle breakdown and degradation) and recycled. Figure reprinted from Meléndez and Levine 2009. Copyright permission granted by a Creative Commons attribution license.

Cells exposed to ischemia show accumulation of protein aggregates in close association with cellular organelles and vesicular structures including APs and ALs, but also mitochondria, fragmented Golgi, and ER (Liu *et al.* 2010; Liu *et al.* 2004; Hu *et al.* 2000). Neurons exposed to ischemia display an increased number of APs and ALs, a phenotype that is especially pronounced in CA1 neurons (Liu *et al.* 2004). APs and ALs are also increased after myocardial ischemia in mice (Ma *et al.* 2012). Chloroquine can be used to measure autophagic flux; as a lysosome inhibitor, it blocks the AP to AL transition. Comparing the effects of a chloroquine challenge between experimental groups (where autophagy might already be inhibited, thereby nullifying the action of chloroquine) and control groups (where flux through the autophagy pathway should be functional and chloroquine should show an effect) can therefore provide insight into the degree to which AP degradation may be blocked in an experimental condition.

Chloroquine challenges have indicated that AP processing, and therefore flux through the autophagy pathway, is impaired after I/R in neurons (especially vulnerable CA1 neurons) and in cardiomyocytes (Liu *et al.* 2010; Ma *et al.* 2012). This flux deficiency may be due at least in part to decreased lysosome-associated membrane protein 2 (LAMP2) levels, as exogenous LAMP2 expression rescues AP processing in cardiomyocytes exposed to I/R (Ma *et al.* 2012). Rescuing autophagy levels through LAMP2 expression or overexpressing the autophagy regulator Beclin1 functions to protect cardiomyocytes from I/R death (Ma *et al.* 2012; Hamacher-Brady *et al.* 2006). Although there isn't evidence to directly link the accumulation of protein aggregates after ischemia to the impairment of autophagic flux, the

failure of autophagy post-ischemia remains a plausible mechanistic hypothesis for the appearance of these aggregates.

In addition to autophagy, the UPS also acts to maintain cellular protein quality control. Interpretation of proteasome dysfunction following ischemia is less ambiguous than autophagy, and there is a general consensus that ischemia disrupts the UPS in both cerebral and myocardial ischemia (Calise and Powell 2013). The degree of impairment varies with the length and duration of ischemic exposure (Keller *et al.* 2000; Ge *et al.* 2007; Kamikubo and Hayashi 1996), and as was seen with autophagy, vulnerable CA1 neurons may be particularly at-risk for proteasome inhibition (Asai *et al.* 2002). Proteasome components can be found in insoluble aggregate fraction (Ge *et al.* 2007), perhaps indicating that proteasomes initially attempt to target the aggregates for degradation, but instead become entangled and cease to function properly.

The two main explanations for UPS dysfunction following ischemia point to ATP depletion and oxidative damage to proteasome and/or regulatory subunits as the causative factors (Calise and Powell 2013). Protein ubiquitination and proteasome function are ATP-dependent processes and ischemia depletes ATP levels within the cell, leading to the intuitive hypothesis that ATP depletion during ischemia is the basis for proteasome inhibition (Calise and Powell 2013). Indeed, I/R decreases ATP-dependent proteasome activity by approximately 50% (Churchill *et al.* 2010). However, this hypothesis predicts that the addition of exogenous ATP would reverse the dysfunction, which was not found true in *ex vivo* post-ischemic cardiac lysates (Powell *et al.* 2007). Alternatively, UPS inhibition might be a result of oxidative damage to the proteasome. The 19S regulatory particle shows signs of oxidative damage in the form of carbonylation following myocardial ischemia in conjunction with a 50% decrease in chymotryptic activity. Furthermore, inhibition of carbonylation was observed with ischemic preconditioning, which also restored the

chymotryptic activity (Divald *et al.* 2010). Mice that overexpress the antioxidant enzyme glutathione peroxidase showed less proteasome inhibition and infarct size following I/R compared to wild-type mice (Keller *et al.* 2000). Although correlative evidence suggests oxidation and/or ATP depletion may be behind UPS impairment in hypoxia, more research will be necessary to make a definitive statement. It is also entirely possible that the dysfunction comes from different sources in different models of hypoxia or different tissues.

Although not part of the cellular proteolytic machinery, chaperones function to support folding of proteins into their correct native structure. They prevent protein aggregation in multiple contexts: they can work on newly synthesized proteins and can also be induced by stressors such as heat and hypoxia (Sun *et al.* 2015; Truettner *et al.* 2009). Some work has been done to investigate whether enhancing chaperone activity is sufficient to protect neurons from ischemic protein aggregation. Ubiquitin-marked protein aggregates appear in primary mouse astrocyte cultures exposed to oxygen-glucose deprivation, an experimental paradigm designed to mimic ischemia, along with cell death. Overexpression of the chaperone HDJ-2 reduces both protein aggregation and cell death (Qiao *et al.* 2003). Similarly, mice with neuronal Hsp70 overexpression show a reduction in ubiquitin-labeled aggregates in CA1 neurons after global ischemia. Heat shock protein overexpression is also able to protect primary astrocyte cultures from cell death after oxygen-glucose deprivation (Giffard *et al.* 2004). Thus, chaperones may be a promising target for reducing hypoxia-induced proteostasis defects.

1.3 INSULIN/IGF-1 SIGNALING (IIS)

AUTHORS NOTE: This subchapter and the following will be dedicated to insulin/IGF-1 signaling (IIS) and AMP-activated protein kinase (AMPK), respectively. IIS and AMPK are

two master regulatory pathways responsible for sensing the metabolic environment and coordinating cellular responses accordingly. As ultimate and penultimate sections (1.4 and 1.5) of this chapter will discuss the intersection between dietary restriction, hypoxic injury, and protein aggregation associated with neurodegeneration, an overview of two of the signaling pathways by which dietary restriction might mediate its effects seems judicious. Furthermore, Chapters 3 and 4 will investigate IIS and AMPK, respectively, as they relate to hypoxia-induced protein aggregation.

Overview of IIS Pathway

Insulin was discovered in 1921 by Frederick Banting and Charles Best, a finding that resulted in a Nobel Prize for Banting (Rosenfeld 2002). Although it is a complex signaling pathway with many nodes of crosstalk with other systems, the fundamentals of the pathway are conserved from yeast through humans (Barbieri *et al.* 2003; Kenyon 2010). This work will focus on mammalian IIS and the corresponding genes, proteins, and pathways in *C. elegans.* At the most basic level IIS involves: autophosphorylation of the IIS tyrosine kinase receptor upon substrate binding, recruitment and phosphorylation of receptor substrates including insulin receptor substrate (IRS) and Shc, and subsequent activation of phosphoinositide 3 kinase (PI3K)/Akt pathway and the Ras/mitogen-activated protein kinase (MAPK) pathway, respectively (Boucher *et al.* 2014). This activation process has a huge variety of differential effects on IIS target tissues (Fig 1.3).

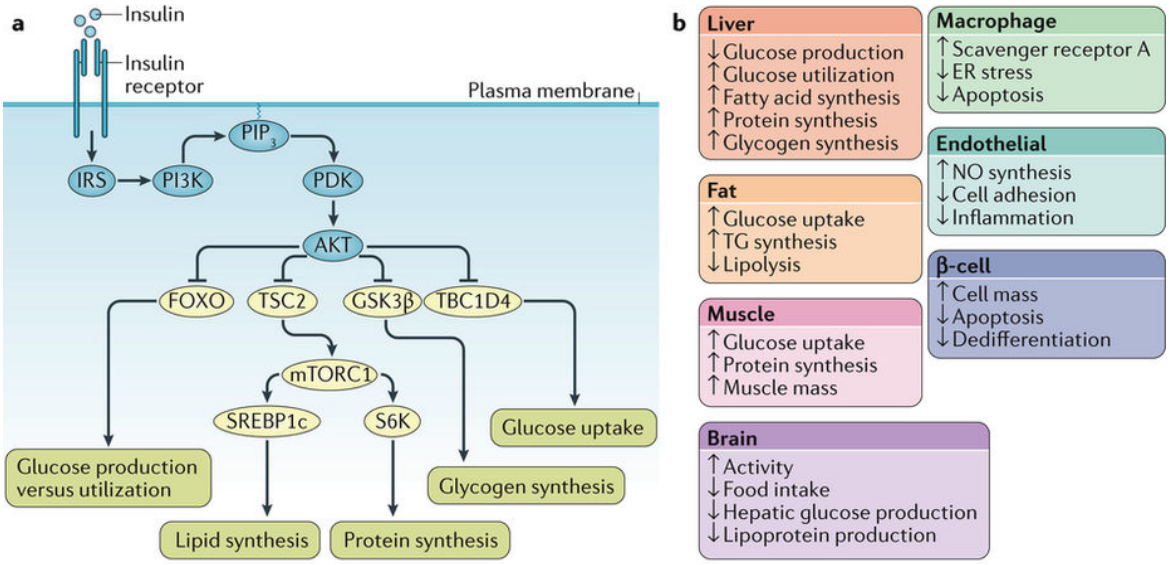


FIGURE 1.3 ACTIVATION OF INSULIN SIGNALING (a) Following insulin binding, the insulin receptor (IR) tyrosine kinase is activated, causing tyrosine phosphorylation of IR and of the IR substrate (IRS) proteins. Phosphotyrosine sites on IRS allow binding of the lipid kinase PI3K, which synthesizes PtdIns(3,4,5)P_3 (PIP_3) at the plasma membrane. This recruits the phosphoinositide-dependent kinase (PDK), which directly phosphorylates the Thr308 residue of AKT. A second phosphorylation of AKT, at the Ser473 residue, is carried out by mTOR complex 2 (mTORC2) (not shown). Activated AKT goes on to phosphorylate a number of substrates at Ser/Thr residues. These include: the forkhead family box O (FOXO) transcription factors; the protein tuberous sclerosis 2 (TSC2), which permits activation of mTORC1 and its downstream targets ribosomal protein S6 kinase (S6K) and sterol regulatory element binding protein 1c (SREBP1c); glycogen synthase kinase 3β (GSK3β) and the RabGAP TBC1 domain family member 4 (TBC1D4). These effector proteins mediate the effects of insulin on glucose production, utilization and uptake, as well as the synthesis of glycogen, protein and lipid. (b) Effects of insulin signalling in various tissues and cell types. ER, endoplasmic reticulum; NO, nitric oxide; TG, triglyceride. Figure reprinted from Haeusler *et al.* 2018. Copyright obtained via Copyright Clearance Center's Rightslink® service.

Insulin/IGF-1 Receptor Binding and Activation of Insulin Receptor Protein

IIS signaling begins with the binding of insulin or IGF-1 to insulin receptors (IRs) and IGF-1 receptors (IGF1Rs). These surface receptors are transmembrane tyrosine kinases (Gammeltoft and Van Obberghen 1986), and are highly homologous (Adams *et al.* 2000). While insulin and IGF-1 will preferentially bind to their cognate receptor, each has the

capability of binding to the alternate receptor, albeit with a reduced affinity (De Meyts *et al.* 1995; Belfiore *et al.* 2009; Xu *et al.* 2018). In *C. elegans*, DAF-2 is the only IIS receptor, and it is equally homologous to human IGF1R and IR (Kimura *et al.* 1997). In both mammals and *C. elegans*, IIS receptor subunits exist as $\alpha\beta$ dimers that interact via disulfide bridges to form a tetrameric receptor complex upon ligand binding (Ullrich and Schlessinger, 1990; Hubbard *et al.* 1994; Kimura *et al.* 1997).

Ligand binding to IIS receptors induces autophosphorylation of the β subunits, providing docking sites for receptor substrates. Receptor tyrosine kinases, including the IIS receptors, use proteins with Src homology 2 (SH2) domains and phosphotyrosine binding (PKB) domains as adaptor signaling proteins; these proteins bind to the phosphorylated tyrosine residues (Ullrich and Schlessinger 1990) and initiate various signal transduction pathways. Within mammalian IIS, these types of proteins are called insulin receptor substrate (IRS) proteins, of which there are four (Sun *et al.* 1991; Sun *et al.* 1995; Lavan *et al*. 1997a; Lavan *et al.* 1997b), though humans appear to lack IRS-3 (Björnholm *et al.* 2002). In *C. elegans*, there don't seem to be any clear homologues to mammalian IRS proteins (Nelson and Padgett 2003). The closest candidates are IST-1 and APP-1, which have a limited sequence homology to other IRS proteins, and aren't essential for DAF-2 signal outputs (Wolkow *et al.* 2002). In addition to IRS proteins, activated IIS receptors can also phosphorylate a variety of other substrates, including Shc protiens. These proteins participate in the signal transduction cascade that leads to Ras-MAPK pathway activation and control over cellular proliferation (Boucher *et al.* 2014; Ravichandran, 2001). *C. elegans* has two Shc homologues (Neumann-Haeflin *et al.* 2008; Mizuno *et al.* 2008), but the Shc proteins in *C. elegans* don't appear to mediate Ras signaling as they do in mammals (Mizuno *et al.* 2008).

Phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) and Akt signaling

Following IRS activation, the next step in the IIS pathway involves the lipid kinase phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K). PI3Ks are heterodimers composed of a regulatory and catalytic subunit (Vadas *et al.* 2011) and are recruited to the plasma membrane upon activation by IRS (Myers *et al.* 1992). PI3K converts phosphatidylinositol (4,5) bisphosphate (PIP_2) to phosphatidylinositol (3,4,5) triphosphate (PIP_3). PIP_3 acts as a lipid second messenger and recruits Akt. Most cellular responses to insulin are abolished when PI3K is inhibited, which has led to the belief that PI3K signaling via PIP_3 plays a crucial role in propagating IIS through the cell (Shepherd *et al.* 1995; Okada *et al.* 1994; Shimizu and Shimazu *et al.* 1994; Sutherland *et al.* 1995). The *C. elegans* homologue of PI3K, AGE-1, was first identified in a forward screen for mutations that increased longevity (Klass *et al.* 1983).

Increased PIP_3 concentrations at the membrane recruits Akt (also known as PKB) from the cytosol. At the membrane Akt binds to PIP_3 via its pleckstrin homology (PH) domain. This interaction results in Akt's phosphorylation at two conserved residues, T308 and S473 in human AKT1, by two other kinases: 3-phosphoinositide-dependent protein kinase 1 (PDK1) and mammalian target of rapamycin complex 2 (mTORC2), respectively (Alessi *et al.* 1996; Bayascas 2010; Sarbassov *et al.* 2005). Activated Akt can then translocate to the nucleus (Andjelković *et al*. 1997) or remain in the cytoplasm to phosphorylate its downstream substrates (Manning and Cantley 2007). Mammals have 3 isofoms of Akt (Schultze *et al.* 2011) while *C. elegans* has 2 (Paradis and Ruvkin 1998; Ailion and Thomas 2003).

Activated Akt has been shown to act on downstream proteins that regulate lipid, glycogen, and protein synthesis, as well as cell survival (Kitamura *et al.* 1999; Cross *et al.* 1995; Scott *et al.* 1998; Datta *et al.* 1999). Akt exerts these cellular effects by phosphorylating a wide

variety of effectors. Of particular note is mTORC1, which is activated by Akt-phosphorylated tuberous sclerosis complex protein 2 (TSC-2) and/or Akt-induced inhibition of its negative regulator PRAS40. mTORC1 ultimately goes on to mediate a wide network of anabolic pathways, partly through the inhibition of 4E-binding protein (4E-BP1) and activation of ribosomal protein S6 kinases (Düvel *et al.* 2010). Glycogen synthase kinase 3 (GSK3) is inactivated by Akt-phosphorylation. GSK3 itself inactivates glycogen synthase, the catalyst for the first step of glycogen synthesis. Thus, Akt ultimately upregulates glycogen synthesis by inhibiting the glycogen synthase inhibitor GSK3 (Lizcano and Alessi 2002).

FOXO Signaling

Another important group of Akt targets are the Forkhead box-containing protein, subfamily O (FOXO) transcription factors (Manning and Cantley 2007). Akt phosphorylates FOXOs at several sites, which ultimately exclude FOXOs from the nucleus and thereby inhibits transcriptional activity (Tzivion *et al.* 2011). This signaling pathway has been particularly well-researched in *C. elegans*, where the sole FOXO transcription factor is DAF-16 (Ogg *et al.* 1997). Many genes in the *C. elegans* IIS pathway, including the insulin receptor *daf-2*, and the FOXO transcription factor *daf-16*, were originally identified for their dauer phenotype. *C. elegans* go through four developmental stages before entering adulthood. When larvae are maintained without sufficient food, at a high density, or in the presence of a specific pheromone, they enter an alternative developmental stage called dauer. This stage is reversible if the environmental conditions improve, and is characterized by increased fat storage, longer lifespan, and reduced metabolic activity (Guarente *et al.* 1998). Mutations can cause a constitutive dauer phenotype or defects in entering dauer, and these dauer-reglating genes are called daf genes in worms (Riddle *et al.* 1981). Mutations in the IIS receptor *daf-2, akt-1*, and *age-1*/PI3K result in a dauer-constitutive phenotype, which can be fully suppressed by mutations in *daf-16*/FoxO (Gottlieb and Ruvkin

1994; Hu *et al.* 2006; Ailion and Thomas 2003; Larsen *et al*. 1995). In worms this, along with suppression of many other *daf-2* phenotypes by *daf-16* mutations, suggests that DAF-16 is the major target of these upstream IIS components (Slack *et al.* 2011).

Negative regulation of IIS

Since the IIS pathway regulates so many metabolic and mitogenic downstream pathways critical to cellular and organismal homeostasis, its regulation must be tightly controlled. There are levels of negative regulation that can act at different points in the signaling cascade. One component of negative regulation involves tyrosine phosphatases. These proteins play a critical role in terminating receptor tyrosine kinase-mediating signals, and can exist both cytoplasmically as well as in the membrane (Goldstein *et al.* 1998). Protein tyrosine phosphatase 1B (PTP1B) is one of the more well-studied. A cytoplasmic tyrosine phosphatase, PTP1B dephosphorylates the tyrosine residues on IRs, IGF-1Rs, as well as IRS proteins (Goldstein *et al.* 1998). In mice, the knockout of PTP1B causes enhanced insulin sensitivity, increased basal metabolic rate, and resistance to both obesity and insulin resistance due to a high-fat diet (Elchebly *et al.* 1999; Klaman *et al.* 2000).

In addition to tyrosine phosphatases, serine/threonine phosphatases can also act on members of the IIS pathway. Protein phosphatase 2A (PP2A) is a family of dimeric and heterotrimeric proteins that accounts for the majority of cellular serine/threonine phosphatase activity (Millward *et al.* 1999). PP2A is usually found as a heterotetramer composed of a catalytic C subunit, structural A subunit, and optional regulatory B subunit (Lambrecht *et al.* 2013). PP2A regulates the phosphorylation of a large number of proteins, including kinases important in the IIS pathway such as Akt and S6K (Millward *et al.* 1999). However, its regulation and mechanisms for specificity are still open questions, as PP2A members are known to function in a huge variety of signaling pathways that regulate

development, immunity, apoptosis, and cell cycle progression (Nematullah *et al*. 2018). In humans, multiple isoforms of each subunit allow for the formation of at least 75 holoenzyme formations (Guergnon *et al.* 2011). In *C. elegans*, *pptr-1* is a PP2A subunit that regulates dephosphorylation of AKT-1 at Thr350 and antagonizes IIS effects (Padmannabhan *et al.* 2009). In cell culture PP2B/calcineurin and PH domain leucine-rich repeat protein phosphatases (PHLPP1 and PHLPP2) have also been shown to dephosphorylate Akt (Ni *et al.* 2007; Brognard and Newton 2008).

Another level of negative regulation for IIS comes at the level of PIP_3 concentration. Phosphatase and tensin homolog (PTEN) is a lipid phosphatase that antagonizes Akt and PI3K by reducing PIP_3 levels via dephosphorylation (Cantley and Neel 1999). PTEN knockout mice have increased insulin sensitivity (Stiles *et al.* 2004). DAF-18 is the worm homolog of PTEN, and mutations in *daf-18* suppress the longevity and dauer-constitutive phenotypes conferred by *akt-1* and *daf-2* mutations (Gottlieb and Ruvkin, 1994; Larsen *et al.* 1995; Ogg and Ruvkin 1998; Gil *et al.* 1999; Mihaylova *et al.* 1999).

IIS clearly touches on many downstream effects and is subject to an intricate level of regulation. As one of the major nutrient-sensing pathways in the cell that also integrates with stress response pathways, I hypothesized that it may be involved in hypoxia-induced protein aggregation or fasting-induced protection against it. This hypothesis is tested in Chapter 3, where I identify a role for the IIS receptor DAF-2 in fasting protection against hypoxia-induced defects in proteostasis.

1.4 AMP-Activated Protein Kinase (AMPK) Signaling

Overview of AMPK Pathway

AMP-activated protein kinase (AMPK) is a metabolic sensor and regulator of cellular energy homeostasis. It monitors fluctuations in the AMP or ADP to ATP ratio in the cell and is activated by nutrient-limiting conditions. It responds to these energy deficits by upregulating catabolic pathways that provide ATP while simultaneously downregulating anabolic pathways and other dispensable processes that consume ATP (Hardie 2014; Mihaylova and Shaw 2011). AMPK is an incredibly conserved protein – its orthologs are found in virtually all eukaryotes, including plants and animals as well as fungi and protists, with the exception of a small number of obligate intracellular parasites (Hardie *et al.* 2012; Miranda-Saavedra *et al.* 2012; Miranda-Saavedra *et al.* 2007). AMPK impinges on a wide variety of cellular processes, perhaps unsurprising given its central role in regulating metabolism. Through its interactions with downstream effectors, AMPK regulates growth, proliferation, metabolism, autophagy, development, and has also been implicated in a number of neurodegenerative disorders (Hardie 2014; Marinangeli *et al.* 2016).

Structural Composition of AMPK

AMPK is a heterotrimeric serine/threonine kinase composed of a catalytic α subunit, and regulatory β and γ subunits. In mammals, each subunit has multiple isoforms (two α, two β, and three γ isoforms) encoded by different genes (Hardie 2014). In *C. elegans*, there are two catalytic α isoforms, called *aak-1* and *aak-2* (Apfeld *et al.* 2004) two β, and five γ subunits (Tullet *et al.* 2014). As its name suggests, AMPK is sensitive to cellular AMP:ATP ratio, though ADP levels can also regulate it to a lesser degree (Gowans *et al.* 2013). Based

on *in vitro* assays, the kinase activity of fully activated AMPK may be almost 1000 fold greater than inactive AMPK; however, AMPK activity in live cells functions at much more modest ranges (Gowans *et al.* 2013; Suter *et al.* 2006; Hardie et al. 2018).

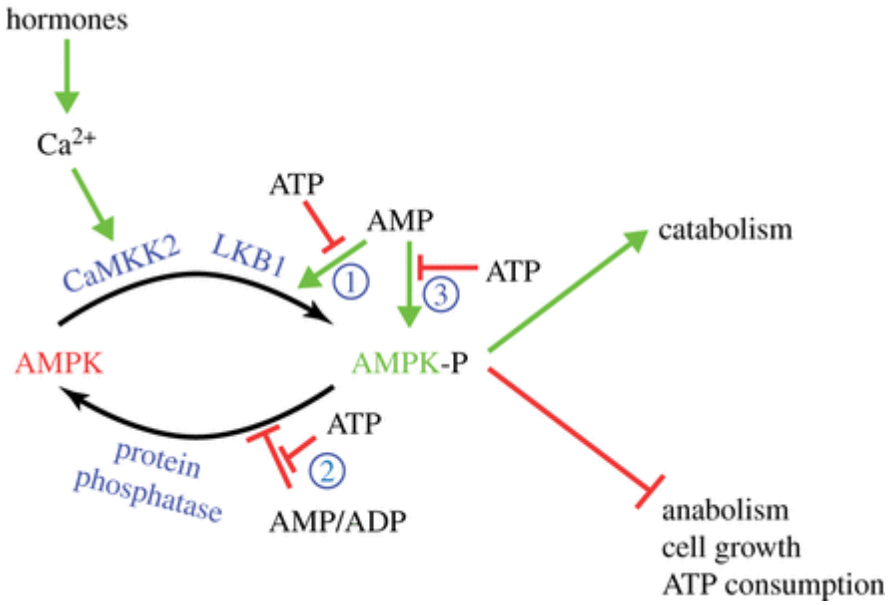


FIGURE 1.4 TRIPARTITE MECHANISM FOR ACTIVATION OF AMPK. Binding of AMP to the AMPK-γ subunit causes activation by (1) promoting phosphorylation by LKB1; (2) inhibiting dephosphorylation by protein phosphatases and (3) allosteric activation. Binding of ADP at higher concentration can mimic effect (2), whereas biding of ATP antagonizes all three effects. Increases in intracellular Ca^{2+} activate CaMKK2, which phosphorylates the same site on AMPK (Thr172) as LKB1. Figure reprinted from Hardie 2018. Copyright permission granted by a Creative Commons attribution license.

Regulation of AMPK activity happens via physical binding of AMP or ADP to AMPK. The regulatory γ subunit contains adenine nucleotide binding sites formed by four tandem cystathionine β-synthase (CBS) repeats (Scott *et al.* 2004). One of these sites is permanently occupied by AMP, one is unoccupied, and the other two sites are capable of binding AMP, ADP, and ATP with similar affinities (Xiao *et al.* 2007; Xiao *et al.* 2011). Binding of AMP to the γ subunit promotes AMPK activation by three mechanisms, diagrammed in Fig 1.4. Firstly, AMP binding promotes phosphorylation by upstream kinases

(Hawley *et al.* 1996). The upstream kinases that phosphorylate AMPK will be described shortly. Secondly, AMP binding inhibits dephosphorylation (Davies *et al.* 1995). This inhibition can also be accomplished when ADP binds to the γ subunit (Xiao *et al.* 2011). Finally, AMP binding causes allosteric activation even at relatively low concentrations (Gowans *et al.* 2013). The degree of allosteric activation varies from 2-5 fold depending on the γ isoform present (Cheung *et al.* 2000).

The α subunit contains a kinase domain at its N terminus. An auto-inhibitory domain composed of α-helices is located immediately adjacent to the kinase domain; constructs containing the kinase domain and the auto-inhibitory domain are approximately 10x less active compared to identical constructs without the auto-inhibitory domain (Pang *et al.* 2007; Chen *et al.* 2009). Within the kinase domain, ATP can bind to the catalytic site, while the substrate protein that will be phosphorylated binds to the nearby target protein binding groove. The activation loop contains a conserved threonine residue that can be phosphorylated by upstream kinases to considerably increase the activity of AMPK. This threonine is typically called Thr172 based on the rat sequence used to identify the site (Hawley *et al*. 1996). Phosphorylation of Thr172 results in a conformational change that allows for the transfer of the terminal phosphate from ATP to the target substrate (Scott *et al.* 2002; Calabrese *et al.* 2014).

The functional importance of the β subunit structure is less well understood. It anchors the α and γ subunits together via its C-terminal domain (Li *et al.* 2015; Xiao *et al*. 2013). The other domain of interest on the β subunit is a carbohydrate binding module, which allows AMPK to bind to glycogen (Hudson *et al.* 2003). Although the significance of this domain remains to be determined, it has been suggested that it may allow AMPK to co-localize with downstream targets like glycogen synthase (Carling and Hardie 1989; Hardie *et al.* 2012),

or that it may allow AMPK to sense glycogen stores as a proxy for cellular energy reserves (McBride *et al.* 2009).

AMPK Upstream Kinases

AMPK is phosphorylated at Thr172 by upstream kinases, including liver kinase B1, LKB1, (Woods *et al.* 2003; Hawley *et al.* 2003; Shaw *et al.* 2004) and Ca^{2+}/calmodulin-activated protein kinase kinase 2, CaMKK2 (Woods *et al.* 2005; Hawley *et al.* 2005; Hurley *et al.* 2005). LKB1 is constitutively active, and its phosphorylation of AMPK is regulated by the binding of AMP to the AMPK γ subunit, as described above (Davies *et al.* 1995). In addition, ADP can also promote LKB1 phosphorylation, and may be the more common activating molecule under moderate energy deficits since its concentrations within the cell are higher than that of AMP (Xiao *et al.* 2011; Oakhill *et al.* 2011). However, only AMP has the potential to act as an allosteric activator for AMPK, and thus may provide additional activation in conditions where there is a more severe lack of energy (Oakhill *et al.* 2011). AMPK phosphorylation by either AMP or ADP requires myristoylation of the β subunit (Oakhill *et al.* 2010).

Interestingly, LKB1 can also phosphorylate AMPK in response to glucose starvation, independent of AMP or ADP concentrations (Zhang *et al.* 2017; Hawley *et al.* 2010). Activation of AMPK via glucose starvation occurs through the enzyme aldolase, which is responsible for splitting fructose-1,6-bisphosphate (FBP) in glycolysis. When the cellular glucose concentration (and therefore flux through glycolysis) is low, aldolase in unbound by its substrate FBP. This unbound aldolase allows for the formation of a super-complex containing a cytoplasmic adaptor protein called Axin and LKB1 to come together with v-ATPase and Ragulator on the lysosomal membrane. AMPK localization to the lysosomal membrane is accomplished via myristoylation of the β subunit as described above (Zhang *et*

al. 2017; Hardie 2018; Oakhill *et al.* 2010). With the LKB1 complex and AMPK brought together by the Ragulator complex, AMPK can be phosphorylated. It is likely that the activated APK subsequently detaches from the membrane, although this has yet to be shown.

In contrast to LKB1, CAMKK2 activates AMPK independently of any AMP or ADP concentrations. Rather, CAMKK2 responds to increased Ca^{2+} levels to phosphorylate AMPK at Thr172 (Fogarty *et al.* 2010). CAMKK2 is activated by the Ca^{2+}-bound form of the regulatory protein calmodulin (Racioppi and Means 2012). As calcium acts in the signal transduction pathway after G protein-coupled receptor activation and ion channel activation, CAMKK2-mediated phosphorylation of AMPK may be especially important as a response to hormones and other signaling molecules like ghrelin and vascular endothelial cell growth factor (VEGF) that act through such systems (Hardie 2018).

AMPK Inhibits TOR Signaling

One of AMPK's major downstream targets is the target-of-rapamycin (TOR) pathway. In mammals, TOR is found in two complexes - mTORC1 and mTORC2. Those protein kinases contain multiple subunits; the major components are mTOR and Raptor and mTOR and Rictor, for mTORC1 and mTORC2, respectively (Laplante and Sabatini 2009). The *C. elegans* ortholog of TOR (ceTOR) is encoded by the *let-363* gene, and the *C. elegans* homologs of Rictor and Raptor are encoded by *rict-1* and *daf-15*, respectively (Jia *et al.* 2004; Soukas *et al.* 2009). In both mammals and *C. elegans*, TOR signaling functionally opposes AMPK signaling by promoting anabolic pathways such as protein synthesis (Laplante and Sabatini 2009; Saxton and Sabatini 2017; Lapierre and Hansen 2012).

In mammals, AMPK inhibits TOR signaling in two ways. Firstly, it phosphorylates the tuberous sclerosis protein (TSC), which has the ultimate effect of inactivating TOR through several other intermediary players (Inoki *et al.* 2003). Secondly, AMPK phosphorylates Raptor itself at two serine residues, Ser722 and Ser792, inhibiting mTOR signaling (Gwinn *et al.* 2008). *C elegans* have no identified TSC proteins, and only the Ser792 residue is conserved in non-mammals (Hindupur *et al*. 2015). The *C. elegans* TOR pathway connects with other nutrient sensing pathways including IIS, but the nodes of this cross-signaling are less well defined than in mammals and needs further study (Long *et al.* 2004).

Effectors of AMPK Signaling

Many of the first identified AMPK substrates were metabolic enzymes. Since AMPK is a master regulator of metabolism and is activated under nutrient-scarce conditions, many of its actions center around the upregulation of catabolic and the downregulation of anabolic processes to restore energy homeostasis. Catabolically, AMPK promotes glucose production in a variety of ways. AMPK acts on glucose transporters to boost cellular glucose uptake (Barnes *et al.* 2002; Jørgensen *et al.* 2004) and promotes uptake and oxidation of fatty acids (Habets *et al.* 2009; O'Neill *et al.* 2013). Other potential major catabolic targets of AMPK are the autophagy initiating kinases Atg-1/ULK proteins, present in *C. elegans* as a single homolog and in mammals as two (Meléndez *et al.* 2003; Hara *et al.* 2008). It's clear in mammals that AMPK phosphorylates ULK1 and ULK2 to promote autophagy (Egan *et al.* 2011; Kim *et al.* 2011). It is less clear that this happens in C*. elegans*, although AMPK and *unc-51*, the *C. elegans* homolog of ULK1, are required for the induction of autophagy in worms with defects in the IIS pathway (Egan *et al.* 2011). Alternatively, AMPK may act to upregulate autophagy in worms via its inhibitory actions on TOR, which itself represses autophagy.

AMPK also represses a number of anabolic pathways. It inhibits the synthesis of a variety of lipids (Carlson and Kim 1973; Fullerton *et al.* 2013; O'Neill *et al.* 2014; Muoio *et al*. 1999; Li *et al.* 2011). Protein synthesis constitutes another major source of energy expenditure for the cell. In addition to inhibiting protein synthesis by repressing TOR activity, AMPK also phosphorylates elongation factor-2 kinase (EF2K), which phosphorylates EF2 to inhibit protein synthesis at the elongation phase (Johanns *et al.* 2017; Browne *et al*. 2004; Horman *et al.* 2003). Finally, AMPK inhibits rRNA synthesis by phosphorylation-induced inactivation of RNA polymerase I-associated transcription factor TIF-IA (Hoppe *et al.* 2009).

AMPK is finely tuned to respond to energy fluctuations within a cell. As both hypoxia and dietary restriction reduce available energy levels, AMPK is an intriguing candidate to play a role in hypoxia-induced protein aggregation and/or fasting protection against hypoxia-induced proteostasis defects. I investigate the role of AMPK in these phenomena in Chapter 4, where I find that AMPK both promotes aggregation in hypoxia and is also required for fasting protection.

1.5 Dietary Restriction and Hypoxic Injury

Dietary restriction protects against I/R in multiple tissues

In Section 1.2, I outlined the defects in proteostasis that can result from hypoxia. Although there is not much research investing dietary restriction (DR) as it relates to hypoxia-induced proteostasis impairments, there are many studies that show that various forms of DR can protect against other kinds of damage associated with hypoxia. For example, mice on an alternate-day feeding regimen have higher survival rates after myocardial ischemia induced via coronary occlusion (Katare *et al.* 2009), and this style of intermittent fasting also

protects rats against cerebral ischemia (Jeong *et al.* 2016). Similar results have been obtained with ischemic damage to the liver (Mauro *et al.* 2016).

Dietary restriction can refer to a number of interventions. Caloric restriction (CR) can vary in intensity but involves some degree of reduction in dietary intake. Fasting most often describes a complete lack of food, although it is sometimes used to describe a very high degree of restriction. In this work, fasting will be used to describe diets where the animals consume only water. Fasting can be prescribed intermittently (intermittent fasting), such as is exemplified with an alternate-day feeding diet. Finally, specific macronutrients can be restricted, with the most well-studied effects having been observed with protein and amino acid restriction (Lee and Longo, 2016). Protein restricted diets sometimes involve concomitant CR, but don't necessarily involve a reduction in food intake. For example, a low protein diet in mice extends lifespan despite increased food intake and higher body fat levels (Solon-Biet *et al.* 2014; Solon-Biet *et al.* 2015). The molecular mechanisms involved in mediating DR and its effects, as well as the degree to which these mechanisms differ between different types of DR, are still being investigated (Lee and Longo, 2016). Although most well-characterized as a method to extend lifespan, accumulating evidence suggests that DR can protect against hypoxic injury.

Mice on a reduced calorie diet have reduced infarct damage compared to *ad-libitum* fed controls (Menezes-Filho *et al.* 2017). CR can also improve outcomes after cerebral ischemic injury by protecting cortical and striatal neurons (Duan *et al.* 2001) and reducing neurological deficits and infarct volume (Ran *et al.* 2015). These observations suggest that understanding the mechanistic basis underlying the protective effects of fasting in hypoxia could provide novel insight into therapeutic strategies to treat pathological conditions associated with I/R injury.

In work done on CR in the context of lifespan extension, some evidence suggests that protein restriction in particular, without an overall reduction in caloric intake, can lead to increased longevity (Speakman *et al.* 2016). Protein restriction may also have the potential to protect against I/R. In mice, CR and protein restriction interventions synergize additively to provide protection against renal ischemia (Robertson *et al.* 2015). Restriction of single amino acids may be able to confer the same benefits as total protein restriction: mice consuming a tryptophan-deficient diet are protected against renal and hepatic I/R injury (Peng *et al.* 2012), and similar results are obtained in 3-6 days via daily injections of the tRNA synthetase inhibitor halofuginone (Peng *et al.* 2012). One week on a protein-free diet reduces kidney damage after renal I/R and liver necrosis after hepatic IR (Mauro *et al.* 2016) and increases survival and reduces damage after renal I/R (Peng *et al*. 2012; Harputlugil *et al.* 2014). It is noteworthy that long-term complete restriction of protein will result in malnutrition and eventual death. Thus, all the work described above used short-term DR protocols.

If shorter DR periods are effective, they may prove more clinically relevant and feasible than long-term DR to confer protection against I/R. Indeed, there are a number of studies showing short stints of CR can be protective. A couple weeks of 30% CR and as little as one day of water-only fasting improved survival and kidney and renal function in mice after I/R (Mitchell *et al.* 2010). Isolated *ex-vivo* rat hearts can be protected from I/R by 11 days with a 70% reduction in food intake (Yamagishi *et al.* 2010) and as little as 16 hours of fasting (Schneider and Taegtmeyer 1991). Mice that have been fasted for 3 days display reduced hepatocellular apoptosis and damage (Qin *et al.* 2016) and also show reduced infarct volume after focal stroke (Varendi *et al*. 2014). Finally, in rats, 48 hours of fasting prior to brain ischemia reduces neuronal necrosis and edema (Marie *et al.* 1990).

However, not all studies have shown reduced cellular damage after I/R as a consequence of DR. Alternatively, some researchers have found comparable levels of neuronal loss between CR and *ad-libitum* fed animals but have rather seen that the CR animals show fewer learning and memory deficits (Roberge *et al*. 2008a; Roberge *et al.* 2008b). These studies suggest that CR may somehow enhance the functionality of the extant neurons following I/R, since the number of remaining neurons isn't different between the two groups.

Alterations in Mitochondrial Structure and Function as Potential Mediators of DR-Induced Ischemia Protection

Multiple mechanisms have been proposed to explain ischemic protection by reduced food intake, with mitochondrial alterations being one of the more well-studied areas of interest. I/R can cause permeabilization of the inner mitochondrial membrane via opening of the mitochondrial permeability transition (MPT) pore. Consequently, the inner membrane of the mitochondria becomes permeable to solutes up to 1.5 kilodaltons in size, leading to depolarization and ATP depletion. Increased concentrations of reactive oxygen species and Ca^{2+} both serve as triggers to open the MPT pore (Carraro and Bernardi 2016). CR increases the capacity for mitochondria to take up Ca^{2+} without opening the MPT pore, which protects against mitochondrial permeability and is correlated with reduced infarct volume in mouse liver (Menezes-Filho *et al.* 2017). The researchers suggest that increased ATP concentrations measured within the CR mitochondria may be the basis for increased Ca^{2+} uptake capability, as its additional negative charge (compared to ADP) gives it a much higher affinity for Ca^{2+} (Menezes-Filho *et al.* 2017). This resultant mitochondrial Ca^{2+} buffering capacity may be one mechanism by which caloric restriction protects against I/R.

The mitochondrial electron transport chain may also play a role. Although glycolysis is the main source of cellular energy in hypoxia (Rohrbach *et al.* 2014), some evidence suggests

that the electron transport chain can be 'primed' for ischemia, preserving its functionality post-I/R. Mice that were protected from cardiac I/R by CR had higher mitochondrial respiration levels and reduced hydrogen peroxide output. These changes may stem from altered Sirtuin3-mediated deacetylation of specific electron transport chain proteins, including NDUFS1 and the Rieske subunit of the cytochrome bc1 complex (Shinmura *et al.* 2011). CR preserves heart function in *ex vivo* rat hearts subjected to global ischemia, and the hearts from the rats on the CR diet showed improved mitochondrial respiration as measured by enhanced pyruvate metabolism, improved mitochondrial coupling, and more efficient energy production (Broderick *et al.* 2002).

In addition to electron chain quality control, mitochondrial fission and fusion dynamics may be important in mediating the protective role of CR in hypoxia. miRNAs are known to be capable of myriad cellular functions through regulation of gene expression, but their role in regulating the mitochondrial fission and fusion machinery in response to DR has only recently begun to be uncovered. Mitochondrial fission 1 protein (Fis1) regulates mitochondrial fission and its upregulation can also regulate apoptosis (Lee *et al.* 2004). Wang *et al.* found that miR-484 can repress Fis1 to inhibit mitochondrial fission, prevent apoptosis, and reduce I/R-induced myocardial infarction volume (Wang *et al.* 2012). Furthermore, FOXO3a can bind to the promoter region of miR-484 to activate it, but this interaction is disrupted in anoxic conditions. In anoxia, FOXO3a expression levels are reduced, resulting in increased mitochondrial fission and apoptosis through Fis1 (Wang *et al.* 2012). As a FOXO transcription factor, FOXO3a would presumably be activated in response to nutrient limiting conditions like CR or fasting, and it is required for CR-induced longevity in mice (Shimokawa *et al.* 2015). Thus, one mechanism by which DR may mediate ischemic resistance is suppression of Fis1 via FOXO3a-induced miR-484 activity. Another miRNA, miR-499, inhibits anoxia-induced cardiomyocyte apoptosis and infarct volume (Wang *et al.* 2011). miR-499 also works by downregulation of mitochondrial fission.

Signaling Through Sirtuins and Adiponectin Pathways may be Necessary for CR Protection Against I/R

Although mitochondrial alterations in structure or function provide many promising leads, the body of research identifying genes that are necessary or sufficient for CR protection against ischemia is much more limited. NAD^+-dependent deacetylases called sirtuins have also been tied to ischemic tolerance, and some studies has found that sirtuin 1 (Sirt1) is required for CR to protect against I/R. For example, although CR reduces infarct size and improves behavioral outcomes after I/R in rats, siRNA knockdown of Sirt1 abolishes the protect effect (Ran *et al.* 2015). Additionally, while *ex vivo* hearts from CR mice are protected against I/R, hearts from Sirt1 knockout mice are not (Yamamoto *et al.* 2016). Rats on a CR diet show an increase in nuclear SIRT1. Its nuclear localization is mediated by nitric oxide and appears to be essential for CR protection (Shinmura *et al.* 2008). Sirt1 is also increased in mouse kidneys as a result of a CR diet that protect the mice against renal I/R, and the kidneys of Sirt1 knockout mice are insensitive to CR protection against hypoxia (Kume *et al.* 2010). Accordingly, overexpression of SIRT1 in myocytes reduces I/R injury (Hsu *et al.* 2010).

Like sirtuins, adiponectin has also been identified in some studies as necessary for DR protection against hypoxia. Adiponectin is a protein hormone involved in regulating glucose and fatty acid metabolism (Chandran *et al.* 2003). Its levels increase as a response to CR (Shinmura *et al.* 2007; Zhu *et al.* 2004; Niemann *et al.* 2008; Wan *et al.* 2010; Ding *et al.,* 2012), it is required for CR to protect against cardiac I/R (Shinmura *et al.* 2007), and can protect against I/R if applied exogenously (Zhang *et al.* 2013). Adiponectin activates AMP-activated protein kinase (AMPK) (Yamauchi *et al.* 2002; Kubota *et al.* 2007; Zhou *et al.* 2009), and AMPK is also required for CR protection against cardiac I/R (Shibata *et al.* 2005; Shinmura *et al.* 2007). In addition to AMPK, nitic oxide may also mediate the effects of

adiponectin in CR. CR and adiponectin induce endothelial nitric oxide (Dolinsky *et al.* 2010; Kondo et al. 2009), and inhibition of nitric oxide ameliorates the protective effect of adiponectin on myocardial infarct volume after I/R (Gonon *et al.* 2008; Shinmura *et al.* 2008).

AMPK and IIS in Ischemia

Although AMPK is canonically thought of as being responsive to AMP/ATP ratios, it can also be activated in response to stress conditions that generate reactive oxygen species (ROS) and reactive nitrogen species (RNS). Depending on the severity of oxygen limitation, hypoxia can lead to a decreased AMP/ATP ratio due to attenuated fatty acid oxidation (Hardie and Hawley *et al.* 2011). Some evidence suggests that the type II diabetes drug and AMPK activator metformin activates AMPK through RNS (Zou *et al.* 2004). Furthermore, ROS generated by hypoxia induce AMPK activation via the opening of calcium release-activated calcium channels without any change in AMP/ATP ratio (Mungai *et al.* 2011). The increase in Ca^{2+} levels result in the phosphorylation of AMPK by CAMKK2 (Mungai *et al.* 2011). Additionally, the Cys130 and Cys174 residues of AMPK's α subunit can be oxidized. Oxidation of these cysteine residues interferes with phosphorylation of AMPK by upstream kinases (Shao *et al.* 2014) and may be one mechanism whereby AMPK is rapidly deactivated upon reperfusion after an ischemic episode.

It is clear that AMPK can be activated in hypoxia, but the role of AMPK signaling in hypoxia and I/R injury is contentious. Some studies investigating the effects of AMPK in the context of hypoxia/ischemia have found a protective role for AMPK (Viollet *et al.* 2011). In the ischemic heart, AMPK has been shown to regulate a number of adaptive metabolic changes, such as increasing production of ATP via glycolysis, mobilizing glycogen stores, and increasing glucose utilization (Qi and Young 2015). Furthermore, AMPK induces autophagy

and suppresses eEF2-regulated protein synthesis, thus generating substrates for cellular metabolism, removing damaged organelles, and reducing ER stress (Qi and Young 2015; Matsui *et al.* 2007; Takagi *et al.* 2007; Terai *et al.* 2005). Finally, mice without AMPK show greater infarct volumes, more cell death, and poorer cardiac function after myocardial I/R (Wang *et al.* 2009; Wang *et al.* 2011; Russell *et al.* 2004; Takagi *et al.* 2007). However, other studies have seen harmful effects from AMPK activation in ischemia. For example, AMPK inhibition is protective in ischemic stroke in mice. Mice deficient in AMPKα2 show reduced infarct volumes (McCullough *et al.* 2005; Li *et al.* 2007). The net effect of AMPK activation in response to ischemia remains open to debate, as a number of studies have found contradictory effects (Li and McCullough 2010; Takagi *et al.* 2007; Viollet *et al.* 2011). Whether AMPK acts to protect cells against ischemia or results in exacerbation of damage may depend on the severity of the ischemic insult, whether cell cultures or whole animals are being utilized, and the tissue being exposed to hypoxia (Li and McCullough 2010).

Although there have been no studies showing that reduced IIS is required for DR protection against hypoxia, it is generally accepted that reduced food intake results in lower insulin/IGF-1 levels (Robertson and Mitchell 2013). Paradoxically, some research has shown that insulin and IIS are beneficial in models of cerebral and cardiac ischemia. Insulin resistance is associated with increased stroke risk and worse outcomes after stroke (Calleja *et al.* 2011; Arenillas et al. 2007). Rats given a dose of insulin prior to cerebral ischemia had reduced infarct volumes (Hamilton et al. 1995). Similarly, rats given insulin prior to cardiac ischemia had improved cardiac function and reduced cell death (Ji *et al.* 2010). Finally, rats with a myocardial specific knockout of insulin receptors have more cardiac dysfunction after myocardial ischemia (Fu *et al.* 2005). The mechanisms by which insulin confers ischemic resistance have not yet been fully elucidated, but may involve reduced formation of peroxynitrite-induced oxidative and nitrative stress (Ji *et al.* 2010) or reduction

in glucose levels (Smit *et al.* 2006; Hamilton *et al.* 1995) As DR also reduces blood glucose (Trepanowki et al. 2011), this mechanism could be one way to reconcile the protective effects of both IIS and DR/fasting protection against ischemia.

It is likely that the protective effect conferred by these DR protocols are not due to a single pathway or gene. Rather, there is probably a range of mechanisms at work in different DR situations, and these may include reduced IIS, upregulated survival signaling, and alterations in cellular metabolism such that fuels are utilized either from different sources or are utilized more effectively (Robertson and Mitchell 2013).

1.6 Altered Metabolism, Dietary Restriction, and Neurodegeneration

Neurodegeneration

The previous section outlined the protective effect of DR against ischemic injury. However, the effects of DR are not limited to hypoxia. A growing body of work investigates the protective effects DR can have against neurodegeneration and its associated proteostasis defects. Neurodegeneration is characterized by neuronal damage and/or death, structural changes to the brain, and impaired cognition (Mattson *et al.* 1999; Camandola *et al.* 2017). In addition to these features, many neurodegenerative disorders including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS) are characterized by misfolded and aggregated proteins (Hadem *et al.* 2017). The rest of this work will focus on the proteostasis defects incurred in neurodegenerative disorders in the context of organismal metabolism and the ways in which insulin signaling, AMPK signaling, and dietary restriction can modulate protein aggregation.

Dysregulated IIS in Neurodegenerative Disorders

Although brain metabolism declines during the course of normal/non-pathological aging (Hoyer 1982; Hoyer 1990), there has been an increasing focus on specific metabolic abnormalities concomitant with neurodegenerative disorders, with dysregulated IIS and AD having received the most attention (Folch *et al.* 2018; Morgen and Frölich 2015). It remains to be determined whether these metabolic defects play a causative role in the etiology of these disorders, synergize with other mechanisms that cause neurodegeneration to exacerbate pathology, or are a secondary result from upstream independent causes, including facets of AD itself such as Aβ deposition. These possibilities aren't mutually exclusive, and untangling these relationships is complicated by the fact that many of the pathways linking metabolic dysregulation and neurodegeneration are bidirectional. Furthermore, cause-and-effect relationships likely differ for separate subgroups (Morgen and Frölich 2015; de la Monte and Wands 2005; Craft *et al.* 2013; Chami *et al.* 2016).

The hypothesis that insulin dysregulation and/or resistance plays a role in the development of AD was originally proposed by Sigfried Hoyer after finding that cerebral glucose metabolism was impaired in patients with AD (Hoyer 1991; Morgen and Frölich 2015). The idea was furthered by the Rotterdam study, which detailed a doubled risk for AD among people with type II diabetes mellitus (Folch *et al.* 2018; Schrijvers *et al.* 2010; Ott *et al.* 1999). Post-mortem analysis of brains from mice with AD also showed decreased IIS signaling (Liu *et al.* 2011), and human post-mortem AD brains have been shown to be insulin and IGF-1 resistant (Talbot *et al.* 2012). Dysregulation of insulin signaling in the brain may result in important cognitive phenotypes that mirror those seen in AD. For example, downregulating IR expression in the rat hippocampus causes long-term potentiation deficits and impairments in spatial learning (Grillo *et al.* 2015). However, the

rest of this work will focus on IIS as it relates to proteostasis, with a focus on proteins known to be involved in neurodegenerative disorders.

The role of IIS in regulating proteostasis and protecting against neurodegeneration is complex; there is no consensus on whether increased or reduced IIS is beneficial (Cohen and Dillin 2008). On the one hand, reduced IIS is generally thought to improve proteostasis in invertebrates (Taylor *et al.* 2014; Cohen and Dillin 2008). For example, in *C. elegans*, mutation of the IR *daf-2* ameliorates Aβ toxicity by enhancing clearance of small toxic aggregates. Interestingly, this mutation enhances the formation of high molecular weight aggregates, suggesting large aggregates are less toxic in this model (Cohen *et al.* 2006). Polyglutamine aggregation can by reduced in worms with enhanced longevity due to mutations in the IIS component *age-1*, the *C. elegans* homologue of PI3K (Morley *et al.* 2002). Neuron specific deletion of the IGF-1R in a mouse model of AD results in reduced Aβ plaque burden and prevents premature mortality. This effect may be independent from FOXO signaling, as no effect is seen from overexpressing or deleting FOXO1, the predominant FOXO form in the hippocampus (Stöhr *et al*. 2013).

However, there are also studies finding detrimental effects on proteostasis from deactivating IIS and/or protective effects from activation of the IIS pathway. IGF-1 reduces apoptotic toxicity in human cells containing familial-Alzheimer's disease mutations in an IGF-1R dependent manner (Niikura *et al.* 2001). Hyperactivation of IIS in adipocytes increases protein stability and folding (Minard *et al.* 2016), and activation of IRS-2 causes clearance of accumulated polyglutamine proteins via autophagy in a mouse model of HD (Yamamoto *et al*. 2006). In *C elegans*, stress response proteins like DAF-21, HSP-1, HSP70, and CDC-48.2 are turned over more slowly in *daf-2* mutants, and DAF-21 and HSP-1 protein levels are downregulated (Depuydt *et al.* 2016; Dhondt *et al.* 2016).

Much of the work on AD and IIS has focused on defining the interactions between IIS and Aβ and tau proteins, which are found in aggregated in plaques and tangles, respectively, in AD brains (Chami *et al.* 2016). Aβ has been found to impair IRS-1 signaling, by inducing aberrant phosphorylation and reducing total IRS-1 levels through activation of the c-Jun N-terminal kinase pathway in mice and monkeys (Ma *et al.* 2009; Bonfim *et al.* 2012) A growing body of work has focused on how Aβ may impair IIS at the receptor level. Aβ and insulin are both amyloidogenic peptides, and soluble Aβ oligomers can bind to IRs causing a decreased IR affinity for insulin, decreased insulin binding, and reduced receptor transduction (Xie *et al.* 2002; Zhao *et al.* 2008; Townsend *et al.* 2007). Binding of Aβ may even cause IRs to be lost from the neuronal cell surface and be instead localized to the interior of the cell (Zhao *et al.* 2008; De Felice *et al.* 2009). Interestingly, this inhibitory relationship appears to be reciprocal, as insulin can also block Aβ from binding to IRs. This effect does not seem to be due to simple competition for binding sites, as IR receptor tyrosine kinase activity is required (De Felice *et al.* 2009). Furthermore, insulin is able to bind to synthetic Aβ peptides and prevent their self-assembly into oligomers and fibrils *in vitro* (Lee *et al.* 2009). It has also been shown in some studies to promote the release of Aβ from the intracellular environment to the extracellular milieu (Gasparini *et al.* 2001; Pandini *et al.* 2013). However, other researchers have found that activation of IRs reduces extracellular levels of soluble Aβ oligomers to prevent IR loss induced by Aβ binding (Zhao *et al.* 2009).

In addition to direct effects on Aβ, inulin appears to also affect the processing of the amyloid precursor protein (APP). As diagrammed in Fig. 1.5, cleavage of APP can follow a non-amyloidogenic pathway in which α-secretase cleaves APP within the Aβ region to produce two fragments that are likely degraded. Alternatively, APP can be processed in a stepwise fashion by β-secretase and γ-secretase in the amyloidogenic pathway to produce Aβ as well as the APP intracellular domain (AICD) that translocates to the nucleus to regulate

transcription of genes involved in Aβ production and aggregation (Hicks *et al.* 2012). Insulin signaling through IRs prevents the translocation of AICD into the nucleus, decreases the transcription of APP, and increases the transcription of anti-amyloidogenic genes including α-secretase and insulin-degrading enzyme (IDE), which is involved in Aβ degradation (Pandini *et al.* 2013).

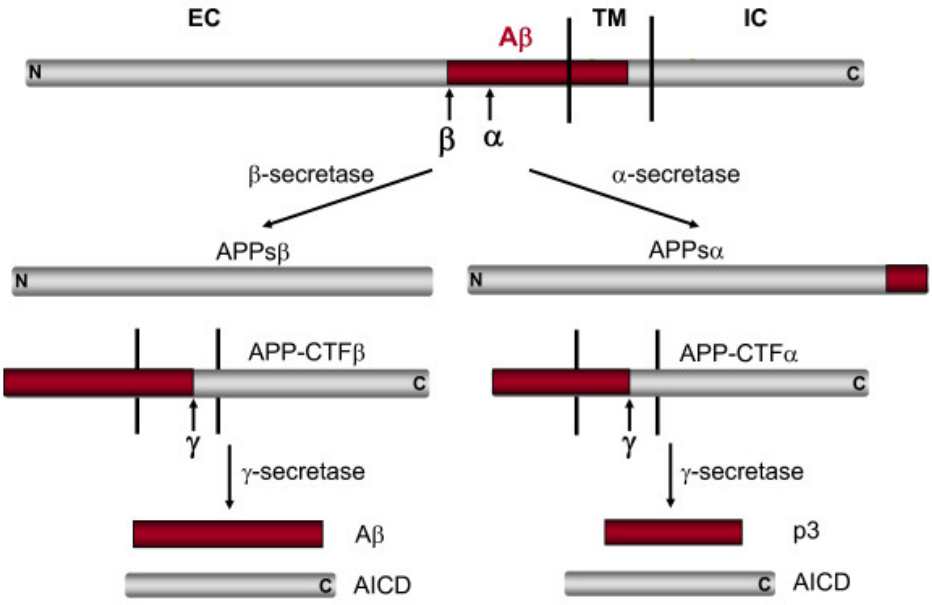


FIGURE 1.5 SCHEMATIC DIAGRAM OF APP PROCESSING PATHWAYS. Aβ domain is highlighted in red. For simplicity, only one cleavage site is shown for each enzyme. EC: extracellular; TM: transmembrane; IC: intracellular. Figure reprinted from Zheng and Koo 2011. Copyright permission granted by a Creative Commons attribution license.

Tau functions as a microtubule stabilizer in normal brains but becomes hyperphosphorylated and aggregates to form neurofibrillary tangles in AD (Iqbal *et al.* 2010). The links between IIS, tau, and AD have yet to be fully elucidated, but the bulk of the research focuses on the enzyme glycogen synthase kinase 3β (GSK3β), which is a tau kinase (Hanger *et al.* 1992; Mandelkow *et al.* 1992; Ishiguro *et al.* 1993) that reduces tau's ability to stabilize microtubules (Utton *et al.* 1997). Neuron-specific IR knockout mice have substantially increased amounts of tau hyperphosphoylation at sites associated with AD due to increased

GSK3β activation (Schubert *et al*. 2004). Interestingly, peripheral insulin stimulation also shows this effect. Mice with peripheral hyperinsulinemia show dose-dependent central nervous system tau phosphorylation, which requires signaling through IRs (Freude *et al.* 2005). Alternatively, it may be that both abnormally high IIS and abnormally low IIS both result in hyperphosphorylated tau and AD symptoms (Stanley *et al.* 2016). Abnormal phosphorylation of IRS-1 at Ser616 was identified in post-mortem AD brains as well as in other tauopathies, and the phosphorylated IRS-1 colocalizes with tau tangles in many of these brains as well as in mice expressing pathological tau (Yarchoan *et al.* 2014). Recently, insulin was shown to oligomerize and accumulate in neurons harboring hyperphosphylated tau in AD and other tauopathies. The insulin accumulation requires tau hyperphosphorylation and often results in insulin resistance and downregulation of IR (Rodriguez-Rodriguez *et al.* 2017). This research is an example of why determining causal relationships between dysregulated IIS and AD is so complex.

Dietary Restriction and Neurodegeneration

Multiple studies have shown that DR is capable of conferring protection against a number of aggregation phenotypes associated with neurodegenerative disorders. CR mice are spared from the accumulation of ubiquitin-reactive proteins observed in aging *ad libitum* fed mice (Opalach *et al.* 2010), and also show decreased levels of Aβ and phosphorylated tau in a mouse model of AD (Halagappa *et al.* 2007; Patel *et al.* 2005). Other researchers have seen similar results, but in a sex-specific fashion. CR protected female, but not male, Tg2576 amyloid mice. The female mice had a reduced Aβ burden, potentially via reduced γ-secretase mediated processing of APP (Schafer *et al.* 2015). However, other studies noted no differences in protein abnormalities. CR in the Tg4510 model of tau deposition rescued some memory defects but did not alter total tau or phospho-tau levels (Brownlow *et al.* 2014). Amyloid fibrils can also be formed via the extracellular deposition of apolipoprotein

A-II, and CR protects against apolipoprotein A-II amyloid aggregates in mice (Li *et al.* 2017).

As was seen with the hypoxia models, some evidence suggests that protein restriction in particular can have beneficial effects on protein aggregation associated with neurodegeneration. Cycles of protein restriction reduce tau phosphorylation but not Aβ levels in mice with cognitive impairments due to expression of genes with human AD-related mutations (Parrella *et al.* 2013). A low protein diet (17% of total calories) is also protective in a mouse model of HD. Mice on a protein-restricted diet show a reduced formation of Huntingtin aggregates and also have ameliorated motor coordination deficits (Chiang *et al.* 2007).

However, CR does not protect against all neurodegenerative disorders. ALS in particular appears to respond poorly to reductions in energy intake. In mouse models of ALS, dietary restriction actually accelerated progression of the disease, and worms expressing human mutated TDP-43 were not rescued from neuronal proteotoxicity by caloric restriction (Patel *et al.* 2010; Tauffenberger *et al.* 2012).

Autophagy and Protein Aggregates Associated with Neurodegenerative Diseases

As one branch in the cell's repertoire of protein degradation machinery, autophagy may play a role in mediating the relationship between caloric restriction and aggregation phenotypes in neurodegeneration. Although basal levels of autophagy occur in nutrient-replete conditions, it is upregulated in response to some stressors. Nutrient-limiting stresses such as starvation, fasting, and caloric restriction are especially potent inducers of autophagy since the products of the autophagic pathway can serve to maintain cellular metabolism

(Ntsapi and Loos 2016). The accumulation of protein aggregates as a feature of neurodegenerative diseases suggests a potential role for autophagy failure in the etiology or progression of these diseases (Ntsapi and Loos 2016; Martinez-Vicente and Cuervo 2007). Age is the biggest risk factor for neurodegenerative diseases, and older mice have hypermethylated and consequent lowly-expressed autophagy-related genes and lower rates of chaperone-mediated autophagy compared to younger populations (Khalil *et al.* 2016; Cuervo and Dice 2000). Autophagy-related genes are also transcriptionally downregulated during the course of aging in humans. Interestingly, it is up-regulated in AD brains, suggesting a compensatory regulation (Lipinski *et al.* 2010).

Although autophagy systems have the potential to regulate aggregating proteins in neurodegeneration, the converse may also be true. Pathogenic α-synuclein variants inhibit their own degradation by blocking uptake into the lysosome by binding to the LAMP-2A receptor (Cuervo *et al.* 2004). Similarly, mutant tau also inhibits chaperone mediated autophagy by blocking the LAMP-2A receptor and preventing full translocation into the lysosome (Wang *et al.* 2010).

Taken together, there seems to be complex and bi-directional relationships governing the interactions between DR, resistance to hypoxia, and protein aggregation as exemplified by the proteostasis defects incurred in a number of neurodegenerative disorders. As key metabolic sensors and regulators, IIS and AMPK are likely to be important players in mediating these relationships, but their exact roles remain undefined. In the next three chapters, I investigate the interactions between hypoxia, proteostasis, and nutrient deprivation. I also identify roles for IIS and AMPK in modulating proteostasis in response to food and oxygen availability.

CHAPTER 2. FASTING PROTECTS AGAINST HYPOXIA-INDUCED PROTEOSTASIS DEFECTS

2.1 SUMMARY

Low oxygen conditions (hypoxia) can impair essential physiological processes and cause cellular damage and death, such as is observed as a result of stroke and cardiovascular disease. We have found that specific concentrations of hypoxia cause a disruption of protein homeostasis in *C. elegans*. Here, we show that nutritional cues regulate the effect of hypoxia on proteostasis. Animals that are fasted prior to hypoxic exposure develop dramatically fewer protein aggregates compared to their fed counterparts. Our results suggest an important role for the nutritional environment experienced at the onset of hypoxia in mediating hypoxia's effect on proteostasis, as fasting protection can be both induced and reversed rapidly. Fasting is effective at protecting against hypoxia-induced proteostasis defects across a variety of developmental stages, tissues, and misfolded or aggregation prone models.

2.2 INTRODUCTION

In order to survive in changing conditions, organisms need to successfully integrate a number of environmental signals and respond appropriately in order to maintain homeostasis. Aerobic heterotrophs must meet their requirements for food and oxygen by taking in these resources from the environment. An inadequate response to low levels of oxygen (hypoxia) can lead to cellular damage or death, an unsurprising outcome given oxygen's central role in cellular metabolism. Like hypoxia, food deprivation presents an

obstacle to homeostasis by impinging on cellular metabolism and disturbing anabolic pathways. However, in many cases food restriction can have beneficial effects, such as extending lifespan and delaying the onset of neurodegenerative diseases and their associated pathologies (Contestabile 2009). In a mouse model of Alzheimer's disease, 12 weeks of caloric restriction reduces amyloid-β plaque burden (Patel *et al.* 2005), and mice expressing human mutant huntingtin maintained on an alternate-day-feeding diet have reduced brain atrophy and decreased huntingtin aggregate formation (Duan *et al.* 2003). Depriving *C. elegans* of their bacterial food source reduces damage associated with expressing polyglutamine proteins (Steinkraus *et al.* 2008).

The protective effect of fasting is not limited to symptoms of neurodegeneration – there are many studies that show fasting can protect against damage associated with hypoxia in mammals. For example, mice on an alternate-day feeding regimen have higher survival rates after myocardial ischemia induced via coronary occlusion (Katare *et al.* 2009). Similar results have been obtained with ischemic damage to the liver. Mice on a calorically restricted diet have reduced infarct damage compared to ad-libitum fed controls (Menezes-Filho *et al.* 2017), and mice that have been fasted for 3 days display reduced hepatocellular apoptosis and damage (Qin *et al.* 2016). Calorie restriction can also improve outcomes after cerebral ischemic injury by protecting cortical and striatal neurons (Duan *et al.* 2001) and reducing neurological deficits and infarct volume (Ran *et al*. 2015). These observations suggest that understanding the mechanistic basis underlying the protective effects of fasting in hypoxia could provide novel insight into therapeutic strategies to treat pathological conditions associated with ischemia and reperfusion injury.

We have previously shown that in *C. elegans* the cellular response to specific hypoxic conditions involves a disruption of proteostasis – the coordination of protein synthesis, folding, degradation, and quality control required to maintain a functional proteome

(Fawcett *et al.* 2015). Here we show that fasting prevents the hypoxia-induced disruption of proteostasis. Our data indicate that the nutritional context of an animal at the onset of hypoxia has the power to alter hypoxia's effect on proteostasis.

2.3 RESULTS

In order to investigate the effect of nutritional status on proteostasis in hypoxia, we first used transgenic *C. elegans* that express yellow fluorescent protein (YFP) fused to a polyglutamine tract in the body wall muscles (Morley *et al.* 2002). We refer to these animals as QX::YFP, where X refers to the number of glutamine residues fused to YFP, such that Q35::YFP animals express YFP with 35 glutamine residues. In these animals, the number of YFP foci, which correspond to large protein aggregates, can be used as an *in vivo* measure of cellular proteostasis (Satyal *et al.* 2000).

Exposing animals to 0.1% oxygen for 24 hours while fed resulted in an increase in the number of YFP foci (Fig. 2.1B-2.1D), consistent with a decrease in proteostasis as has been demonstrated previously (Fawcett *et al.* 2015). However, we found that the number of YFP foci that formed in hypoxia was dramatically reduced if the animals were removed from food for six hours before the hypoxic exposure and remained off of food for the duration of hypoxia (Fig. 2.1A). Hypoxia-induced protein aggregation (HIPA) was prevented by fasting in fourth-stage larvae (L4) Q35::YFP animals (Fig. 2.1C) as well as in first-stage larvae (L1) Q40::YFP (Fig. 2.1D). We conclude that fasting prevents HIPA and that this effect persists across development.

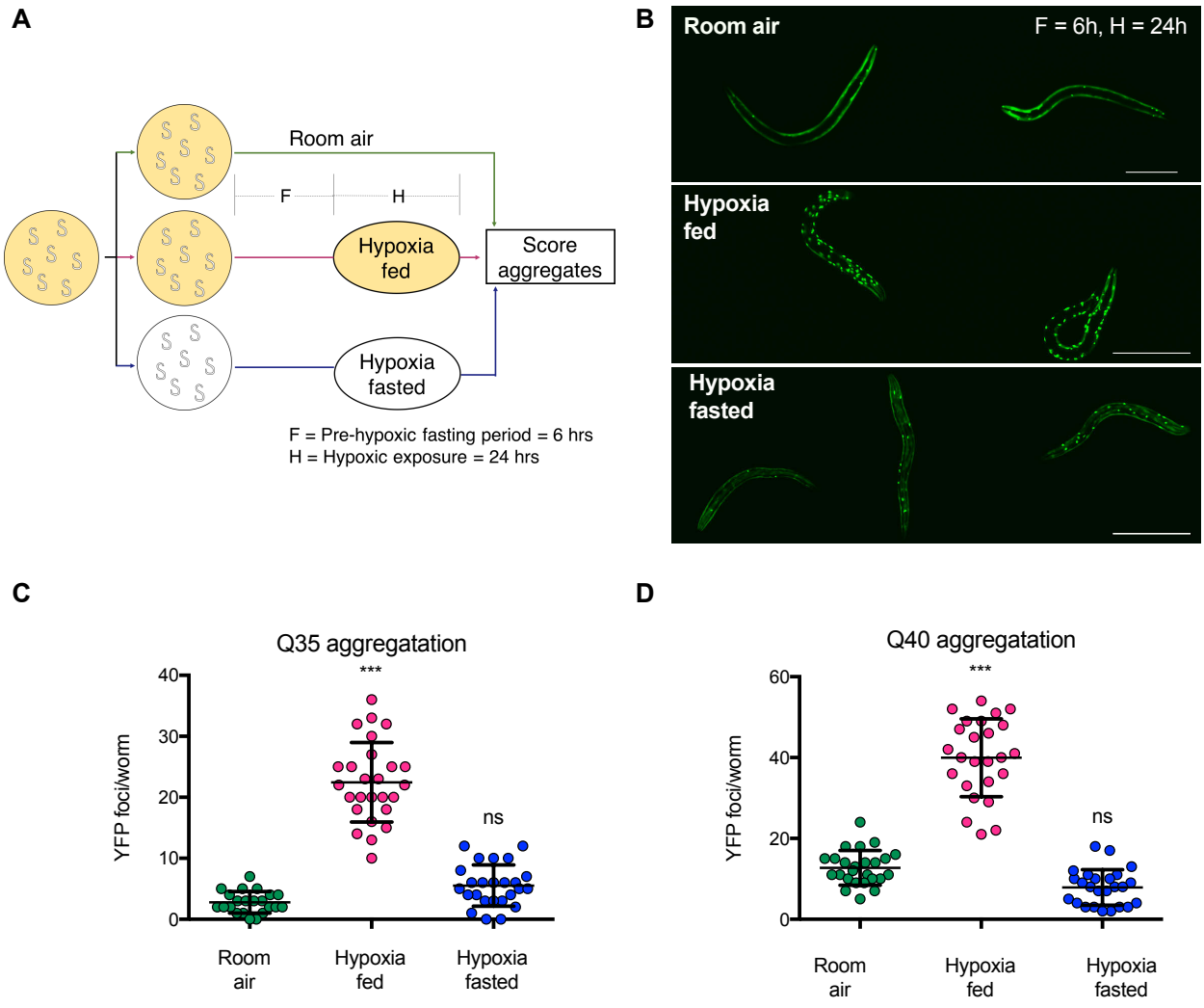


FIGURE 2.1 FASTING PROTECTS AGAINST HYPOXIA-INDUCED PROTEIN AGGREGATION. (A) Experimental schematic. Cohorts of age-synchronized animals were split into three groups: the first was maintained on food in room air, the second was maintained on food before and during exposure to hypoxia, and the third was 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.

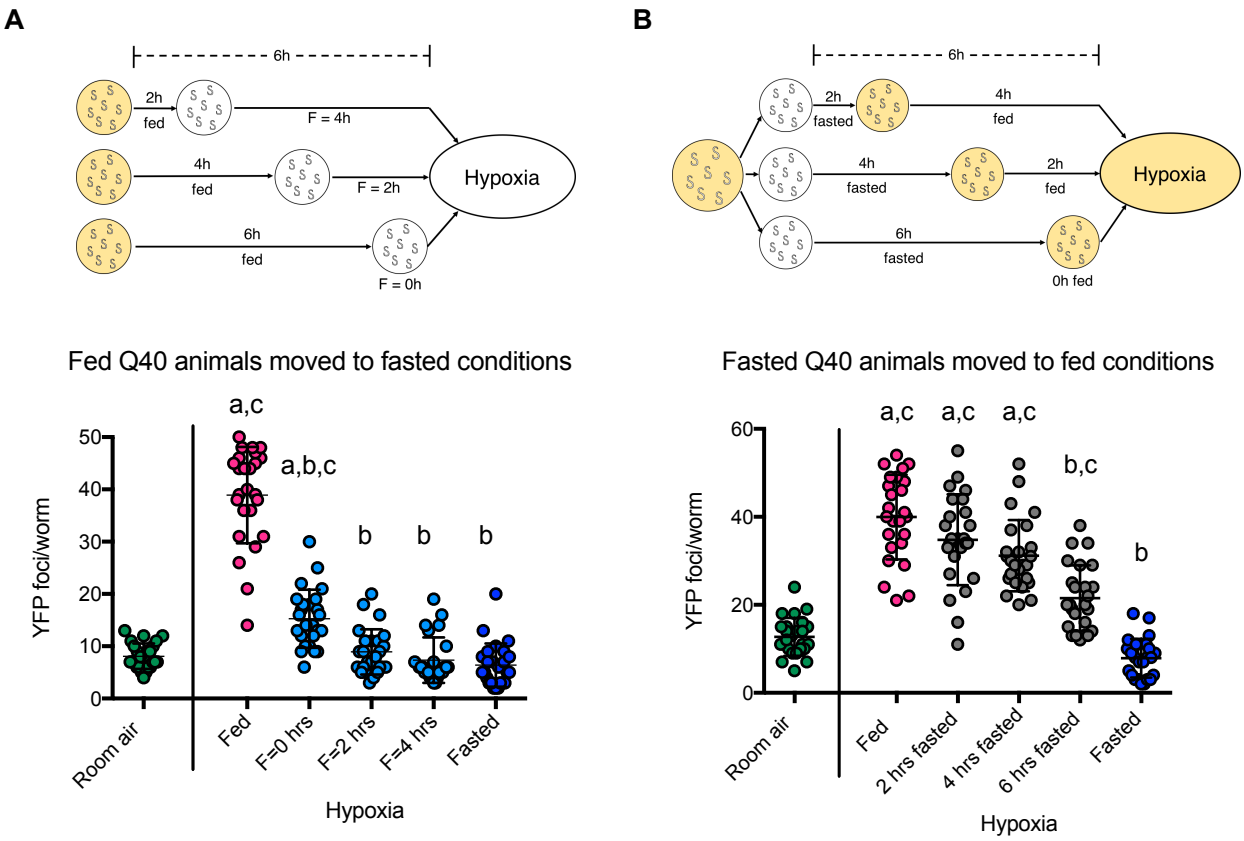


FIGURE 2.2 FASTING PROTECTION AGAINST HIPA IS QUICKLY INDUCED AND REVERSED. (A) Effect of fasting occurs rapidly in hypoxic conditions. Cohorts of L1 Q40::YFP animals were removed from food before exposure to hypoxia (F = 0, 2, or 4 h; H=24 h). All animals were off of food when exposed to hypoxia and 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, error bars are the standard deviation. Statistical comparisons were made between animals fasted for the indicated amount of time and controls maintained in room air, fed controls exposed to hypoxia after being continuously on food, and fasted controls that were not returned to food before hypoxia. 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.

We originally chose to fast animals for 6h before exposure to hypoxia to allow animals time to alter gene expression (Van Gilst *et al.* 2005), and this period of time off of food is sufficient to deplete stored glycogen as measured by iodine staining (DLM unpublished). However, there is no evidence to suggest that the protective effects of fasting in hypoxia requires changes in gene expression or glycogen stores. Therefore, we next measured how long of a fasting period was required to mitigate the effects of hypoxia on aggregation of polyglutamine proteins.

To determine the pre-hypoxia fasting duration required to protect against HIPA, we removed Q35::YFP animals from food for varying lengths of time before being exposed to hypoxia (as diagrammed in Fig. 2.2A). We found that animals removed from food immediately before exposure to hypoxia developed significantly fewer YFP foci in hypoxia as compared to controls that remained on food in hypoxia (Fig. 2.2A, 6h fed compared to fed). We conclude that extended fasting before exposure to hypoxia is not required to prevent HIPA. Instead, our data show that the protective effects of fasting occur very rapidly. In fact, the full protection against HIPA is realized with only 2h fasting before exposure to hypoxia (Fig. 2.2A). These results suggest that at least some of the protective effects of fasting are due to the absence of food directly, rather than metabolic changes or alterations in gene expression that occur during fasting prior to the hypoxic insult.

Work in other systems has shown that fasting can have a protective effect that persists even after animals are returned to food (Robertson and Mitchell 2013). To further explore the requirements for fasting to protect against HIPA we next asked whether the protective

effects of fasting against HIPA could be reversed. In these experiments (Fig. 2.2B), we began fasting animals 6h before exposure to hypoxia but then returned the animals to food prior to initiation of hypoxia. We observed that animals fasted for a full 6h and then returned to food immediately before exposure to hypoxia (Fig. 2.2B, 6h fasted) developed significantly more YFP foci than animals that were fasted for 6h and then exposed to hypoxia in the absence of food (Fig 2.2B, fasted), suggesting that the nutritional context of an animal as it experiences hypoxia is able to mediate the effect of hypoxia on proteostasis. Furthermore, we found no protection from HIPA if animals were fasted for 4h, but then fed for 2 h before exposure to hypoxia (Fig. 2.2B, 4h fasted), even though 4h of fasting was sufficient for complete protection against HIPA in the absence of food (Fig. 2.2A, 2h fed). This result indicates that the protective effects of fasting are fully reversed within 2h of return to food. We conclude that the protective effects of fasting in hypoxia are rapidly reversed.

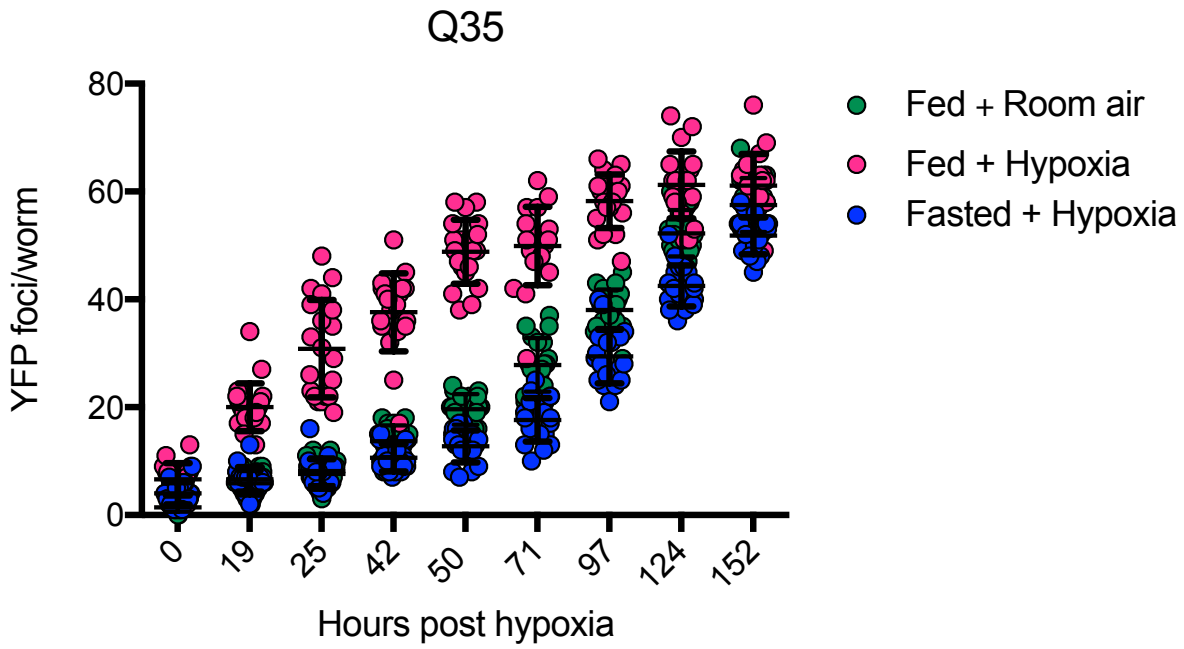


FIGURE 2.3 FASTING PROTECTS AGAINST LONG-TERM EFFECTS OF HYPOXIA ON PROTEOSTASIS. Cohorts of L4 Q35::YFP animals were exposed to hypoxia (H=10 h) on food (magenta) or fasted (blue, F=6h). Controls remained in room air on food (green). The

number of YFP foci was scored after return to room air as indicated. Data from one representative experiment is shown. The experiment was repeated at least 3 times. Each cohort included at least 20 animals per time point.

Shorter exposures to hypoxia that do not immediately increase the number of polyglutamine protein aggregates still disrupt long-term proteostasis, as evidenced by the increased rate of age-associated protein aggregation after return to room air (Fawcett *et al.* 2010). We therefore asked whether fasting could protect against these long-term proteostasis deficits in addition to aggregates accrued immediately after hypoxia. We exposed Q35::YFP L4 animals to hypoxia for only 10h either in the fed state or after fasting for 6h (F = 6 hours, H = 10 hours as per Fig. 2.1A). Control animals remained on food in room air. Immediately after this short hypoxic exposure, there was no observed increase in the number of YFP foci in animals exposed to hypoxia regardless of whether food was present (Fig. 2.3, 0 hours post-hypoxia). As expected, the animals exposed to hypoxia in the fed state accumulate aggregates faster than control animals. In contrast, animals exposed to hypoxia while fasted accumulate YFP foci at the same rate as control animals. These data indicate that fasting both prevents HIPA and protects against the long-term effects on proteostasis induced by a short exposure to hypoxia.

The cellular role of protein aggregates is controversial, with some reports finding a protective role and others suggesting a cytotoxic effect (Soto 2003). We have previously shown that aggregates induced by hypoxia are cytotoxic, resulting in accelerated paralysis after animals are returned to room air (Fawcett *et al.* 2015). We therefore next asked if fasting would protect against increased proteotoxicity in addition to HIPA. To address this, we exposed cohorts of L1 Q40::YFP animals to hypoxia for 24 hours while fed or fasted, then returned the animals to room air and measured the onset of paralysis in each cohort. We found that fasting slowed the rate at which paralysis developed relative to animals

exposed to hypoxia while fed (Fig. 2.4A). This result indicates that fasting protects against hypoxic effects of increased protein aggregation and proteotoxicity.

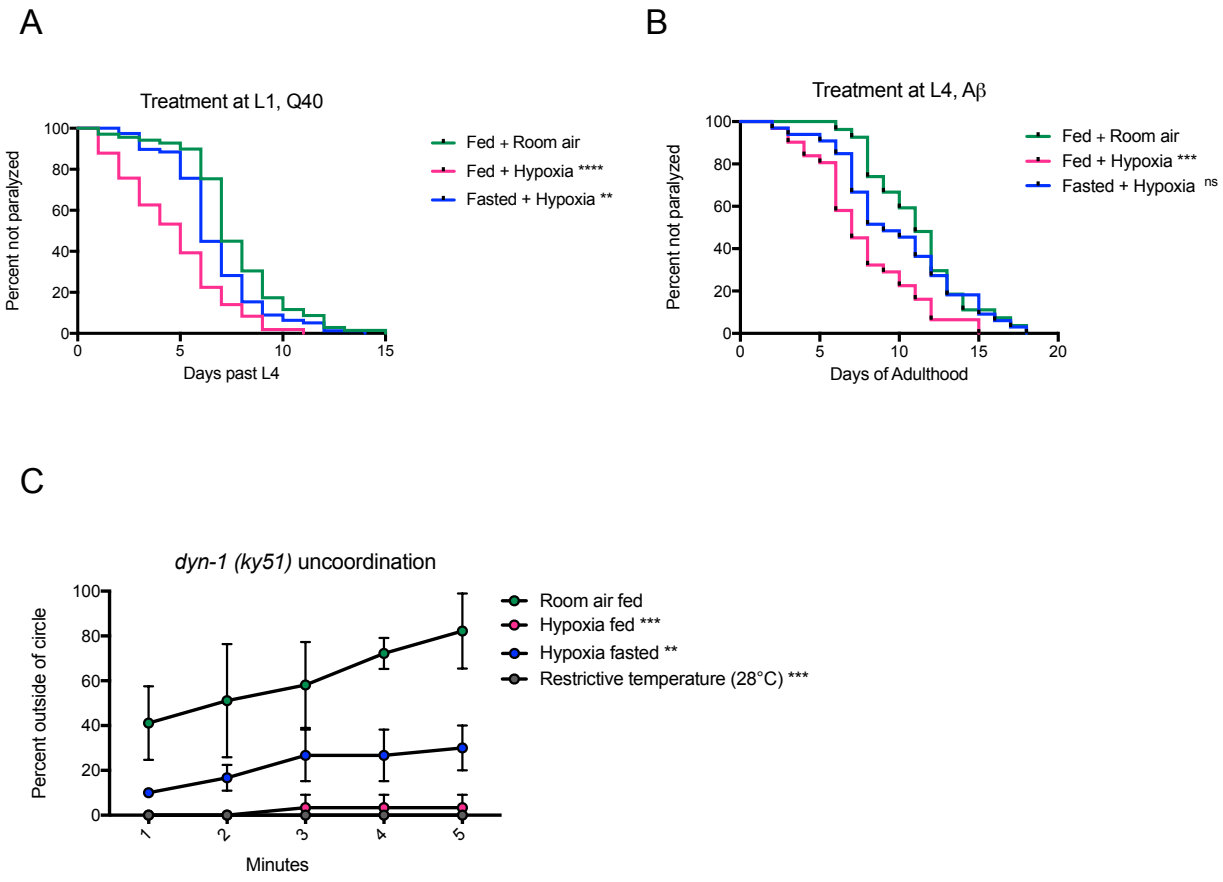


FIGURE 2.4 FASTING HAS GENERAL PROTECTIVE EFFECTS AGAINST HYPOXIA-INDUCED DEFECTS IN PROTEOSTASIS. (A) Fasting protects against toxicity of Q40::YFP. Cohorts of L1 animals expressing Q40::YFP were exposed to hypoxia on food (magenta), or fasted (blue) before exposure to hypoxia (F=6h, H=24 h). Paralysis was scored after return to room air, beginning the first day of adulthood. Controls remained on food in room air (green). Data from one representative experiment is shown, each cohort included at least 70 animals. Each experiment was repeated at least 3 times. Significance was calculated using a Log-rank (Mantel-Cox) test with a Bonferroni correction for multiple comparisons. Statistical comparisons were made between animals exposed to hypoxia and animals maintained in room air. **** $p < 0.0001$; ** $p < 0.01$. (B) Fasting protects against toxicity of A_{1-42}. Cohorts of L4 animals expressing A_{1-42} were exposed to hypoxia on food (magenta) or fasted (blue) before exposure to hypoxia (F=6h, H=24h). Paralysis was scored after return to room air, beginning at the first day of adulthood. Controls remained on food in room air (green). Data from one representative experiment is shown, each cohort included at least 70 animals. Each experiment was repeated at least 3 times. Significance was calculated using a Log-rank (Mantel-Cox) test with a Bonferroni correction for multiple comparisons. Statistical comparisons were made between animals exposed to hypoxia and animals

maintained in room air. *** $p < 0.001$. (C) Fasting protects against hypoxia effects on metastable DYN-1. Temperature-sensitive *dyn-1(ky51)* mutant animals were exposed to hypoxia at the permissive temperature on food (magenta), or after fasting (blue). Controls remained on food in room air at the permissive temperature (green) or on food at the non-permissive temperature (28°C, gray). Paralysis was scored 1h after return to room air. Average data from 3 independent experiments is shown, each cohort included 10 animals. Significance was calculated using a repeated measures two-way ANOVA and Dunnett's multiple comparisons test. Statistical comparisons were made between animals exposed to hypoxia or animals maintained at the restricted temperature and animals maintained in room air. Significance: *** $p < 0.001$; ** $p < 0.01$

We next sought to determine whether fasting's protective effects on proteostasis extend to other models of proteotoxicity. Human amyloid β $(A\beta)_{1-42}$ peptide expressed in the body wall muscles of *C. elegans* results in cytoplasmic plaque formation, with a subsequent phenotype of progressive paralysis (Link 1995). *C. elegans* expressing $A\beta_{1-42}$ in their body wall muscles become paralyzed more quickly when they are exposed to hypoxia (Fawcett *et al*. 2015). We found that this effect of hypoxia was reversed by fasting, as the rate that paralysis develops is slowed if animals expressing $A\beta_{1-42}$ are exposed to hypoxia while fasting (Fig. 2.4B). Because $A\beta_{1-42}$ and Q40::YFP are both expressed in body wall muscles, we also evaluated if fasting protected animals expressing a metastable version of the neuronal dynamin protein DYN-1 from the effects of hypoxia. The *dyn-1(ky51)* mutant contains a temperature-sensitive (ts) mutation, such that the DYN-1 protein is functional and *dyn-1(ky51)* mutant animals exhibit wild-type motility at the permissive temperature (20°C) but become uncoordinated at the restrictive temperature (28°C) due to improper folding of the DYN-1 protein (Clark *et al.* 1997). Genetic and environmental factors that disrupt proteostasis, including hypoxia, prevent the proper folding of the DYN-1 protein at the permissive temperature, thereby rendering the *dyn-1(ky51)* animals uncoordinated (Ben-Zvi *et al.* 2009; Fawcett *et al.* 2015). Similar to our experiments with Q40::YFP and $A\beta_{1-42}$, we found that fasting *dyn-1(ky51)* mutant animals before exposure to hypoxia results in a partial rescue of hypoxia-induced uncoordination at the permissive temperature (Fig. 2.4C). Together, our results suggest that fasting has a general protective effect against

proteostasis defects induced by hypoxia, and that this protective effect is not specific to a particular tissue, developmental stage, or misfolded/aggregation prone model.

2.4 DISCUSSION

This study illustrates the power of fasting to ameliorate the deleterious effects of hypoxia on proteostasis. These findings are consistent with phenomena that have been observed in mammals – fasting mice for a single day increases survival after kidney ischemia and also reduces ischemic damage to the liver (Mitchell *et al.* 2010). Our results suggest that the nutritional milieu present at the onset of hypoxia can dictate the effect of hypoxia on proteostasis, as fasting protection against hypoxia can be induced quite quickly. Animals that are removed from food immediately before hypoxia are protected against HIPA to a significant degree, even after being maintained on food for the entire pre-hypoxic period. This implies that worms are integrating information about their environment, including nutrient availability, right as they sense hypoxia. The importance of the nutritional environment of the animal as it experiences hypoxia is further supported by the fact that we also see a rapid reversal of fasting protection. Worms fasted for six hours but that are moved onto food immediately preceding hypoxia are not as protected against HIPA compared to worms that were fasted and remained off of food for the duration of hypoxia.

The speed with which fasting protection can be induced and reversed indicates that protection cannot be explained solely by changes in gene expression resulting in a hypoxia-resistant pre-adapted state. Furthermore, the rapidity with which fasting protection can be reversed suggests that altered gene expression or metabolism resulting from the fasting period is alone insufficient to protect against HIPA. Although *C. elegans* enter a reproductive and developmental diapause in 0.1% oxygen (Miller and Roth 2009), the protection

conferred by fasting does not represent a simple delay in the onset of proteostasis decline due to the time spent in hypoxia. Rather, fasting provides long-term protection against the accrual of protein aggregates and toxicity even after the return to room air.

Other environmental stresses including oxidative stress, heavy metal stress, and osmotic stress can induce protein aggregation (Weids *et al*. 2016; Moronetti Mazzeo *et al.* 2012; Burkewitz *et al.* 2012; Tamás *et al.* 2014) Additionally, multiple studies have shown that resistance to these stress-induced protein aggregates can be achieved through a preconditioning or acclimation period prior to the onset of the stress. The most well-known preconditioning paradigm is ischemic preconditioning. Ischemic preconditioning involves exposing animals to short sublethal ischemic bouts to induce ischemic tolerance prior to a longer or more severe ischemic period (Murry *et al.* 1986; Yang *et al.* 2010). Preconditioning most often focuses on outcomes like survival and tissue/cellular damage as the metric for success, but protection against protein aggregation has also been reported. For example, preconditioning via exposure to mildly hypertonic conditions suppresses osmotically induced aggregates (Burkewitz *et al.* 2012), and ischemic preconditioning can protect against aggregation due to cerebral I/R (Liu *et al.* 2005).

The mechanisms through which preconditioning events exert their effects vary with the method. Reduced protein synthesis, an upregulation of chaperone heat shock proteins, and increased proteasome activity have all been reported (Burkewitz *et al.* 2012; Ge *et al.* 2008; Liu *et al.* 2012; Badawi *et al.* 2014). Understanding the mechanisms and signaling pathways underlying fasting-mediated protection against hypoxia-induced proteostasis defects may therefore provide insights into potential avenues for therapeutics for stroke and heart attacks. In the following two chapters I will investigate two signaling pathways that are involved in fasting protection against hypoxia: insulin/IGF-1 (IIS) signaling, and AMPK-activated protein kinase (AMPK) signaling.

CHAPTER 3. INSULIN/IGF-1 SIGNALING IS REQUIRED FOR FASTING PROTECTION AGAINST HYPOXIA

3.1 SUMMARY

Oxygen is essential for the survival of virtually all metazoans (Danovaro *et al.* 2010). We have found that specific concentrations of hypoxia cause a disruption of protein homeostasis in *C. elegans*, and that dietary restriction in the form of fasting is able to protect against these hypoxia-induced proteostasis defects. The role of insulin/IGF-1 signaling (IIS) in mediating proteostasis and resistance to hypoxia is unclear. Insulin resistance has been implicated in protein aggregation in the context of neurodegeneration (Cohen *et al.* 2006), but reduced IIS is also associated with fasting-mediated resistance to hypoxia (Mitchell *et al*. 2012). I set out to clarify whether IIS has a role in hypoxia-induced protein aggregation or fasting protection. I found that that the aggregation phenotype incurred as part of the fed response to hypoxia is independent of IIS, but that IIS is required for fasting protection, as animals with mutations in *daf-2*, the *C. elegans* insulin/IGF-1-receptor, display wild-type levels of hypoxia-induced protein aggregation when fed, but are not protected by fasting. However, this requirement for IIS is independent of the downstream transcription factor DAF-16/FOXO. Taken together, my results highlight a non-canonical role for the IIS pathway in coordinating the effects of both hypoxia and nutritional state on proteostasis.

3.2 INTRODUCTION

Insulin/IGF-1 signaling (IIS) is a conserved pathway that has been extensively studied for its role in longevity (Barbieri *et al.* 2003). The main components of the pathway are

conserved from yeast through humans. In each system, the IIS pathway is activated by the binding of a ligand to the IIS receptor. In *C. elegans* and *Drosophila*, there are multiple insulin-like peptides, called ILPs and Dilps, respectively (Duret *et al.* 1998; Hua 2003; Slaidina *et al.* 2009; Kannan and Fridell 2013). In mammals, including mice and humans, insulin and insulin-like growth factor types 1 and 2 (IGF1 and IGF2) receive the vast majority of attention and research. Although seven insulin-like peptides also exist, virtually nothing about their function is known. (Pollak 2008; Fernandez and Torres-Alemán; Bathgate *et al.* 2013).

Upon ligand binding, IIS receptors (IR, IGF1R in humans; DAF-2 in *C. elegans*), initiate a phosphorylation cascade with a number of downstream effects. In mammals, the downstream effects of IIS are mediated by a number of well-characterized molecules. IIS results in the activation of AKT, which then inhibits FOXO transcription factors, TSC2 (an activator of TOR signaling), GSK3β, and TBC1D4. The net result of this signaling cascade is a decrease in glucose production and uptake, and decreased lipid, protein, and glycogen synthesis. (Haesler *et al.* 2017). In *C. elegans*, only the IIS-AKT-FOXO/DAF-16 pathway is well characterized.

In both mammals and *C. elegans*, FOXO/DAF-16 is a master regulator of many genes that regulate longevity, growth and development, metabolism, autophagy, stress resistance, learning, and memory (Kaletsky *et al.* 2016; Murphy *et al.* 2003; Tepper *et al.* 2013; Zhang *et al.* 2013; Zhao *et al.* 2007; Shimokawa *et al.* 2015; Yamazowa et al. 2010; Warr *et al.* 2013; Yim and Webb 2017, Haeusler *et al.* 2018). The role of FOXOs in particular, and IIS in general, in promoting longevity is a complex and pleiotropic process; despite this fact, a number of IIS effects and mechanisms have been well-characterized because this system has been such a popular target for investigations.

However, although it has been implicated in both protein aggregation and hypoxia, its role in these phenomena are much less clear. Research has been conducted on the effect of IIS on both hypoxia and proteostasis, but a number of conflicting results have been obtained. On the one hand, in invertebrates it is generally agreed up on that reduced IIS improves proteostasis. In *C. elegans*, reduction of IIS via knockdown of DAF-2 reduces Aβ toxicity through DAF-16 and HSF-1 (Cohen et al. 2010). Similarly, reduction in DAF-2 signaling decreases the aggregation and toxicity of SOD-1, and is also dependent upon DAF-16 (Boccitto *et al.* 2012). Proteostasis benefits from reducing IIS is not limited to invertebrates. Mice without neuronal IGF1R are protected against aggregation and mortality in a mouse model of AD (Stöhr *et al.* 2013), and FOXO3 activity protects neurons against α-synuclein aggregation and toxicity (Pino *et al.* 2014). Interestingly, FOXO3 inhibition protects against neuronal death in this same model (Pino *et al.* 2014). On the other hand, there are a multitude of studies that have found protective effects against protein aggregation by upregulation of IIS (Bedse *et al.* 2015; Athauda and Foltynie 2016; Bassil *et al.* 2014). Overall, the evidence that decreased IIS leads to increased proteostasis is much less convincing in mammals than it is in invertebrates.

With regard to IIS in hypoxia, there is not much to suggest that IIS is either protective or harmful. In *C. elegans*, decreased IIS due to mutations in *daf-2* are resistant to hypoxia, exhibiting higher survival rates (Scott *et al.* 2002). DAF-16 is required for hypoxia to increase lifespan in *C. elegans*, which suggests that the molecular mechanisms involved in adaptive responses to hypoxia might require decreased IIS (Leiser *et al.* 2013). In contrast, mice with a cardiomyocyte-specific knockout of the insulin receptor show more dysfunction compared to controls after I/R (Fu *et al.* 2015). Additionally, insulin treatment has been shown to confer resistance to cerebral and cardiac ischemia (Ji *et al.* 2010; Fu *et al.* 2015).

We sought to test the hypothesis that changes in IIS are involved in fasting protection against hypoxia-induced proteostasis defects. Our data indicate that that the IIS pathway plays a role in fasting's ability to protect against proteostasis decline independently of the canonical downstream transcription factor DAF-16/FOXO.

3.3 RESULTS

Dysregulation of IIS has been tied to protein aggregation and neurodegeneration in a number of model organisms (Cohen *et al.* 2006). As the IIS pathway links food availability to growth, development, stress resistance, and aging, we hypothesized that changes in IIS could explain how fasting modulates the effect of hypoxia on proteostasis. The IIS pathway is widely conserved in metazoans (Piñero González *et al.* 2009). We therefore explored the hypothesis that IIS would mediate the effects of fasting to prevent HIPA.

We first looked at the localization of DAF-16::GFP in animals exposed to hypoxia to determine if IIS is active in hypoxia. DAF-16 is the *C. elegans* ortholog of the FOXO transcription factor. When active, the insulin/IGF-like receptor DAF-2 initiates a phosphorylation cascade that results in the phosphorylation and nuclear exclusion of DAF-16 protein (Lin *et al.* 2001; Henderson and Johnson 2001). Conversely, when nutrients are scarce, DAF-16 remains unphosphorylated by upstream kinases and is able to enter the nucleus and bind to its target genes (Lin *et al.* 2001; Murphy *et al.* 2003). We found that DAF-16::GFP remained diffuse and cytoplasmic in control worms maintained in room air on food (Fig 3.1B, 3.1C), but accumulated in the nucleus of animals that were removed from food in room air (Fig. 3.1B, 3.1C) or were exposed to hypoxia on food (Fig. 3.1B, 3.1C). These results suggest that IIS activity is reduced by fasting and hypoxia, consistent with previous reports (Honjoh *et al.* 2009; Leiser *et al.* 2013). Surprisingly, DAF-16::GFP did not

accumulate in the nuclei of animals exposed to hypoxia after fasting (Fig 3.1B, 3.1C), despite hypoxia and fasting both individually resulting in nuclear accumulation.

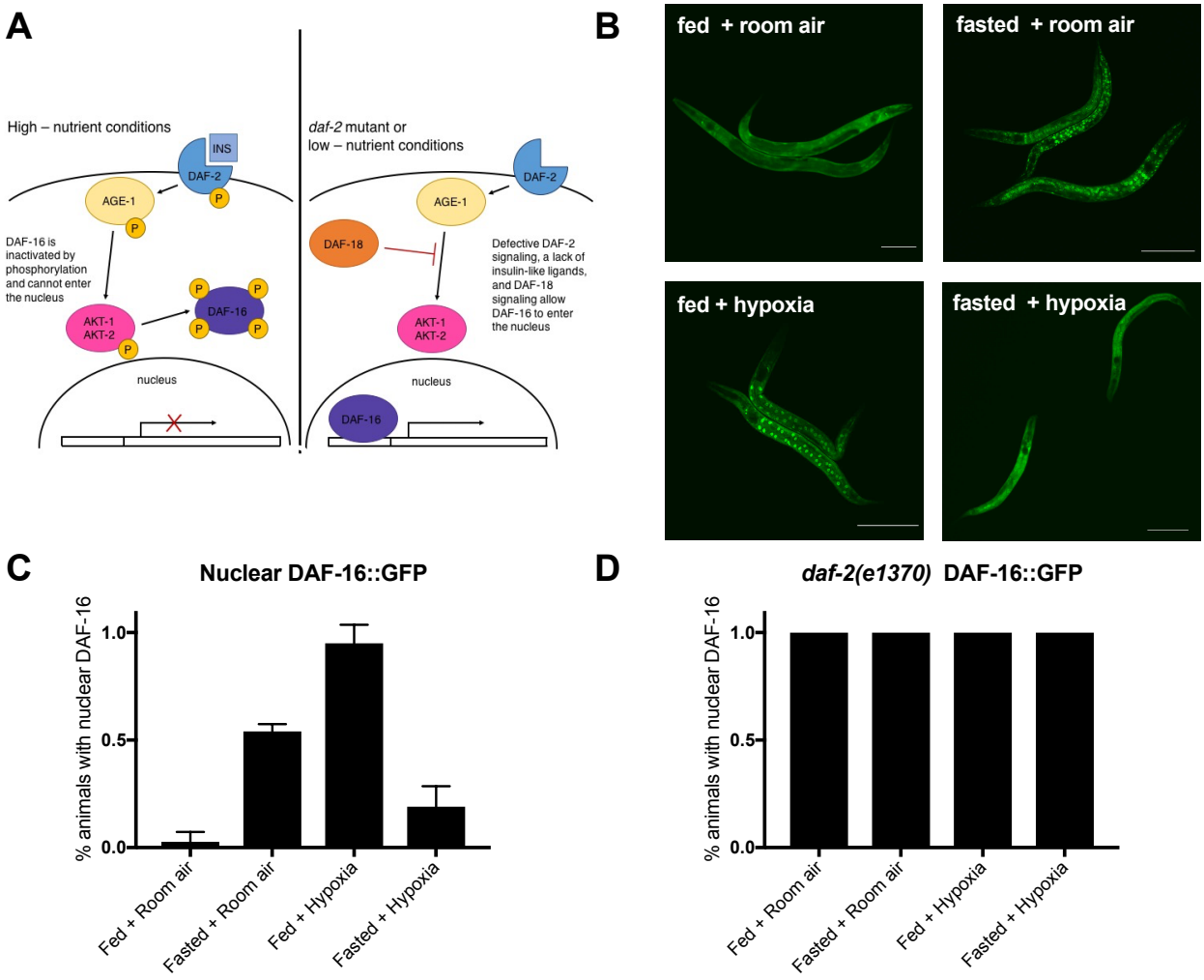


FIGURE 3.1 INSULIN/IGF-1 SIGNALING IS REDUCED BY HYPOXIA AND FASTING. (A) Schematic of key insulin-signaling pathway members in *C. elegans*. Under nutrient-rich conditions, insulin-like peptides bind to the insulin receptor DAF-2, initiating a phosphorylation cascades that ultimately leads to the phosphorylation of the FoxO transcription factor DAF-16, excluding it from the nucleus. Conversely, when nutrients are scarce, DAF-16 remains unphosphorylated and is able to enter the nucleus and bind to its target genes. (B) DAF-16 is not localized to the nucleus in fasted animals exposed to hypoxia. Cohorts of 20 DAF-16::GFP animals were maintained in room air on food for 24 hrs (fed + room air), fasted in room air for 24 hrs (fasted + room air), exposed to hypoxia for 24 hrs on food (fed + hypoxia), or exposed to hypoxia after fasting (fasted + hypoxia; F=6h, H=24hr). Scale bars = 100μm. (C-D) Quantification of DAF-16::GFP nuclear accumulation in wild-type and *daf-2(e1370)* backgrounds. Cohorts of 20 animals expressing DAF-16::GFP were maintained in room air on food for 24 hours (Fed + Room air), fasted in room air (Fasted + Room air), exposed to hypoxia for 24 hours on food (Fed + Hypoxia), or

exposed to hypoxia after fasting (Fasted + Hypoxia; F=6h; H=24h). The percent of animals with nuclear GFP was scored immediately post hypoxia. Average data from 3 independent experiments is shown. The bar height indicates the mean. Error bars (present, but not visible in D) are the standard deviation.

These DAF-16::GFP localization patterns led us to interrogate requirements for DAF-16 and the upstream IIS receptor DAF-2 in mediating fasted and fed responses to hypoxia. To this end, we crossed the Q35::YFP transgene into *daf-2(e1370)* and *daf-16(mu86)* backgrounds. The fact that DAF-16::GFP is localized to the nucleus in fed animals exposed to hypoxia suggests the possibility that DAF-16 facilitates HIPA. We found that *Q35::YFP; daf-16(mu86)* mutant animals exhibit robust HIPA on food (Fig 3.2), indicating that DAF-16 is not required for HIPA despite its nuclear accumulation in fed hypoxic animals. We also asked if there was a genetic requirement for the IIS receptor DAF-2. Our data indicate that IIS does not mediate the effects of hypoxia on proteostasis in fed animals, as *Q35::YFP; daf-2(e1370)* mutant animals exhibit robust HIPA when fed (Fig. 3.2). Thus, neither DAF-16 nor DAF-2 activities are required for HIPA in fed animals.

Given the IIS-independent nature of HIPA in fed animals, we next investigated whether fasting protection requires IIS. We discovered that DAF-2, but not DAF-16 is required for fasting protection against HIPA. Fasting protects the *Q35::YFP; daf-16(mu86)* similar to wild-type (Fig 3.2); however, we observe significant HIPA when *Q35; daf-2(e1370)* and *Q35; daf-2(e1368)* mutant animals are exposed to hypoxia when fasted (Fig 3.2 and Supplemental Fig C1, Appendix C). These results show that protective effects of fasting in hypoxia require DAF-2, but not DAF-16. This is consistent with our observation that DAF-16::GFP is not localized to the nucleus in fasted animals exposed to hypoxia (Fig 3.1B, 3.1C).

We found that the IIS receptor DAF-2 mediates the protective effects of fasting on HIPA, while the the FOXO transcription factor DAF-16 is not required for protection. Given this finding, we sought to investigate whether the nuclear localization of DAF-16 in *daf-2(e1370)* mutants, which are not protected by fasting, would be altered by hypoxia. We checked the DAF-16::GFP localization pattern in worms with a *daf-2(e1370)* mutation. These mutants have constitutively nuclear DAF-16 in the fed state due to decreased signaling through the IIS pathway (Lin *et al.* 2001). Although DAF-16::GFP is not localized to the nucleus in fasting-protected wild-type animals exposed to hypoxia,. We found that DAF-16::GFP is fully nuclear in all conditions, including fasted hypoxia, in these *daf-2(e1370)* mutants (Fig. 3.1D).

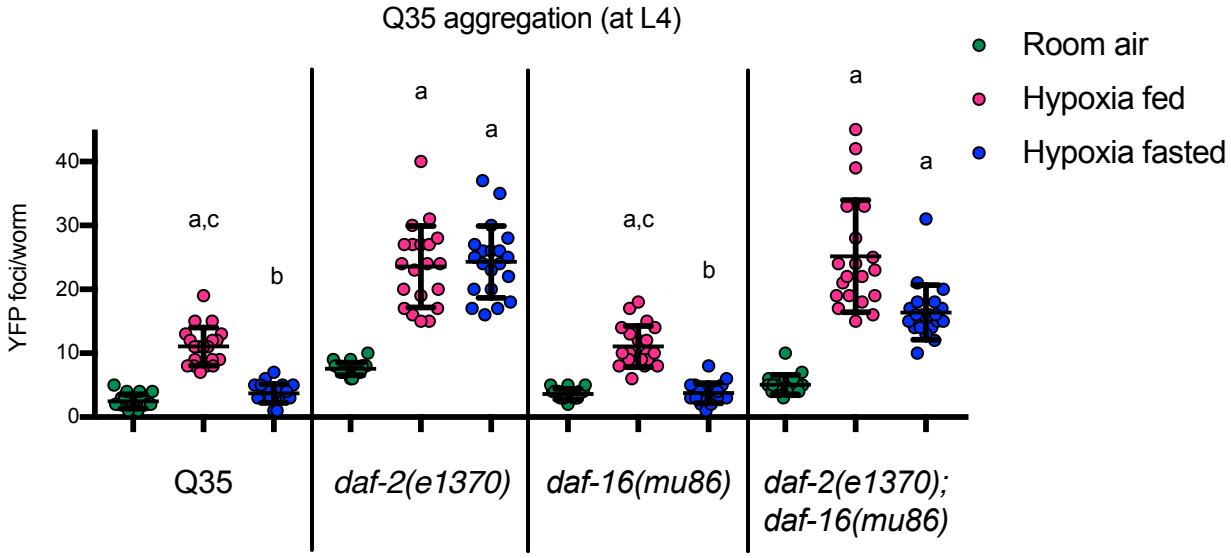


FIGURE 3.2 THE INSULIN/IGF-1 SIGNALING PATHWAY IS REQUIRED FOR FASTING PROTECTION. Fasting does not protect *daf-2* mutants against HIPA. Aggregation measurements (F=6h, H=24h) for L4 Q35::YFP animals with mutations in *daf-2(e1370)*, *daf-16(mu86)*, and the *daf-2(e1370); daf-16(mu86)* double mutant. Animals were maintained on food in room air (room air, green), were exposed to hypoxia on food (hypoxia fed, magenta), or were exposed to hypoxia after removal from food (hypoxia fasted, blue). 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. Data from one representative experiment is shown. Each cohort included at least 20 animals, and each experiment was repeated at least 3 times. Significance was calculated using a Kruskal-Wallis test and Dunn's multiple comparisons post hoc analysis. Significant differences ($p < 0.05$) in aggregation for a given

strain 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.

In *C. elegans*, DAF-16 mediates the effects of decreased signaling through DAF-2. Mutations in *daf-16* suppress most *daf-2* mutant phenotypes including increased lifespan, enhanced dauer formation, increased fat storage, reproductive delays, and increased resistance to heat and oxidative stress. (Ogg *et al.* 1997; Zhou *et al.* 2011). This coupled with the nuclear localization of DAF-16::GFP in *daf-2* mutants led us to hypothesize that *daf-16* would be required for the HIPA in fasted *Q35; daf-2(e1370)* mutant animals. While *Q35; daf-16(mu86)* mutant animals were protected from HIPA by fasting similar to wild-type controls, *Q35; daf-2(e1370);daf-16(mu86)* animals still exhibit significant HIPA when fasted (Fig. 3.2). These results indicate that DAF-2 mediates the effects of fasting to prevent HIPA at least partly independently of DAF-16.

Given that fasting protection against HIPA exhibits a genetic requirement for *daf-2*, we sought to determine whether *daf-2* was required for fasting protection against the long-term effects of hypoxia on proteostasis. As discussed in Chapter 2 (see Fig 2.3), short exposures to hypoxia still result in an accelerated rate of age-associated protein aggregation after return to room air, despite the fact that there is no increase the number of polyglutamine protein aggregates immediately after hypoxia, indicating a disruption of long-term proteostasis. To determine if DAF-2 is required for fasting-mediated resistance against long-term proteostasis defects induced by hypoxia, we exposed Q35::YFP and Q35; *daf-2(e1370)* L4 animals to hypoxia for only 10h either in the fed state or after fasting for 6h (F = 6 hours, H = 10 hours as per Fig. 2.1A). Control animals remained on food in room air. Immediately after this short hypoxic exposure, there was no observed increase in the number of YFP foci in animals exposed to hypoxia regardless of whether food was present (Fig. 2.3, t=0 hours post-hypoxia). As we saw previously, the Q35::YFP animals exposed to

hypoxia in the fed state accumulate aggregates faster than control animals, and this was prevented by fasting (Figure 3.3, compare t=24 hrs and t=48 hrs for "Room air Q35" and "Hypoxia fasted Q35" versus "Hypoxia fed Q35"). Surprisingly, Q35; *daf-2(e1370)* animals were also protected against long-term proteostasis defects by fasting (Fig 3.3), suggesting that while DAF-2 is required for fasting-mediated protection against aggregation present immediately after hypoxia, it is not required for protection against long-term defects in proteostasis conferred by fasting.

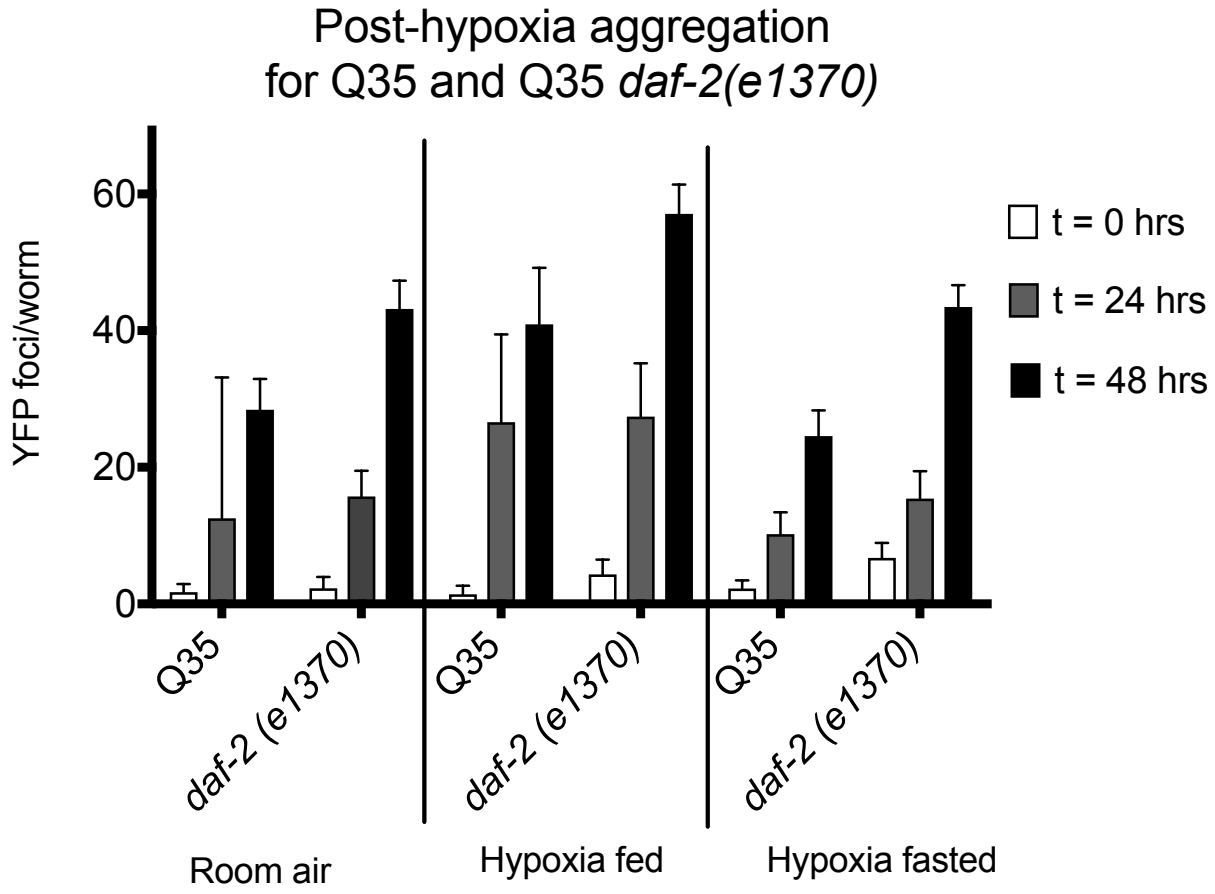


FIGURE 3.3 DAF-2 IS NOT REQUIRED FOR FASTING-MEDIATED RESISTANCE TO LONG-TERM EFFECTS OF HYPOXIA ON PROETOSTASIS. Fasting protects *daf-2* mutants against post-hypoxia aggregation. Cohorts of L4 Q35::YFP and Q35; *daf-2(e1370)* animals were exposed to hypoxia (H=10 h) on food (Hypoxia fed) or fasted (Hypoxia fasted, F=6h). Controls remained in room air on food (Room air). The number of YFP foci was scored after return to room air, as indicated by t – immediately after hypoxia (t=0hrs) 1 day after

hypoxia (t=24 hrs) and 2 days after hypoxia (t=48 hrs). Data from one representative experiment is shown. Each cohort included at least 20 animals per time point.

3.4 Discussion

We found that IIS mediates fasting protection against HIPA. Notably, IIS is not required for the fed response to hypoxia, as fed IIS mutants show increased aggregate levels comparable to wild-type animals. In worms and flies, mutations in the insulin receptor are generally considered protective against hypoxia. In *C. elegans, daf-2* mutants have a hypoxia-resistant phenotype, displaying reduced muscle and neuronal cell death following hypoxia (Scott *et al.* 2002; Mabon *et al.* 2009), while flies with defective insulin signaling (mutations in the insulin receptor *InR*, or *Chico*, the insulin receptor substrate) are protected against anoxia/reoxygenation injury (Vigne *et al.* 2009). The *daf-2* phenotype uncovered here is therefore distinct in that these mutants are sensitive to hypoxia in the fasted state, with fasted *daf-2* mutant animals exhibiting increased HIPA compared to wild-type controls. These results contradict the *a priori* expectation that *daf-2* mutants might be resistant to hypoxia even in the fed state due to their inability to detect insulin-like peptides.

Mammalian systems offer precedents of insulin receptor mutations causing sensitivity to hypoxic stress. Knockdown of neuronal insulin-like growth factor 1 receptor (IGF-1R) exacerbates hypoxic injury and increases mortality in mice (Liu *et al.* 2011), and IGF-1R is required in order for IGF-1 to protect myocardial cell exposed to ischemia (Liu *et al.* 2016). However, data on the role of mammalian IIS in response to hypoxia are mixed, and are complicated by the fact that different types of insulin receptors mediate distinct cellular functions (Cai *et al.* 2017). As such, the simplified *C. elegans* IIS system may be useful for understanding contextual inputs that alter IIS outputs.

Interestingly, we found that while DAF-2 is required for fasting protection against HIPA, it is not required for protection against exacerbated age-associated aggregation induced by a short exposed to hypoxia. Other studies have shown that stress and aging-induced polyQ aggregates are distinct morphologically, biophysically, and biochemically (Moronetti Mazzeo *et al.* 2012). Insulin signaling in particular has been shown to act differentially in healthy versus proteotoxically stressed neurons containing Q128. The polyQ-containing neurons are protected by DAF-16 activity, whereas the neurons without polyQ are not. Furthermore, DAF-2 signaling enacted different patterns of neuronal branching in Q128 neurons and healthy neurons (Scerbak *et al.* 2014). These studies provide support for a model in which hypoxia/stress-induced aggregates are influenced by different cellular/mechanistic aggregation pathways compared to age-associated aggregates, and thus may provide the basis for the differential requirement for DAF-2 in fasting protection against HIPA versus age-associated aggregation.

DAF-16 is believed to be the main nexus of IIS (Lin *et al.* 2001; Dillin *et al.* 2002; Hsu *et al*. 2003; Honda and Honda 1999), which makes the DAF-2-dependent, but DAF-16-independent nature of the protective effect of fasting described here unusual in *C. elegans*. Decreased DAF-2 activity results in phenotypes such as increased lifespan, reproductive delays, and increased resistance to heat and oxidative stress, all of which require DAF-16 (Zhou *et al.* 2011). However, a few other examples exist in the literature of DAF-2 dependent, DAF-16 independent phenomena, including dauer formation at 27°, salt chemotaxis learning, and of particular interest: α-synuclein aggregation and dopaminergic neurodegeneration in a *C. elegans* model of Parkinson's (Ailion and Thomas 2000; Lopez *et al.* 2013; Tomioka *et al.* 2006; Vellai *et al.* 2006; Yu and Larson 2001; Knight *et al.* 2014). In chemotaxis learning, DAF-2 acts on learning through phosphatidylinositol 3,4,5-triphosphate (PIP_3), but not DAF-16. In promoting α-synuclein aggregation, DAF-2 instead works through the glycolytic enzyme glucose 6-phosphate isomerase (GPI-1). Similar to

these studies, fasting-mediated protection against HIPA supports the existence of downstream targets of DAF-2 separate from DAF-16 that are capable of influencing stress responses and proteostasis.

Although there haven't been studies showing that reduced IIS is required for DR protection against hypoxia, it is generally accepted that reduced food intake results in lower insulin/IGF-1 levels (Robertson and Mitchell 2013). Paradoxically, there have been studies showing that insulin and IIS are beneficial in models of cerebral and cardiac ischemia. Insulin resistance is associated with increased stroke risk and worse outcomes after stroke (Calleja *et al.* 2011; Arenillas et al. 2007). Rats given a dose of insulin prior to cerebral ischemia had reduced infarct volumes (Hamilton et al. 1995). Similarly, rats given insulin prior to cardiac ischemia had improved cardiac function and reduced cell death (Ji *et al.* 2010). Finally, rats with a myocardial specific knockout of insulin receptors have more cardiac dysfunction after myocardial ischemia (Fu *et al.* 2005). The mechanisms by which insulin confers ischemic resistance have not yet been fully elucidated, but may involve reduced formation of peroxynitrite-induced oxidative and nitrative stress (Ji *et al.* 2010) or reduction in glucose levels (Smit *et al.* 2006; Hamilton *et al.* 1995). As DR also reduces blood glucose (Trepanowki et al. 2011), this mechanism could be one way to reconcile the protective effects of both IIS and DR/fasting protection against ischemia.

CHAPTER 4. AMP-ACTIVATED PROTEIN KINASE MEDIATES HYPOXIA-INDUCED PROTEIN AGGREGATION AND FASTING PROTECTION

4.1 SUMMARY

In order to survive in changing environmental conditions, organisms must be able to successfully sense and integrate diverse environmental signals and respond appropriately. We are interested in how the energy sensor AMP-activated protein kinase (AMPK) integrates environmental cues regarding oxygen and nutrient availability to regulate proteostasis. We have found that specific concentrations of hypoxia cause a disruption of protein homeostasis in *C. elegans*, as measured by increased aggregation and toxicity of aggregation-prone proteins across a variety of tissues and developmental stages. We have also shown that nutritional cues regulate the effect of hypoxia on proteostasis, as animals that are fasted develop dramatically fewer protein aggregates compared to their fed counterparts when exposed to hypoxia. Here, I show that the effects of hypoxia and nutrient deprivation on protein aggregation are mediated through AMPK, a cellular nutrient sensor that regulates energy balance. AMPK is required for both hypoxia-induced protein aggregation as well as the protective effect of fasting against hypoxia. AMPK mutant animals do not display increased protein aggregation in hypoxia. Moreover, fasting does not protect against hypoxia-induced aggregation in these mutant animals. Taken together, our results underscore AMPK's role in modulating cellular pathways that maintain proteostasis in response to a complex interaction of environmental cues.

4.2 INTRODUCTION

A well-folded proteome is essential to organismal survival, as cellular structure and function is dependent upon the ability of proteins to obtain their native conformations. As animals age, the capacity to sustain proteostasis declines, leading to increased morbidity and mortality (Labbadia and Morimoto 2014; Morimoto and Cuervo 2014; Taylor and Dillin 2011). Correspondingly, the incidence of diseases associated with the accumulation of protein aggregates, including Huntington's, Alzheimer's, and Parkinson's disease, increase with age (Hung *et al.* 2010). Environmental stresses can challenge cellular maintenance of a stable proteome, as stressful conditions may interfere with proper protein folding or interfere with protein quality control mechanisms. In order to cope with stressful environments, the expression of chaperone proteins is often upregulated to help proteins fold into their correct structures (Feder and Hofmann 1999; Voth and Jakob 2017). However, the mechanisms that coordinate proteostasis with environmental stress responses are not well characterized.

Oxygen is an essential environmental resource that is required by virtually all animals, as it plays a central role in cellular metabolism (Danovaro *et al.* 2010). We have found that oxygen is capable of modulating protein aggregation in *Caenorhabiditis elegans*, but that this effect is dependent upon nutritional status. Fasting protects against hypoxia-induced protein aggregation (HIPA), suggesting that nutritional cues can change the organismal response to hypoxia. As a cellular energy sensor that responds to both fasting and hypoxia (Emerling *et al.* 2009; Hao *et al.* 2015; Mungai *et al.* 2011; Hardie, 2011; Cantó and Auwerx 2011; Cantó *et al.* 2013) AMP-activated protein kinase (AMPK) is an attractive candidate as a mediator of the cellular response to oxygen and food deprivation.

AMPK is a heterotrimeric kinase activated by changes in the cellular AMP/ATP ratio. In conditions where energy is limited, AMPK upregulates energy-producing processes and downregulates catabolic processes that consume energy (Hardie *et al.* 2017). AMPK has been implicated in promoting protein aggregation in a number of neurodegenerative disorders. Activated AMPK accumulates in neurons with tau tangles and pre-tangles across a variety of tauopathies, and AMPK can directly phosphorylate tau on two residues (Vingtdeux *et al.* 2011). In mouse models of Huntington's disease, mutant huntingin activates the α1-containing version of AMPK, resulting in its translocation into the nucleus. Activation of AMPK with the drug AICAR results in neuronal death and formation of huntintin aggregates via reduction of the anti-apoptotic gene Bcl2 (Ju *et al.* 2011). However, overexpression of AMPKα1 or AMPKα2 protects against α-synuclein aggregation *in vitro*, although the highest degree of protection was obtained with an AMPKα1 variant that has a constitutively low level of activation (Boblea *et al.* 2017). Thus, the role of AMPK in promoting or perturbing proteostasis is complex and deserves further investigation.

Here, we investigated the role of AMPK on protein aggregation and toxicity in response to hypoxia and food deprivation. We have preliminarily found that AMPK has dual opposing roles in mediating proteostasis in response to hypoxia. As part of the fed response to hypoxia, AMPK promotes the aggregation of polyglutamine proteins, whereas AMPK acts to protect against hypoxia-induced proteostasis defects in fasted worms. These divergent roles may be mediated in part by tissue specific activity. Our results suggest that AMPK in the excretory canal may work to augment aggregation in fed animals exposed to hypoxia, while AMPK in the neurons may mediate fasting protection against aggregation. Taken together, our results underscore a context-dependent, cell non-autonomous role for AMPK in integrating hypoxia, nutrient status, and proteostasis.

4.3 RESULTS

AUTHORS NOTE: Some of the data contained in this chapter are preliminary.

AMP-activated protein kinase (AMPK) is a conserved nutrient sensor and regulator of energy homeostasis (Hardie 2007). It is activated in response to energetic stress, which can include both changes in the AMP:ATP ratio (Mihaylova and Shaw 2011) as well as hypoxia or ischemia (Emerling *et al.* 2009; Hao *et al.* 2015; Mungai *et al.* 2011). Activated AMPK exerts a number of actions to restore energy homeostasis to the cell, in general upregulating catabolic processes and downregulating energy-consuming pathways (Hardie 2007). As a protein kinase, AMPK has at least 60 known phorsphorylation target proteins (Hardie 2018) including enzymes, transcription factors, cell cycle regulators, and neuronal ion channels (Hardie 2018).

In order to determine if AMPK is involved in coordinating the response to hypoxia with proteostasis, we utilized the *aak-2(ok524)* mutant, in which one of the two catalytic α subunits of AMPK is disrupted. The *aak-2(ok524)* background is a null allele containing a 409bp deletion, resulting in a truncated protein without a complete kinase domain (Apfeld *et al.* 2004). We crossed Q35::YFP into the *aak-2(ok524)* background and exposed the animals to hypoxia (H=24h, as per Figure 2.1A). We found that Q35::YFP does not aggregate in hypoxia in fed *aak-2(ok524)* worms (Fig. 4.1, compare Q35 Hypoxia fed to *aak-2* Hypoxia fed – both in magenta). These data suggest that AMPK promotes the normal disruption of proteostasis we see in hypoxia.

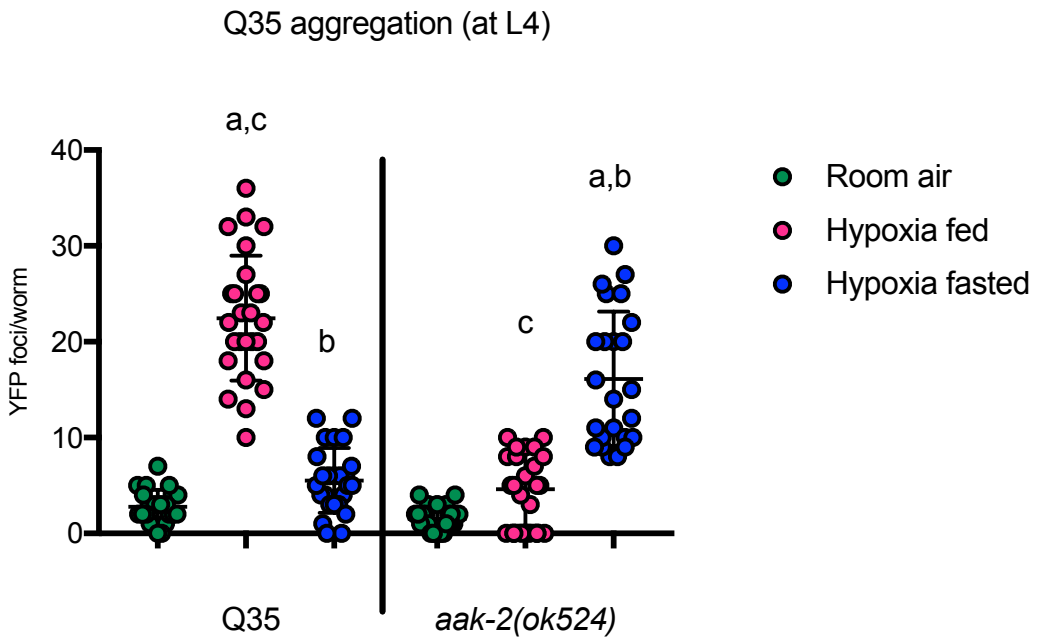


FIGURE 4.1 AAK-2 IS REQUIRED FOR BOTH HIPA AND FASTING PROTECTION AGAINST HIPA. Hypoxia does not induce aggregation in fed *aak-2* mutants, and fasting does not protect *aak-2* mutants from HIPA. Aggregation measurements (F=6h, H=24h) for L4 Q35::YFP animals with mutations in *aak-2(ok524)*. Animals were maintained on food in room air (room air, green), were exposed to hypoxia on food (hypoxia fed, magenta), or were exposed to hypoxia after removal from food (hypoxia fasted, blue). 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. Data from one representative experiment is shown. Each cohort included at least 20 animals. Significance was calculated using a Kruskal-Wallis test and Dunn's multiple comparisons post hoc analysis. Significant differences ($p < 0.05$) in aggregation for a given strain 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.

AMPK is activated in response to hypoxia in cell culture and mice, (Emerling *et al.* 2009; Mungai *et al.* 2011; McCullough *et al.* 2005), but this method of activation has not been demonstrated *C. elegans*. Our observation that AAK-2 is required for the aggregation of Q35::YFP in hypoxia suggests that the fed response to hypoxia involves activation of AMPK, and that this AMPK-mediated hypoxic response promotes the loss of proteostasis. Importantly, DR and fasting can also activate AMPK (Hardie, 2011; Cantó and Auwerx 2011; Cantó *et al.* 2013). However, since fasting helps to maintain proteostasis and prevent proteotoxicity, we hypothesized that the protective effects of fasting would not require AAK-

2. Surprisingly, we found that Q35::YFP aggregates in *aak-2(ok524)* mutants that are fasted before exposure to hypoxia, despite the fact that fasting is normally protective in a wild-type background (Fig. 4.1 compare Q35 Hypoxia fasted to *aak-2* Hypoxia fasted – both in blue). This result demonstrates that AMPK is required for fasting protection against HIPA and indicates that AMPK exerts different effects in fed and fasted animals exposed to hypoxia. In fed animals, AMPK contributes to the protein aggregation induced by hypoxia, while it acts to prevent hypoxia-induced proteostasis decline in fasted animals.

Our data suggest that AMPK is at least partially responsible for the breakdown of proteostasis in hypoxia and consequent accumulation of protein aggregates in fed animals. One potential explanation for AMPK promoting HIPA is the preferential allocation of resources away from proteostasis in metabolically stressful conditions. Protein quality control is energetically expensive (Díaz-Villanueva *et al.* 2015). In severe hypoxia, it may be that organisms divert the limited supply of available energy toward surviving the hypoxic insult, at the expense of maintaining proteostasis. This model would explain the appearance of polyglutamine aggregates in wild-type animals. Because AMPK is a crucial energy sensor, AMPK mutants may not be as percipient of the threat that severe hypoxia presents; accordingly, they may not allocate resources in the same way as do wild-type animals, instead electing to maintain proteostasis rather than using more energy to survive the unfavorable environment. Although *aak-2* is not required for animals to survive a 24-hour exposure to hypoxia and develop to adulthood (Miller and Roth 2009), it may be that AMPK mutants' allocation of energy toward maintaining proteostasis in hypoxia has consequences later in life.

To investigate whether maintenance of proteostasis in hypoxia affects longevity, we measured the lifespan of animals exposed to hypoxia for 24 hours at the L1 stage with or without 6 hours of fasting compared to controls maintained in room air (F=6h; H=24h, as

per Fig. 2.1A). All animals were moved back onto food and into room air after the hypoxic exposure. We used wild-type (N2) animals and *aak-2* mutants without the polyQ::YFP transgene. We observed that control room air *aak-2* mutants had shorter lifespans across conditions compared to N2 animals (Figure 4.2, see solid and dashed green lines), as has been described by other researchers (Apfeld *et al.* 2004). This trend held even for the groups exposed to hypoxia on food and animals exposed to hypoxia in the fasted state. The *aak-2* mutants had shorter lifespans than the N2 worms, and there were no differences between animals maintained in room air, exposed to hypoxia with food, or exposed to hypoxia in the fasted state. These data suggest that a relatively brief 24-hour hypoxic exposure does not appreciably affect the lifespan of *aak-2* or N2 animals, regardless of the nutritional status of the animal for the duration of the hypoxic bout.

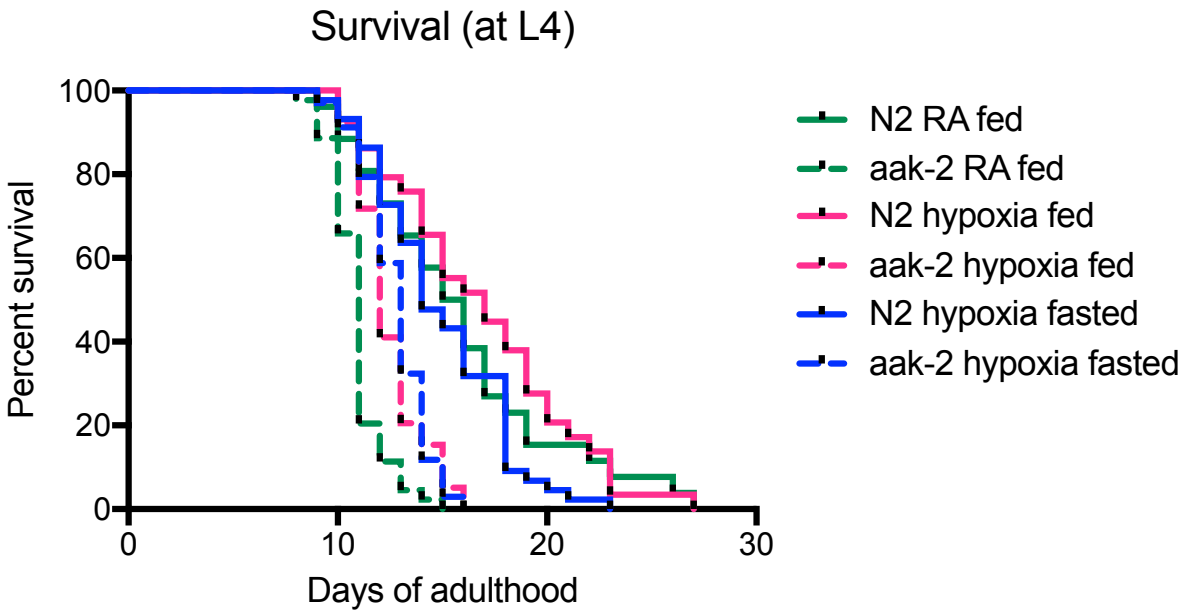


FIGURE 4.2 FED AND FASTED HYPOXIC EXPOSURES DO NOT ALTER LIFESPAN. Exposure to hypoxia in the fed or fasted state does not extend or shorten the lifespan of N2 or *aak-2* animals. Cohorts of N2 and *aak-2* animals were exposed to hypoxia on food (magenta), or fasted (blue) before exposure to hypoxia (F=6h, H=24 h). All animals were moved back onto food and into room air after the hypoxic exposure. Survival was scored once per day

after return to room air, beginning the first day of adulthood. Controls remained on food in room air (green). N2 animals are represented by solid lines, while *aak-2* animals are represented by dashed lines. Data from one representative experiment is shown, each cohort included at least 50 animals.

Although there are no lifespan effects resulting from hypoxic exposure, it is still possible that the allocation of energy towards maintaining proeotstasis in hypoxia results will alter polyQ::YFP proteotoxiciy and consequent paralysis rates. To investigate the relationship between AMPK, fasting, hypoxia, and paralysis, we used Q40 and Q40; *aak-2* animals and exposed them to hypoxia for 24 hours at the L1 stage in the fed and fasted state. All animals were moved back onto food and into room air after the hypoxic exposure.
We previously showed that polyglutamine aggregation in the body wall muscles is cytotoxic, resulting in uncoordination and eventual paralysis (Fawcett *et al.* 2015), and that this accelerated paralysis rate can be partially rescued by fasting (Fig 2.3A). Indeed, we saw that fed Q40 worms exposed to hypoxia have an accelerated rate of paralysis compared to controls maintained in room air, and that fasted Q40 animals exposed to hypoxia were partially protected (Fig 4.3, compare solid green, magenta, and blue lines). Based on these data and the data from Fig 4.1, the two conditions that result in proteostasis maintenance throughout hypoxia are fed *aak-2* mutants and fasted wild-type animals (Fig 4.1). Since hypoxia-induced protein aggregates are proteotoxic and induce paralysis, we expected that fed *aak-2* mutants would also be protected from accelerated paralysis, while fasted *aak-2* mutants would become paralyzed more quickly. Unexpectedly, we saw that while fed Q40; *aak-2* worms displayed an accelerated paralysis rate similar to fed Q40 animals exposed to hypoxia, fasted *aak-2* animals were protected against accelerated paralysis despite the fact that these animals have higher aggregate levels after hypoxia. This result indicates that while Q40::YFP aggregates are toxic in a wild-type background, they may not be in *aak-2* mutants. Fasted *aak-2* mutants with higher aggregate levels immediately after hypoxia are nonetheless protected against proteotoxicity in the long-term, showing slowed paralysis rates compared to fed *aak-2* mutants with low aggregate levels after hypoxia. This suggests

that paralysis may be uncoupled from aggregation. Alternatively, the long-term aggregation kinetics may switch after the return to room air in these animals, such that fed animals accumulate age-associated aggregates more quickly than fasted animals, despite starting off with fewer aggregates immediately post-hypoxia.

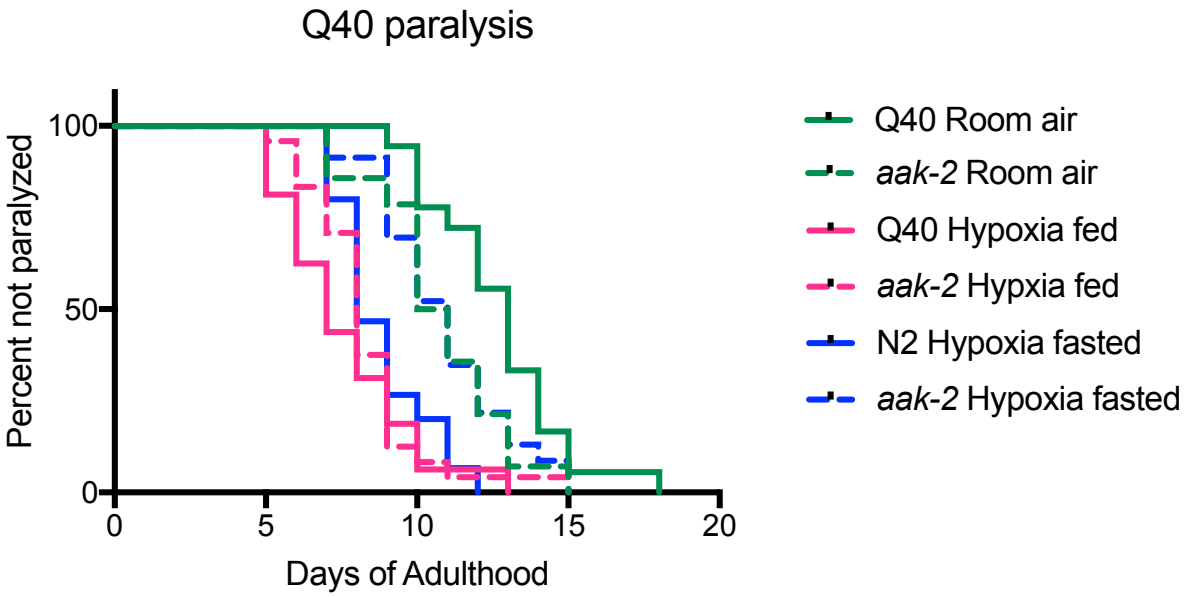


FIGURE 4.3 AAK-2 IS NOT REQUIRED FOR FASTING PROTECTION AGAINST ACCELERATED PARALYSIS INDUCED BY HYPOXIA. Fasting protects against toxicity of Q40::YFP in N2 and *aak-2* backgrounds. Cohorts of L1 Q40 and Q40; *aak-2* animals were exposed to hypoxia on food (magenta), or fasted (blue) before exposure to hypoxia (F=6h, H=24 h). All animals were moved back onto food and into room air after the hypoxic exposure. Paralysis was scored once per day after return to room air, beginning the first day of adulthood. Controls remained on food in room air (green). N2 animals are represented by solid lines, while *aak-2* animals are represented by dashed lines. Data from one representative experiment is shown, each cohort included at least 50 animals.

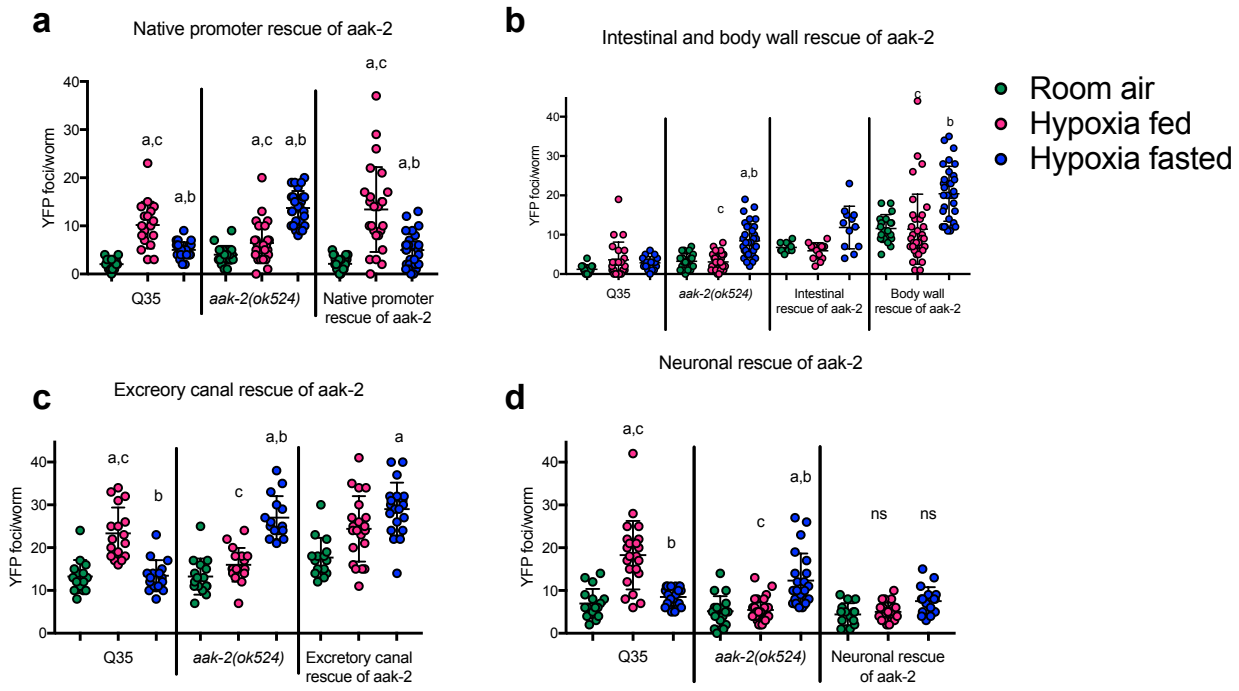


FIGURE 4.4 AAK-2 IS REQUIRED IN DIFFERENT TISSUES TO MEDIATE ITS EFFECTS ON HIPA AND FASTING PROTECTION. (A) Expression of aak-2 under its endogenous promoter recapitulates a wild-type phenotype. Animals exhibit HIPA when fed and are protected by fasting. (B) Expression of aak-2 limited to the intestine or the body wall has no effect on aggregation compared to the *aak-2* background. (C) Expression of aak-2 in the excretory canal is required for promotion of HIPA by AMPK. Animals exhibit HIPA when fed but are not protected by fasting (D) Expression of aak-2 in neurons is required for fasting protection. Animals do not exhibit HIPA when fed but are protected by fasting. (A-D) Aggregation measurements (F=6h, H=24h) for L4 Q35::YFP animals with mutations in *aak-2(ok524)*, but with aak-2 rescued in the tissue specified. Animals were maintained on food in room air (room air, green), were exposed to hypoxia on food (hypoxia fed, magenta), or were exposed to hypoxia after removal from food (hypoxia fasted, blue). 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. Data from one representative experiment is shown. Each cohort included at least 20 animals. Significance was calculated using a Kruskal-Wallis test and Dunn's multiple comparisons post hoc analysis. Significant differences ($p < 0.05$) in aggregation for a given strain 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.

Finally, we investigated the hypothesis that AMPK exerts its conflicting effects on proteostasis through differential tissue activation. It has been shown that heat shock can regulate proteostasis in a non-cell autonomous manner via the AFD sensory neuron (Prahlad

et al. 2008). Furthermore, AMPK activity can act non-autonomously in the hypodermis and excretory system to regulate dauer life span (Narbonne and Roy 2009). AMPK targets and regulation can vary in different tissues, cell-types, or developmental contexts (Mantovani and Roy 2011). To define AMPK tissue specificity in HIPA and fasting protection, we used transgenic *aak-2* mutant strains, in which GFP-tagged AAK-2 is rescued in specific tissues including body wall muscle, neurons, hypodermis, excretory canal, intestine, and under the endogenous *aak-2* promoter (Fukuyama *et al.* 2012). These strains were crossed with the Q35::YFP reporter, allowing for quantification of aggregation with AMPK activity limited to specific tissues.

Animals were exposed to hypoxia in the fed and fasted state (F=6h; H=24h). Although the data shown above are preliminary, there appears to be differential requirements for AMPK in HIPA and fasting protection against HIPA. As a positive control, we used a strain in which *aak-2* is expressed under its endogenous promoter. We expected this strain to exhibit a wild-type response to hypoxia when fed (increased aggregation) and fasted (reduced aggregation). This is indeed what we saw when we tested this strain (Figure 4.4A). In contrast, rescue of *aak-2* activity in the body wall or intestine had reduced HIPA when fed and increased aggregation when fasted, as *aak-2* mutants do (Fig 4.4B). This suggests *aak-2* activity in these tissues isn't important for regulating HIPA or fasting protection. However, animals with rescue of *aak-2* in the excretory canal showed HIPA when fed, like a wild-type animal, but were not protected by fasting, like an *aak-2* mutant (Fig 4.4C). This indicates that AMPK activity in the excretory canal is required for AMPK's promotion of HIPA as part of the fed response to hypoxia. Finally, we saw the reverse pattern with rescue of *aak-2* in the neurons. These animals showed reduced HIPA when fed, like an *aak-2* mutant, but also seem to be protected by fasting, like a wild-type animal (Fig 4.4D) This finding suggests that neuronal AMPK activity is required for fasting protection against HIPA. Taken together,

our results underscore the non-cell autonomous requirement for AMPK in different tissues to integrate proteostasis with fed and fasted responses hypoxia.

4.4 Discussion

We found that AMPK regulates proteostasis by integrating both oxygen and nutrient availability. Strikingly, AMPK exerts opposite effects on proteostasis in the fed and fasted state. In the fed state, AMPK promotes aggregation, as *aak-2* mutants don't exhibit increased aggregate levels after hypoxia. In the fasted state, AMPK is required for fasting-mediated proteostasis maintenance, as *aak-2* mutants are not protected against HIPA by fasting.

Early work investigating the effects of AMPK in the context of hypoxia/ischemia considered AMPK activation to be protective (Viollet *et al.* 2011). In the ischemic heart, AMPK has been shown to regulate a number of adaptive metabolic changes, such as increasing production of ATP via glycolysis, mobilizing glycogen stores, and increasing glucose utilization (Qi and Young 2015). Furthermore, AMPK induces autophagy and suppresses eEF2-regulated protein synthesis, thus generating substrates for cellular metabolism, removing damaged organelles, and reducing ER stress (Qi and Young 2015; Matsui *et al.* 2007; Takagi *et al.* 2007; Terai *et al.* 2005). Mice without AMPK show greater infarct volumes, more cell death, and poorer cardiac function after myocardial I/R (Wang *et al.* 2009; Wang *et al.* 2011; Russell *et al.* 2004; Takagi *et al.* 2007). However, in our model, AMPK works to promote the loss of proteostasis as part of the fed response to hypoxia. This finding is more in line with work showing that AMPK inhibition is protective in ischemic stroke in mice (McCullough *et al.* 2005; Li *et al.* 2007). The net effect of AMPK activation in response to ischemia remains open to debate, as a number of studies have found contradictory effects (Li and McCullough

2010; Takagi *et al.* 2007; Viollet *et al.* 2011). Whether AMPK acts to protect cells against ischemia or results in exacerbation of damage may depend on the severity of the ischemic insult, whether cell cultures or whole animals are being utilized, and the tissue being exposed to hypoxia (Li and McCullough 2010).

The idea that AMPK may have differing functions depending on its site of activity is consistent with our results showing that AMPK is differentially required in the excretory canal and the neurons to mediate HIPA and fasting protection against HIPA, respectively. In *C. elegans*, the excretory system regulates osmotic balance and is responsible for waste removal, analogous to the renal system of higher animals (Nelson and Riddle *et al.* 1994). AMPK has previously been shown to be required in the excretory canal (and hypodermis) to permit long-term dauer survival by rationing fat reserves and maintaining osmoregulation (Narbonne and Roy 2006). In mammals, AMPK is differentially regulated by hormones in a tissue specific fashion. For example, AMPK is activated by leptin in muscle cells but inhibited by leptin in the hypothalamus, resulting in fatty acid oxidation and feeding inhibition respectively (Minokoshi *et al.* 2002; Minokoshi *et al.* 2004). Endocannabinoids and ghrelin inhibit AMPK in the liver (promoting fatty acid synthesis), but activate AMPK in the hypothalamus (inhibiting fatty acid synthesis) (Kola *et al.* 2005; Lage *et al.* 2008). These studies provide other examples of tissue-specific AMPK regulation, but our results are unique in that AMPK exerts opposing effects (promotion versus prevention of protein aggregation) through its activity in separate tissues, rather than differential regulation of its activation or inhibition.

In addition to highlighting a contextual requirement for AMPK in different tissues, our results also highlight a non-cell autonomous role for AMPK in the regulation of proteostasis in response to food and oxygen availability. AMPK action in the excretory system and neurons is able to exert pro- and anti-aggregation effects on Q35::YFP expressed in the body wall.

Non-cell autonomous action of AMPK has been demonstrated to control aging in *Drosophila* via modulation of autophagy. Neuronal activation of AMPK induces autophagy in the intestine, potentially via a whole-body increase of 4E-BP (Ulgherait *et al.* 2014). There is a growing body of evidence reinforcing cell non-autonomous activation of stress response pathways that are capable of modulating proteostasis (Taylor *et al.* 2014). For example, expression of the chaperone HSP90 in intestinal cells or neurons confers resistance to protein misfolding in muscle cells (van Oosten-Hawle *et al.* 2013), and knockdown of the electron transport chain protein cytochrome c oxidase in neurons upregulates mitochondrial chaperones in the intestine (Durieux *et al.* 2011). Notably, a panel of ER and mitochondrial stress proteins, as well as proteasomal subunits, TOR-related, and autophagy proteins don't appear to be differentially upregulated as part of the fasted response to hypoxia compared to the fed response (Supplemental Figures D1 and D2, Appendix D). However, the candidate genes we examined don't represent an exhaustive list of potential molecular mechanisms responsible for enhanced proteostasis. There is also growing evidence that the neuronal death and dysfunction observed in neurodegenerative diseases is also at least partially regulated in a non-cell autonomous manner (Ilieva *et al.* 2009; Hult *et al.* 2011). Elucidating the mechanisms and signaling pathways through which non-autonomous regulation of proteostasis occurs may therefore provide insight into potential therapeutics for these diseases.

CHAPTER 5. CONCLUSIONS AND FUTURE DIRECTIONS

5.1 SUMMARY

This thesis work is organized around fasting's ability to provide protection against hypoxia-induced defects in proteostasis, including aggregation and toxicity. I showed that the removal of food prior to the onset of hypoxia is able to protect *C. elegans* from hypoxia-induced proteostasis defects. In characterizing the requirements for fasting protection, I found that fasting protection can be both induced and reversed rapidly: animals that are removed from food immediately prior to a hypoxic exposure are still protected, whereas animals that are fasted for a full six hours but returned to food immediately prior to hypoxia are not protected. This suggests that animals are capable of integrating information about nutrient availability into their response to hypoxia, and that fasting protection is not merely a pre-conditioning effect due to alterations in metabolism or gene expression. I determined that fasting protection is not limited to the duration of hypoxia. Animals that are only exposed to short bout of hypoxia do not show hypoxia-induced protein aggregation (HIPA) immediately afterward, but they accumulate age-associated aggregates more quickly than room air controls. Fasting is able to protect against this accelerated age-associated protein aggregation, indicating that short periods without food can protect against long-term proteostasis defects. Fasting protection is not limited to a particular tissue or developmental stage, as I observed protection in both L1 and L4 animals, and across a variety of aggregation-prone and metastable protein models.

I also discovered a role for both insulin/IGF-1 signaling (IIS) and AMP-activated protein kinse (AMPK) in HIPA and fasting protection. Both IIS and AMPK are required for fasting protection against HIPA, as mutations in the IIS receptor *daf-2* or the AMPK catalytic

subunit *aak-2* abolish the resistance fasting usually confers against HIPA. The requirement for *daf-2* is independent of the canonical downstream transcription factor *daf-16*, as *daf-2; daf-16* double mutants are responsive to fasting and show reduced aggregation after hypoxia without food. This DAF-16 independence is consistent with my observation that DAF-16::GFP is not localized to the nucleus in fasted hypoxic conditions, despite following a nuclear localization pattern after both fasting and hypoxia individually. Thus, both genetic and localization data suggest that an alternative pathway downstream from DAF-2 is responsible for mediating the IIS role in fasting protection.

AMPK plays both pro-and anti-aggregation roles in the response to hypoxia, depending on the nutritional state of the animal. In the fed state, AMPK supports the formation of protein aggregates, as fed animals with mutations in an AMPK catalytic subunit, *aak-2*, do not display HIPA. AMPK may be acting in the excretory canal to mediate this effect, as *aak-2* mutants with *aak-2* rescued specifically in the excretory canal do display polyQ::YFP aggregation after hypoxia. In contrast, fasted *aak-2* mutants are not protected against HIPA by fasting, suggesting that AMPK is required for the resistance to HIPA conferred by fasting. AMPK may be working in neurons to mediate this effect, as *aak-2* mutants with neuronal rescue of *aak-2* can be protected by fasting.

5.2 Paradoxical Roles for IIS and AMPK in Lifespan Extension and Proteostasis Impairment

IIS has a well-defined role in longevity. In *C. elegans*, reducing IIS increases longevity and general stress resistance (Kenyon *et al.* 2005). The same is true in mice – mice with a heterozygous knockout of IGF1R have 18% longer lifespans (Holzenberger *et al.* 2003). The ability to maintain proteostasis is intimately correlated with lifespan and aging (Kaushik and

Cuervo 2015). However, as was discussed in Chapter 1.5, there is no consensus on whether increased or decreased IIS is protective against proteotoxicity. In invertebrate models, a vast majority of the literature points to proteostasis improvements when IIS is downregulated. However, in mammals, there are many studies that suggest increased IIS can be protective against protein aggregation and toxicity associated with neurodegenerative diseases (Cohen and Dillin 2008). Our studies point toward IIS as being beneficial in proteostasis maintenance as part of the fasted response to hypoxia.

How then, should IIS be modulated for optimum longevity *and* proteome health? Does lifespan extension come at the expense of protein quality control and *vice versa* (at least in mammals) or is there a way to improve both simultaneously? Before we can consider IIS as a therapeutic target, these are questions that must be considered. One possibility is that IIS has effects that are specific to particular tissues. For example, while mice lacking insulin receptors specifically in their fat cells are long-lived and protected against age-associated metabolic dysfunction (Blüher *et al.* 2003), mice without hepatic insulin receptors are insulin resistant, glucose intolerant and hyperinsulemic (Michael *et al.* 2000). Circulating insulin/IGF-1 levels are not necessarily reflective of brain IIS, as reflected by the fact that AD patients have high plasma insulin levels combined with low cerebrospinal fluid levels (Craft *et al*. 1998).

Alternatively, work in worms and flies has shown that the timing of IIS reduction is important to control aging. Although *daf-2* mutants are long-lived, loss of *daf-2* specifically during development does not extend lifespan and reduction of *daf-2* throughout the reproductive period of adulthood was sufficient to extend lifespan (Dillin *et al.* 2002). In *Drosophila*, activation of dFOXO in the fat body during adulthood increases longevity (Hwangbo *et al.* 2004). Thus, it may be possible to alter IIS with spatial and temporal specificity to both increase lifespan and ameliorate neurodegeneration.

Many of the issues described above regarding the complications of reduced IIS as it applies to lifespan and proteostasis maintenance apply equally to activation of AMPK. Much of the interest in AMPK comes from its potential to extend healthy aging. AMPK has been shown to regulate multiple pathways that are known to play a role in longevity, including autophagy and mitochondrial and energy homeostasis (Burkewitz *et al.* 2014). Activation of AMPK increases lifespan in worms and flies (Apfeld *et al.* 2004; Stenesen *et al.* 2013), and while direct activation hasn't been investigated in mice, treatment with the type II diabetes drug and AMPK activator metformin does extend lifespan in mice (Martin-Motalvo *et al*. 2013). Additionally, AMPK is less responsive to activation signals in aged mice than young mice. While young mice show increased AMPK activity in response to exercise, older mice show a blunted response (Reznick *et al.* 2007), further supporting the idea that AMPK signaling becomes less functional with age.

However, like IIS, it's not at all clear that activation of AMPK is beneficial in neurodegeneration. Protective effects of AMPK activation have been observed in a mouse model of AD. Oral ingestion of resveratrol was shown to activate AMPK and reduce Aβ accumulation in the cortex (Vingtdeux *et al.* 2010). In a *C. elegans* model of Hungtinton's disease (HD) where animals express Q128::YFP in touch receptor neurons, overexpression of *aak-2* restores the touch response, while *aak-2* mutants have further reductions in function. Interestingly, this effect is dependent upon DAF-16 (Vázquez-Manrique *et al.* 2016). Transfection with an AMPK gain-of-function allele reduces soluble mutant huntingtin *in vitro*, and slightly reduces neuronal loss without a concomitant decrease in huntingtin aggregate levels (Vázquez-Manrique *et al.* 2016).

Additionally, AMPK has been shown to be a tau kinase that is activated by Aβ-induced CaMKK2 signaling *in vitro* (Thornton *et al.* 2011). Metformin activates AMPK and increases both intracellular and extracellular Aβ levels along with an upregulation of β-secretase, and

this effect is inhibited with application the AMPK inhibitor Compound C (Chen *et al.* 2009). Mice heterozygous for AMPKα2 have reduced insoluble tau in a mouse tauopathy model (Domise *et al.* 2016), and aberrant AMPKα1 activation is found in human and mouse HD brains. In a mouse model of Huntington's disease, AMPK activation cause increased huntingtin aggregate formation, neuron loss, and brain atrophy, which was dependent on AMPKα1 translocaltion into the nucleus (Ju *et al.* 2011).

AMPK expression also has the potential to be regulated with temporal and spatial specificity in order to maximize the its beneficial effects. For example, the AMPK overexpression-mediated lifespan extension in flies was seen when AMPK was overexpressed in only either muscle or the fat body (Stenesen *et al.* 2013). Additionally, AMPK may be differentially regulated based on its subunit composition (Lage *et al.* 2008). More research into how AMPK subunit composition dictates its effects, along with the different effects of AMPK in separate tissues, are needed in order to harness AMPKs beneficial effects without the risk of augmenting neurodegenerative pathways.

5.3 Future Directions

Extensions of this Project

There are multiple avenues for extending the work presented in this dissertation. One of the most critical next steps will be to identify a mechanism for the IIS-dependent nature of fasting protection. We showed that fasting protection against HIPA requires the IIS receptor DAF-2 but is independent of the canonical downstream transcription factor DAF-16. In *C. elegans,* some work in learning and memory have identified PIP_3 signaling as downstream of DAF-2, but parallel to DAF-16 (Vellai *et al.* 2006). Other candidates include SKN-1 and HSF-

1. Identifying the signaling pathway that occurs downstream of DAF-2 in fasting protection will clarify the outputs of IIS in *C. elegans* and also prove important for delineating the points of integration between IIS and other signaling pathways that are important for proteostasis maintenance in response to fasting.

Additionally, as discussed above, we need a more delicate understanding of the temporal and spatial requirements for IIS and AMPK. How do the effects of these signaling pathways change based on tissue type or with the age of an animal? How do their effects change in the presence of morbidities like cancer, diabetes, or neurodegenerative disorders? Currently, our understanding of the effects of these pathways are too coarse to fully utilize their therapeutic potential. Although my experiments with tissue-specific rescue of AMPK has started to unravel the nature of AMPK effects in specific locations, one limitation is that these rescue strains use *aak-2* driven off of an unintegrated array. As such, the levels of AMPK are overexpressed and may not be consistent between strains. Single-copy insertion of *aak-2* rescued under tissue-specific promoters would represent a much more physiologically relevant system to investigate AMPK's role in HIPA and fasting protection. Similar tissue-specific rescues can be made for *daf-2* in order to determine tissue specificity for its actions in fasting protection.

Finally, as the insulin receptor and AMPK both transduce their effects to downstream targets via phosphorylation, a phosphoproteomics approach could reveal insight into novel effectors downstream of DAF-2 and AMPK that are responsible for mediating the effects of AMPK and IIS on proteostasis in the context of hypoxia. Proteins that are detected to be regulated by phosphorylation can then be functionally tested by assaying their role in HIPA and fasting protection. Identifying differential phosphorylation patterns in response to fed and fasted hypoxic exposure will elucidate new components of these pathways in the *C. elegans* stress response network.

IIS and AMPK Signaling as Therapeutic Targets for Neurodegeneration

Evidence that IIS and AMPK are involved in both proteostasis pathways and appear to be dysregulated in neurodegenerative diseases suggests that both pathways may be promising targets for therapeutics. With a growing body of research showing that IIS is impaired in AD, a number of groups have attempted to use drugs originally developed for use in type II diabetes to treat AD. For example, thiazolidinediones (TZDs) are anti-diabetic compounds that agonize PPAR-γ to regulate lipid and glucose metabolism. Pioglitazone and rosiglitazone are both TZDs that have been tested for their efficacy in patients with mild-to-moderate AD. A small clinical trial found pioglitazone was able to improve memory retention and cognition (Sato *et al.* 2011). Mixed results were found for rosiglitazone: a phase II trial found that 6 months of rosiglitazone improved memory and attention in late-onset AD patients, but a later phase III trial did not find rosiglitazone to be effective in ameliorating AD symptoms (Risner *et al.* 2006; Gold *et al.* 2010). Importantly, the dosages used for these trials were much lower than the dosages reported to improve AD pathology in mice.

Alternatively, insulin itself has been considered as a therapeutic option for AD. Intranasal insulin has received particular attention because this method of delivery bypasses the blood brain barrier. Intranasal insulin has been shown to be effective at improving spatial memory, working memory, decision making, motor memory, and reducing Aβ in animal models, and current human clinical trials are ongoing (Chapman *et al.* 2017). Short-term studies have shown that intranasal administration of insulin improves verbal memory and reduced Aβ levels in the plasma in memory-impaired adults without a genetic predisposition to AD (Reger *et al.* 2008). IIS as a target for therapeutics in neurodegenerative diseases is reviewed in more detail elsewhere (Athuada and Foltynie 2016; Torres-Aleman 2007; Folch *et al*. 2016; de la Monte 2012).

Data on the use of drugs modulating AMPK activity are inconclusive. Metformin is a diabetes drug that is also an activator of AMPK. Activation of AMPK may promote Aβ clearance via upregulation of microglial phagocytosis (Labuzek *et al.* 2010). Kickenstein *et al.* showed that treatment with metformin reduces tau phosphorylation, but that its protective effects were dependent on PP2A and not AMPK (Kickenstein *et al.* 2010). However, another study showed that metformin increased Aβ production in an AMPK-dependent manner in mice (Chen *et al*. 2009.) The AMPK inhibitor Compound C has been investigated as a therapeutic for cancer, but some of the beneficial effects seen with this drug seem to work independently of its effects on AMPK (Vucicevic *et al.* 2011; Liu *et al.* 2014). This is a general problem with the pharmacological activators and inhibitors of AMPK that are currently most widely available – they exert a number of off-target effects and lack specificity (Zaha and Young 2012). Finding more specific ways to regulate AMPK activity that take into consideration its distinct roles in different tissues and perhaps the effects of differential subunit composition will be key to utilizing AMPK signaling as a therapeutic target for neurodegenerative disorders and other human health problems.

APPENDIX A: 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). See Supplemental Table A1 for worm strains. Strains were obtained from the *Caenhorabditis* Genetics Center at the University of Minnesota. Double and triple mutants were generated using standard genetic techniques, and genotypes were verified using PCR.

Construction of hypoxic chambers

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

YFP::polyQ aggregation assays

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

cohorts of L4 YFP::polyQ$_{35}$ animals were generated by picking L4 animals from well-fed, logarithmically growing populations.

Cohorts of 25-35 YFP::polyQ animals were exposed to hypoxia for approximately 24 h at 20°C on unseeded 3 cm NGM plates with 40mg/mL carbenicillin or NGM plates seeded with live OP50 food. Plates were ringed with palmitic acid (10mg/mL in ethanol), creating a physical barrier around the edge of each plate to discourage animals from leaving the surface of the agar.

To quantify the number of YFP foci, worms were mounted a 2% agar pad in a drop of 50mM sodium azide as anesthetic. Control experiments showed that azide did not affect the aggregation of YFP::polyQ$_{35}$ or YFP::polyQ4$_0$ (Moronetti Mazzeo et al. 2012). YFP foci were identified and quantified as described in Morley et al. (2002) and Silva et al. (2011). A Nikon 90i fluorescence microscope with the YFP filter and 10x objective (Nikon Instruments Inc., Melville, NY, USA) was used to visualize and quantify aggregates. In all experiments, the number of aggregates was counted blind to 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) In all cases, $P < 0.05$ was considered statistically significant.

Paralysis and uncoordination assays of proteotoxicity

Animals expressing A$\beta_{1\text{-}42}$ or YFP::polyQ$_{40}$ were exposed to 1000 ppm O_2 for 24 at 20°C as L4 or L1, respectively. For both, animals were grown on seeded NGM plates until 6 hrs before hypoxic exposure, at which point fasted animals were transferred to unseeded NGM plates, where they remained until the end of the hypoxic exposure. Fed animals were transferred to new seeded NGM plates. After hypoxic exposure, all animals were returned to

food and normoxia, and incubated at 20°C. Paralysis was scored daily. Worms were considered paralyzed if they failed to respond, other than with movement of the nose or pharyngeal pumping, when tapped with a platinum wire pick 3 consecutive times. Dead or 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 scored as either paralyzed or dead. Statistical significance was calculated using Kaplan-Meier log-rank (Mantel-Cox) tests and a Bonferroni correction for multiple comparisons using GraphPad Prism version 7.0c for Mac OSX (GraphPad Softare, San Diego, California, USA).

DAF-16::GFP localization

Synchronous cohorts of L2 animals expressing DAF-16::GFP were exposed to hypoxia for 24 h at 20°C on unseeded 3 cm NGM plates with 40mg/mL carbenicillin or NGM plates seeded with live OP50 food. Plates were ringed with palmitic acid (10mg/mL in ethanol), creating a physical barrier around the edge of each plate to discourage animals from leaving the surface of the agar. To visualize the localization of DAF-16::GFP, worms were mounted a 2% agar pad in a drop of 10mM levamisole as anesthetic. A Nikon 90i fluorescence microscope with the GFP filter and 10x objective (Nikon Instruments Inc., Melville, NY, USA) was used to visualize DAF-16::GFP. For quantification, percent of animals with nuclear GFP was scored immediately after removal from hypoxia. In all experiments, the GFP localization was scored blind to 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.

Lifespan analysis

Animals were exposed to 1000 ppm O_2 for 24 at 20°C at L1. Animals were grown on seeded NGM plates until 6 hrs before hypoxic exposure, at which point fasted animals were transferred to unseeded NGM plates, where they remained until the end of the hypoxic exposure. Fed animals were transferred to new seeded NGM plates. After hypoxic exposure, all animals were returned to food and normoxia, and incubated at 20°C. Survival was scored daily. Worms were considered dead if they failed to respond to tapping with a platinum wire pick 3 consecutive times. Bagged worms were censored from the experiment on the day of bagging. Dead worms were removed from the plate on the day of paralysis. Live worms were moved to a new plate each day until all worms were scored as either bagged or dead.

qRT-PCR

Animals were grown on NGM plates seeded with OP50 *E. coli* at 20°C*.* When animals reached gravid adult, synchronized embryos were obtained by a 5-minute bleach in 1:1:5 water:KOH:hypochloric acid solution. For each strain/condition, ~9,000 embryos were plated onto a 150 mM NGM plate seeded with live OP50 *E. coli*. Animals were not allowed to starve out the plate at any time during the experiment. When animals reached L4, they were exposed to hypoxia with or without a 6 hour fasting period, or were left in room air as controls. Animals were harvested into 1 mL Trizol solution and immediately frozen in liquid nitrogen, as described previously (Fawcett *et al.* 2012). RNA was isolated from the Trizol preparation as described previously (Chomczynski 1993).cDNA was made using Invitrogen SuperScript III First Strand Synthesis System. qPCR was performed using Kappa SYBR FAST qPCR Kit. PCR cycle was as follows: 95C for 3 min, 95C for 15 sec, 55C for 15 sec x40. 4°C to hold. qRT-PCR values were analyzed as described in (Miller *et al.* 2011). In summary, ΔCt for each gene product was calculated by subtracting Ct values from the geometric mean of

the control targets that are not altered in response to fasting or hypoxia (HIL-1, IRS-2, and TBA-1). ΔCt were averaged across experiments. Student's t-test was used to evaluate differences between ΔCt values of treated samples and untreated controls. For differences between genotypes, p-values were calculated with a one-way ANOVA from summary statistics (mean, standard error, n). Reported fold-changes were calculated as 2^-ΔΔCt where ΔΔCt = ΔCt(experimental condition) - ΔCt(control condition). Error bars on graphs represent standard error of the mean.

Strain	Genotype	Reference
AM140	rmIs132 [unc-54p::Q35::YFP]	Saytal et al. 2000
AM141	rmIs133 [unc-54p::Q40::YFP]	Saytal et al. 2000
CX51	dyn-1 (ky51)	Clark et al. 1997
CL2006	dvIs2 [pCL12(unc54/human Abeta peptide 1-42 minigene) + pRF4]	Link, 1995
CB1370	daf-2(e1370)	Kimura et al. 1997
CF1038	daf-16(mu86)	Lin et al. 1997
TJ356	zIs356 [daf-16p::daf-16a/b::GFP + rol-6(su1006)]	Lin et al. 2001
GR1895	daf-2(e1370); mgIs67 [daf-16p::daf-16::GFP + rol6(su1006)]	Riedel et al. 2013
RB754	aak-2(ok524)	Apfeld et al. 2004
YB1018	aak-1(tm1944);aak-2(ok524); pKS19 [aak-2p::aak-2::GFP+ rol6(su1006)] (aak-2 promoter rescue)	Fukuyama et al. 2012
YB904	aak-1(tm1944);aak-2(ok524); pMF342 [myo-3p::aak-2::GFP+ rol6(su1006)] (aak-2 body wall rescue)	Fukuyama et al. 2012
YB969	aak-1(tm1944);aak-2(ok524); pMF308 [rgef-1p::aak-2::GFP+ rol6(su1006)] (aak-2 neuronal rescue)	Fukuyama et al. 2012
YB1045	aak-1(tm1944);aak-2(ok524); pMF380 [pgp-1p::aak-2::GFP+ rol6(su1006)] (aak-2 intestinal rescue)	Fukuyama et al. 2012
YB1023	aak-1(tm1944);aak-2(ok524); pMF381 [myo3pgp-12p::aak-2::GFP+ rol6(su1006)] (aak-2 excretory canal rescue)	Fukuyama et al. 2012
	daf-2(e1370); Q35::YFP	*
	daf-2(e1368); Q35::YFP	
	daf-16(mu86); Q35::YFP	*
	daf-2(e1370); daf-16(mu86); YFP::Q35	*
	aak-2(ok524); Q35::YFP	*
	aak-2(ok524); Q40::YFP	

	aak-2(ok524); pKS19 [aak-2p::aak-2::GFP+ rol6(su1006)]; Q35::YFP (aak-2 promoter rescue)	*
	aak-2(ok524); pMF342 [myo-3p::aak-2::GFP+ rol6(su1006)]; Q35::YFP (aak-2 body wall rescue)	*
	aak-2(ok524); pMF308 [rgef-1p::aak-2::GFP+ rol6(su1006)]; Q35::YFP (aak-2 neuronal rescue)	*
	aak-2(ok524); pMF380 [pgp-1p::aak-2::GFP+ rol6(su1006)]; Q35::YFP (aak-2 intestinal rescue)	*
	aak-2(ok524); pMF381 [myo3pgp-12p::aak-2::GFP+ rol6(su1006)]; Q35::YFP (aak-2 excretory canal rescue)	*

SUPPLEMENTAL TABLE A1. *C. ELEGANS* EXPERIMENTAL STRAINS. Strains used for experiments described in this dissertation. * indicates that strain was created by crossing AM140 with indicated genetic background. Mutant alleles were verified using PCR genotyping.

APPENDIX B: SUPPLEMENTAL MATERIAL FOR CHAPTER 2

	Room air			Hypoxia fed				Hypoxia fasted			
Strain	mean #YFP foci	SD	n	mean #YFP foci	SD	n	p-value	mean #YFP foci	SD	n	p-value
Q35::YFP (AM140)	2.8	1.80	22	22.5	6.50	26	****	5.5	3.40	25	ns
Q35::YFP (AM140)	5.1	1.80	25	20.6	7.60	20	****	8.9	2.72	20	**
Q35::YFP (AM140)	2.3	1.10	22	17.5	6.50	20	****	4.2	1.80	20	ns
Q40::YFP (AM141)	12.7	4.30	25	40	9.6	25	****	7.9	4.40	25	ns
Q40::YFP (AM141)	10.7	3.30	20	45.8	9	20	***	5.2	4.30	20	*
Q40::YFP (AM141)	9.2	3.40	25	41.1	6.80	25	****	5.3	2.40	25	*

SUPPLEMENTAL TABLE B1. SUMMARY OF EXPERIMENTS IN FIGURE 2.1. Statistical comparisons between animals exposed to hypoxia ('Hypoxia fed' and 'Hypoxia fasted') and controls maintained in room air ('Room air'). p-value: ns = >0.05, * = ≤0.05, ** = ≤0.01, *** = ≤0.001, **** = ≤0.0001

	Room air			
Strain	**mean #YFP foci**	**SD**	**n**	
Q40::YFP (AM141) (r1)	8.1	2.4	25	
Q40::YFP (AM141) (r2)	17.1	6.9	20	
Q40::YFP (AM141) (r3)	28.0	7.3	18	
	Hypoxia fed			
Strain	**mean #YFP foci**	**SD**	**n**	**significance**
Q40::YFP (AM141) (r1)	38.9	9.2	25	a, c
Q40::YFP (AM141) (r2)	33.3	9.3	18	a, c
Q40::YFP (AM141) (r3)	41.0	8.0	20	a, c
	Hypoxia X=0 hrs			
Strain	**mean #YFP foci**	**SD**	**n**	**significance**
Q40::YFP (AM141) (r1)	15.5	6.3	25	a, b, c
Q40::YFP (AM141) (r2)	19.7	7.7	20	b
Q40::YFP (AM141) (r3)	28.2	5.4	20	c
	Hypoxia X=2 hrs			
Strain	**mean #YFP foci**	**SD**	**n**	**significance**
Q40::YFP (AM141) (r1)	8.9	4.3	25	b
Q40::YFP (AM141) (r2)	14.1	5.9	20	b
Q40::YFP (AM141) (r3)	19.8	7.9	20	b
	Hypoxia X=4hrs			
Strain	**mean #YFP foci**	**SD**	**n**	**significance**
Q40::YFP (AM141) (r1)	7.4	4.3	25	b
Q40::YFP (AM141) (r2)	21.7	11.4	19	b
Q40::YFP (AM141) (r3)	23.6	8.8	20	b
	Hypoxia fasted			
Strain	**mean #YFP foci**	**SD**	**n**	**significance**
Q40::YFP (AM141) (r1)	6.4	4.1	25	b
Q40::YFP (AM141) (r2)	16.4	11.1	17	b
Q40::YFP (AM141) (r3)	14.2	5.9	20	a, b

SUPPLEMENTAL TABLE B2. SUMMARY OF EXPERIMENTS IN FIGURE 2.2A. Statistical comparisons between animals exposed to hypoxia ('Hypoxia fed' and 'Hypoxia fasted') and controls maintained in room air ('Room air'). The first row in each condition represents data from the first biological replicate (r1), the second row in each condition represents data from the second biological replicate (r2), and the third row in each condition represents data from the third biological replicate (r3). 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

	Room air			
Strain	**mean #YFP foci**	**SD**	**n**	
Q40::YFP (AM141) (r1)	12.7	4.3	25	
Q40::YFP (AM141) (r2)	12.0	5.1	25	
Q40::YFP (AM141) (r3)	13.1	4.2	20	
	Hypoxia fed			
Strain	**mean #YFP foci**	**SD**	**n**	**significance**
Q40::YFP (AM141) (r1)	40	9.6	25	a, c
Q40::YFP (AM141) (r2)	35	7.9	25	a, c
Q40::YFP (AM141) (r3)	35.1	6.8	20	a, c
	Hypoxia 2 hrs fasted			
Strain	**mean #YFP foci**	**SD**	**n**	**significance**
Q40::YFP (AM141) (r1)	34.8	10.3	25	a, c
Q40::YFP (AM141) (r2)	35.0	6.6	25	a, c
Q40::YFP (AM141) (r3)	32.3	7.4	20	a, c
	Hypoxia 4 hrs fasted			
Strain	**mean #YFP foci**	**SD**	**n**	**significance**
Q40::YFP (AM141) (r1)	31.2	8.1	25	a, c
Q40::YFP (AM141) (r2)	33.0	5.9	25	a, c
Q40::YFP (AM141) (r3)	31.3	6.5	20	a, c
	Hypoxia 6 hrs fasted			
Strain	**mean #YFP foci**	**SD**	**n**	**significance**
Q40::YFP (AM141) (r1)	21.5	7.5	25	b, c
Q40::YFP (AM141) (r2)	25.0	5.7	25	a, b, c
Q40::YFP (AM141) (r3)	25.1	6.3	20	b, c
	Hypoxia fasted			
Strain	**mean #YFP foci**	**SD**	**n**	**significance**
Q40::YFP (AM141) (r1)	7.9	4.4	25	b
Q40::YFP (AM141) (r2)	9.3	3.6	25	b
Q40::YFP (AM141) (r3)	8.5	3.8	20	b

SUPPLEMENTAL TABLE B3. SUMMARY OF EXPERIMENTS IN FIGURE 2.2B. Statistical comparisons between animals exposed to hypoxia ('Hypoxia fed' and 'Hypoxia fasted') and controls maintained in room air ('Room air'). The first row in each condition represents data from the first biological replicate (r1), the second row in each condition represents data from the second biological replicate (r2), and the third row in each condition represents data from the third biological replicate (r3). 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

	Room air			Hypoxia fed			Hypoxia fasted		
Hours post hypoxia	mean #YFP foci	SD	n	mean #YFP foci	SD	n	mean #YFP foci	SD	n
0	1.4	0.8	20	6.65	3.0	20	4	2.1	20
19	5.9	2.3	20	20	4.5	20	6.65	2.3	20
25	8.15	2.6	20	30.85	9.0	20	7.65	2.7	20
42	13.7	2.9	20	37.6	7.3	20	10.65	2.5	20
50	19.6	2.8	20	48.9	5.9	20	12.8	3.0	20
71	27.9	5.0	20	49.9	7.2	20	17.7	4.0	20
97	38.0	3.9	20	58.2	5.0	20	29.5	5.0	20
124	52.3	4.3	20	61.3	6.2	20	42.5	3.8	20
152	57.5	5.0	20	61.1	5.9	20	51.8	3.4	20

	Room air			Hypoxia fed			Hypoxia fasted		
Hours post hypoxia	mean #YFP foci	SD	n	mean #YFP foci	SD	n	mean #YFP foci	SD	n
0	1.72	1.2	25	1.4	1.3	25	2.2	1.2	25
28	8.5	2.0	25	26.6	12.8	25	10.2	3.2	25
49	28.4	4.5	25	40.9	8.3	25	24.6	3.8	25

	Room air			Hypoxia fed			Hypoxia fasted		
Hours post hypoxia	mean #YFP foci	SD	n	mean #YFP foci	SD	n	mean #YFP foci	SD	n
0	1.4	0.8	20	6.65	3.0	20	4	2.1	20
8	5.9	2.3	20	20	4.5	20	6.65	2.3	20
24	8.15	2.6	20	30.85	9.0	20	7.65	2.7	20
48	13.7	2.9	20	37.6	7.3	20	10.65	2.5	20

SUPPLEMENTAL TABLE B4. SUMMARY OF EXPERIMENTS IN FIGURE 2.3

Strain	Condition (24 hours at L1)	Median onset of paralysis (days)	Hazard ratio (Mantel-Haenszel)	n	p-value
Q40::YFP(*rmIs133*)	Room air	7		69	
Q40::YFP(*rmIs133*)	Hypoxia fed	7	0.30	107	****
Q40::YFP(*rmIs133*)	Hypoxia fasted	6	9.58	78	**
Q40::YFP(*rmIs133*)	Room air	8		87	
Q40::YFP(*rmIs133*)	Hypoxia fed	8	0.37	79	****
Q40::YFP(*rmIs133*)	Hypoxia fasted	8	0.46	98	****
Q40::YFP(*rmIs133*)	Room air	9		82	
Q40::YFP(*rmIs133*)	Hypoxia fed	7	0.24	98	****
Q40::YFP(*rmIs133*)	Hypoxia fasted	8	0.36	81	****

SUPPLEMENTAL TABLE B5. SUMMARY OF EXPERIMENTS IN FIGURE 2.4A. Statistical comparisons made against room air controls. p-value: ns > 0.05, * < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001

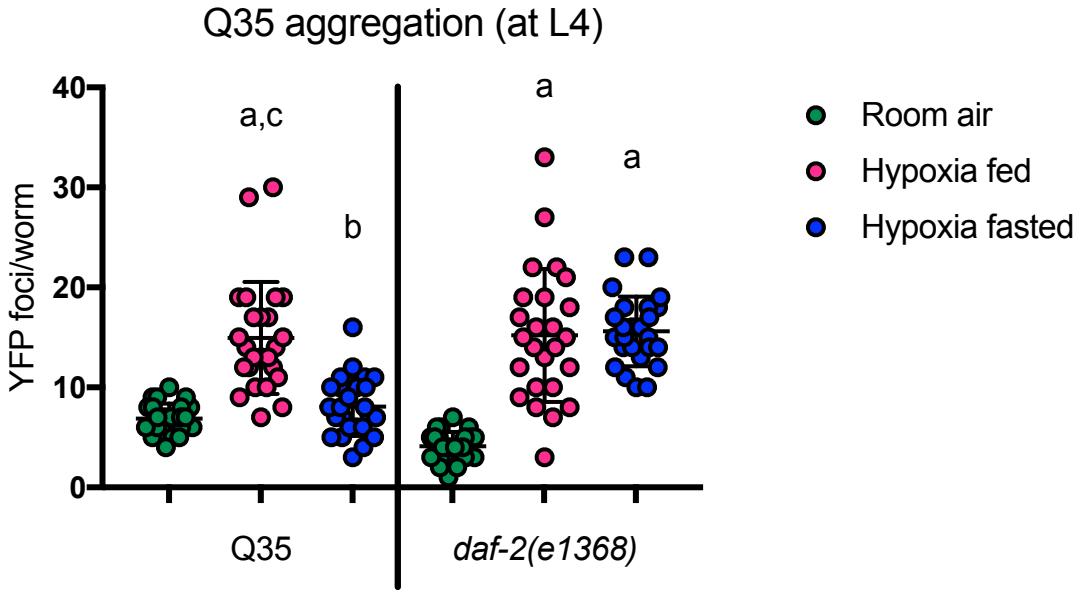
Strain	Condition (24 hours at L4)	Median onset of paralysis (days)	Hazard ratio (Mantel-Haenszel)	n	p-value
$A\beta_{1-42}$(*dvIs2*)	Room air	11		27	
$A\beta_{1-42}$(*dvIs2*)	Hypoxia fed	7	0.35	31	***
$A\beta_{1-42}$(*dvIs2*)	Hypoxia fasted	9	0.80	33	ns
$A\beta_{1-42}$(*dvIs2*)	Room air	8		37	
$A\beta_{1-42}$(*dvIs2*)	Hypoxia fed	6	0.52	54	**
$A\beta_{1-42}$(*dvIs2*)	Hypoxia fasted	7	0.87	40	ns
$A\beta_{1-42}$(*dvIs2*)	Room air	8		37	
$A\beta_{1-42}$(*dvIs2*)	Hypoxia fed	6	0.41	52	***
$A\beta_{1-42}$(*dvIs2*)	Hypoxia fasted	8	0.85	39	ns

SUPPLEMENTAL TABLE B6. SUMMARY OF EXPERIMENTS IN FIGURE 2.4B. Statistical comparisons made against room air controls. p-value: ns > 0.05, * < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001

	Room air			
Minutes	**% outside of circle**	**SD**	**n**	**p-value**
1	41.1	16.5	30	
2	51.1	25.3	30	
3	58.1	19.1	30	
4	72.2	6.9	30	
5	82.2	16.8	30	
	Hypoxia fed			
Minutes	**% outside of circle**	**SD**	**n**	**p-value**
1	0	3.0	30	
2	0	3.0	30	
3	3.3	5.8	30	***
4	3.3	5.8	30	
5	3.3	5.8	30	
	Hypoxia fasted			
Minutes	**% outside of circle**	**SD**	**n**	**p-value**
1	10	0.0	30	
2	16.7	5.8	30	
3	26.7	11.5	30	**
4	26.7	11.5	30	
5	30.0	10.0	30	
	Restrictive temperature			
Minutes	**% outside of circle**	**SD**	**n**	**p-value**
1	0	0.0	30	
2	0	0.0	30	
3	0	0.0	30	***
4	0	0.0	30	
5	0.0	0.0	30	

SUPPLEMENTAL TABLE B7. SUMMARY OF EXPERIMENTS IN FIGURE 2.4C. Statistical comparisons made against room air controls. p-value: ns > 0.05, * < 0.05, ** < 0.01, *** < 0.001, **** < 0.0001

APPENDIX C: SUPPLEMENTAL MATERIAL FOR CHAPTER 3

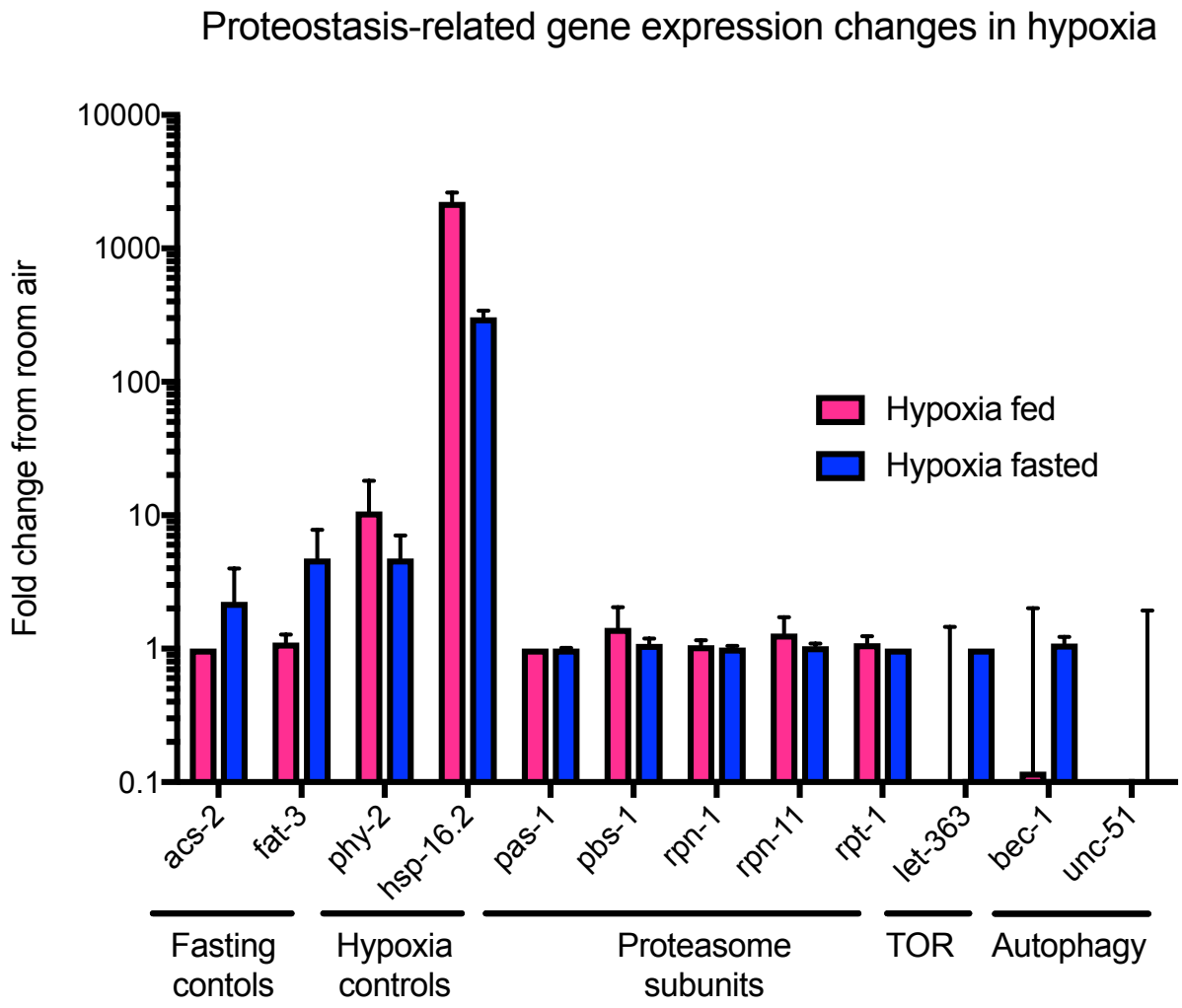


SUPPLEMENTAL FIGURE C1 FASTING DOES NOT PROTECT *DAF-2(E1368)* MUTANTS AGAINST HIPA. Aggregation measurements (F=6h, H=24h) for L4 daf-2(e1368) Q35::YFP animals. Animals were maintained on food in room air (green), were exposed to hypoxia on food (magenta), or were exposed to hypoxia after removal of food (blue). Each circle is the number of YFP foci in a single animal. The mean in indicated by the line, error bars are the standard deviation. Data from one representative experiment is shown. Each cohort included at least 20 animals, and the experiment was repeated at least 3 times. Significance was calculated using a Kruskal-Wallis test and Dunn's multiple comparisons post hoc analysis. Signficant differences ($p<0.05$) in aggregation for a given strain 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 controls.

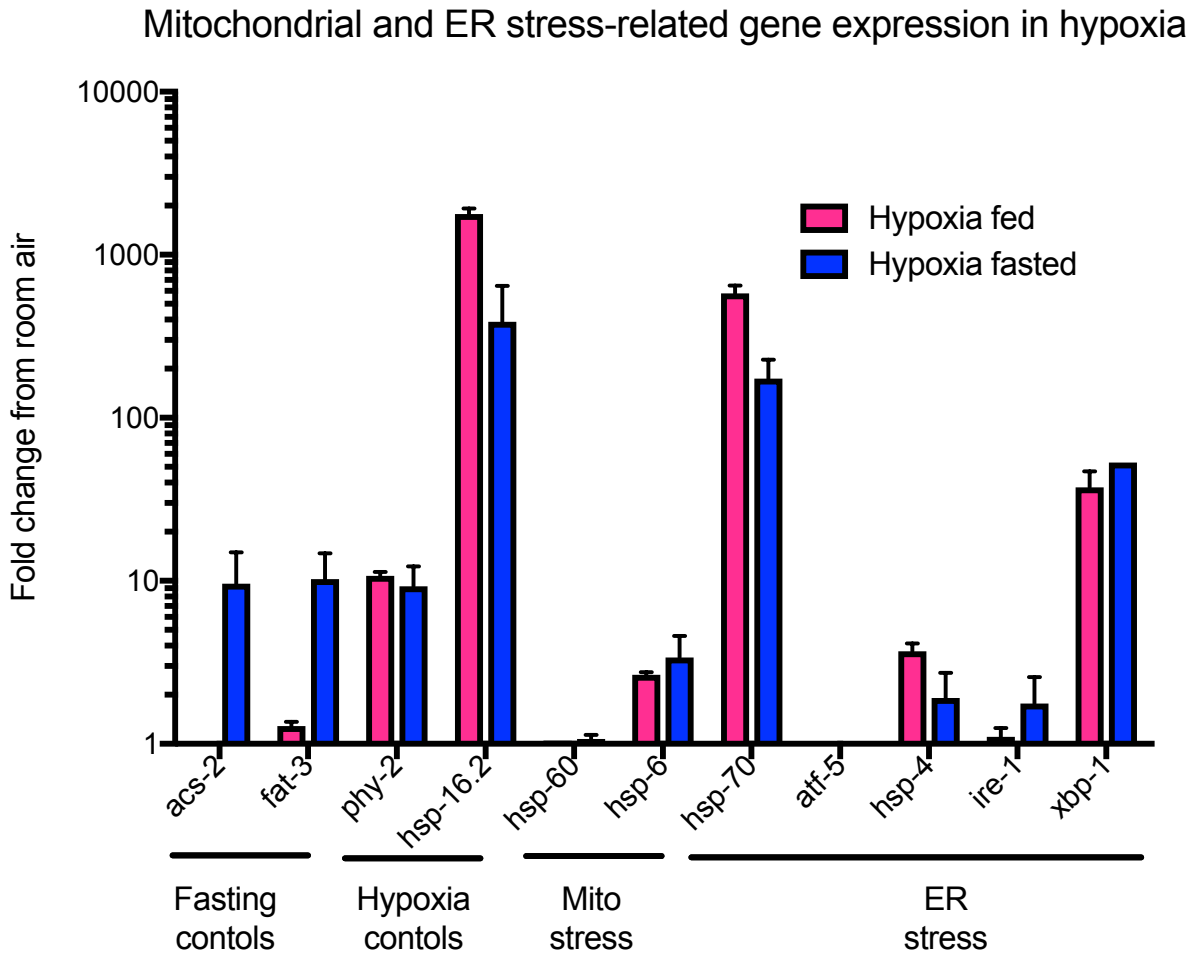
	Room air			
Strain	**mean #YFP foci**	**SD**	**n**	
Q35::YFP (AM140)	2.5	1.1	20	
Q35::YFP (AM140)	2.2	1.6	20	
Q35::YFP (AM140))	1.7	1.3	20	
daf-2(e1370) Q35::YFP	7.6	1.0	20	
daf-2(e1370) Q35::YFP	4.8	1.6	20	
daf-2(e1370) Q35::YFP	7.5	2.4	20	
daf-16(mu86) Q35::YFP	3.6	0.8	20	
daf-16(mu86) Q35::YFP	4.1	1.9	20	
daf-16(mu86) Q35::YFP	0.6	0.8	20	
daf-2(e1370); daf-16(mu86) Q35::YFP	5.1	1.5	20	
daf-2(e1370); daf-16(mu86) Q35::YFP	3.5	1.6	20	
daf-2(e1370); daf-16(mu86) Q35::YFP	2.5	1.3	20	
	Hypoxia fed			
Strain	**mean #YFP foci**	**SD**	**n**	**p-value**
Q35::YFP (AM140)	11.1	3.0	20	a, c
Q35::YFP (AM140)	18.9	8.5	20	a, c
Q35::YFP (AM140))	19.9	6.5	20	a, c
daf-2(e1370) Q35::YFP	23.6	6.3	20	a
daf-2(e1370) Q35::YFP	28.0	7.8	20	a
daf-2(e1370) Q35::YFP	22.1	7.7	20	a
daf-16(mu86) Q35::YFP	11.1	3.2	20	a, c
daf-16(mu86) Q35::YFP	16.8	7.4	20	a, c
daf-16(mu86) Q35::YFP	16.3	7.2	20	a, c
daf-2(e1370); daf-16(mu86) Q35::YFP	25.2	8.8	20	a
daf-2(e1370); daf-16(mu86) Q35::YFP	29.4	9.6	20	a
daf-2(e1370); daf-16(mu86) Q35::YFP	21.2	5.5	20	a
	Hypoxia fasted			
Strain	**mean #YFP foci**	**SD**	**n**	**p-value**
Q35::YFP (AM140)	3.7	1.5	20	b
Q35::YFP (AM140)	6.5	1.9	20	a, b
Q35::YFP (AM140))	6.1	2.0	20	b
daf-2(e1370) Q35::YFP	24.3	5.6	20	a
daf-2(e1370) Q35::YFP	27.4	6.0	20	a
daf-2(e1370) Q35::YFP	22.2	6.8	20	a
daf-16(mu86) Q35::YFP	3.8	1.5	20	b
daf-16(mu86) Q35::YFP	5.7	2.2	20	b
daf-16(mu86) Q35::YFP	3.8	1.3	20	b
daf-2(e1370); daf-16(mu86) Q35::YFP	16.4	4.3	20	a
daf-2(e1370); daf-16(mu86) Q35::YFP	29.9	6.5	20	a
daf-2(e1370); daf-16(mu86) Q35::YFP	20.5	4.7	20	a

SUPPLEMENTAL TABLE C1. SUMMARY OF EXPERIMENTS IN FIGURE 3.2. Statistical comparisons between animals exposed to hypoxia ('Hypoxia fed' and 'Hypoxia fasted') and controls maintained in room air ('Room air'). 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

APPENDIX D: SUPPLEMENTAL MATERIAL FOR CHAPTER 4



SUPPLEMENTAL FIGURE D1 EXPOSURE TO HYPOXIA IN THE FASTED OR FED STATE DOES NOT RESULT IN THE UPREGULATION OF GENES INVOLVED IN PROTEOSTASIS MAINTENANCE. qRT-PCR analysis of genes commonly identified as upregulated in response to hypoxia and fasting, as well as proteostasis maintenance. ΔΔCt were calculated as described in (Miller *et al*. 2011). Upregulated hypoxia controls were selected from microarray data published in (Shen *et al*. 2005). Upregulated fasting controls were selected from genes published in (Van Gilst *et al*. 2005). 9,000 synchronized L4 animals were exposed to hypoxia for 24 hours and harvested into Trizol.



SUPPLEMENTAL FIGURE D2 EXPOSURE TO HYPOXIA IN THE FASTED OR FED STATE DOES NOT RESULT DIFFERENTIALLY UPREGULATE MITOCHONDRIAL OR ENDOPLASMIC RETICULUM STRESS GENES. qRT-PCR analysis of genes commonly identified as upregulated in response to hypoxia and fasting, as well as genes involved in the mitochondrial and endoplasmic reticulum stress responses. ΔΔCt were calculated as described in (Miller *et al*. 2011). Upregulated hypoxia controls were selected from microarray data published in (Shen *et al*. 2005). Upregulated fasting controls were selected from genes published in (Van Gilst *et al*. 2005). 9,000 synchronized L4 animals were exposed to hypoxia for 24 hours and harvested into Trizol.

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