Chapter 9
Stress Response Pathways

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Abstract Physiological stress occurs when conditions perturb homeostasis. There are a multitude of stressors commonly encountered by organisms, including environmental factors such as temperature, pathogens, toxins, and food or oxygen availability, or internal disturbances caused by genetic defects or damage accumulated over the course of ageing. In this chapter, we discuss the fundamental relationships between stress and homeostasis. We then focus on various stress response strategies and highlight established molecular genetic stress response pathways. Many experiments, particularly those in *C. elegans*, have highlighted the intimate relationship between stress resistance and longevity. As such, a deeper understanding of the fundamental nature of stress and homeostatic stress responses is essential to fully appreciate the causes and consequences of ageing in animals.

Keywords *C. elegans* • Ageing • Stress response • Oxidative stress • Thermal stress • Hypoxia • Protein folding stress • Lifespan

9.1 Introduction

In order to consider stress responses, it is important to first understand the concept of stress. In biology, stress is sometimes not clearly defined. For instance, Selye suggested that stress is a situation that elicits a stress-response (in [1]). From this standpoint, the word stress can be used to describe emotional or psychological states, injury, illness, and even normal developmental processes. Physiological stress, which is the focus of this chapter, can be understood by analogy with mechanical stress [1]. In this framework, stress is an applied force, and strain is the deformation resulting from the application of the stress force. As stress force is applied, the system deforms, or is strained. In biological systems, strain is the physiological effect of the stressor. Initially, the deformation is elastic – if the stress force is removed the system will return to its original form. However, as the stress force

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increases, the system yields and deformation becomes inelastic. Finally, application of even greater stress force results in failure, or rupture. This would be a lethal physiological stress.

Stress, defined as force(s) that perturb physiological systems, is a common occurrence. Forces that cause stress can derive from fluctuations in environmental conditions such as changes in temperature, food availability, pathogens, toxins, or oxygen availability. Stress can also be induced by changes in internal physiological conditions, which can result as a consequence of cellular dysfunction due to genetic mutations, illness, or injury. Even ageing may cause stress, as cellular processes become dysfunctional or less efficient. A general consequence of stress is to perturb homeostasis, defined by Cannon as the ability of organisms to maintain an internal equilibrium when external conditions are altered [2]. In Cannon's paradigm, the limits of homeostatic mechanisms are revealed at the point at which physiological stress results in failure (rupture), often manifest as cellular death or damage [3].

Stress response pathways can therefore be defined as cellular and organismal mechanisms to resist the effects of stress and/or restore homeostasis when conditions change. Biological stressors come in many flavours, such as thermal stress, oxidative stress, xenobiotic stress, proteotoxic stress, and osmotic stress. Each of these perturb the physiological system differently, and as such a variety of stress responses have evolved to defend homeostasis in these different conditions. In effect, stress response pathways highlight the weaknesses in physiological networks revealed by the application of stress. Understanding how stress-response pathways buttress cellular physiology to maintain homeostasis and ensure survival provides powerful insight into fundamental pro-survival mechanisms.

9.2 The Relationship(s) Between Stress Response Pathways and Ageing

A common feature of ageing is a progressive decline in the ability to survive stress. Older *C. elegans* are less resistant to heat stress, the superoxide-generating compounds paraquat and juglone, anoxia, osmotic stress, and pathogenic bacteria, but more resistant to UV [4–8]. One hypothesis is that ageing increases the basal level of stress being experienced, which decreases the ability to induce an additional response to external conditions. Arguing against this possibility, no increase in expression of eight different stress-responsive reporters was observed in older *C. elegans* in the absence of stress; in fact, the expression of several actually decreased as animals aged [6]. Instead, older animals exposed to stress were not able to effectively induce expression of stress response markers, including *hsp-6*, a marker of mitochondrial unfolded protein response (UPR), *hsp-4*, a marker of the ER UPR, *gst-4*, a glutathione S-transferase, *gcs-1*, the rate-limiting enzyme in glutathione

synthesis, or *sod-3*, a superoxide dismutase [6]. These results suggest there is a defect in engaging stress-responsive pathways in aged animals. The mechanistic basis of this defect is not yet understood, though several possibilities have been proposed. One possibility is that the transcription factors required to direct stress responses are poorly expressed in older animals. Alternatively, it could be that altered chromatin structure blunts the ability to induce stress response genes, or that ageing results in the dysregulation of transcriptional networks that interfere with normal stress responses.

It was recognized early that animals with increased lifespan were resistant to a variety of stresses. The first long-lived mutants cloned, *age-1*, *daf-2*, and *spe-26*, were found to be resistant to oxidative stress and/or thermal stress [9, 10], and 88 of 160 RNAi gene inactivations that increase lifespan induce expression of at least one stress-responsive reporter [11]. The converse is also true, as long-lived mutants were enriched in screens that selected mutations that conferred increased resistance to heat stress [12–14] or juglone [15]. There is also functional correlation between stress resistance and longevity. Loss-of-function mutation in the *hsf-1* gene, a key transcription factor for inducing heat-shock proteins, reduces the increased lifespan of *daf-2* mutants, dietary restricted adults, and mutations in the target of rapamycin (TOR) kinase pathway [16–19]. Moreover, RNAi knockdown of genes required for expression of stress-responsive reporters reduces the lifespan of animals long-lived as a result of mitochondrial dysfunction, decreased insulin signalling, and/or reduced feeding [11]. Thus, there is significant overlap in genetic perturbations that increase lifespan and those that enhance cytoprotective stress-response mechanisms.

The correlation between increased longevity and stress resistance suggests the possibility that activation of stress-response pathways is a key aspect of increased lifespan. This is consistent with the somatic maintenance theory of lifespan [20]. However, the correlation between lifespan and stress resistance is not absolute. Many mutations that increase resistance to thermal or oxidative stress do not increase lifespan [9, 15, 21]. Even in genetic backgrounds that are long-lived and stress resistant the correlation is not simple. For example, when comparing the various *daf-2* mutant alleles, the magnitude of increase in lifespan is not perfectly correlated with the increase in stress resistance [22]. These data suggest that although stress response pathways are necessary in some situations for increased lifespan, activation of these pathways is not sufficient to increase lifespan. This interpretation is complicated, however, by the fact that simply overexpressing stress-responsive transcription factors such as *hif-1*, *daf-16*, or *hsf-1* alone can increase lifespan [19, 23–27]. Moreover, though long-lived mutant animals are generally resistant to at least one type of stress, the specific spectrum of *which* stress responses are enhanced varies between different mutant strains. This could indicate that there are multiple mechanisms by which stress response pathways contribute to lifespan. A better understanding of how different stresses perturb cellular physiology is necessary to reveal the mechanistic relationship between lifespan and stress biology.

9.3 Stress Response Strategies

9.3.1 Avoidance

When animals encounter stressful conditions, a common response is to attempt escape. *C. elegans* have aversive behavioural responses to a variety of stimuli associated with physiological stress, including hypoxia [28], hyperoxia [29], high temperature [30], pathogenic bacteria [31], and ultraviolet light [32]. It has also been observed that animals tend to avoid the food when they are grown on RNAi of essential genes that, when depleted, extend lifespan [33]. These authors suggest that the animals perceive the internal disturbances from the RNAi as indicative of environmental stress, which the animals attempt to avoid.

When it is impossible to avoid the physical location of stress, animals can instead attempt to temporally avoid the stress by entering into a quiescent, often stress-resistant state. The best-known example of this in *C. elegans* is the dauer larval state. Dauer is an alternative third larval stage that is entered when animals experience high temperature, crowding, and food restriction. Dauer larvae can persist for months until conditions improve, and are resistant to a variety of environmental stresses [34]. The decision to enter dauer is regulated by the insulin signalling pathway and TGF-β signalling, which converge on the DAF-12 nuclear hormone receptor (NHR). The dauer is quiescent in that development is paused, and metabolic rate is lowered [35]. Arrest of germline development in dauer requires the AMP-activated kinase (AMPK) *aak-2* and *daf-18*/PTEN [36]. Dauer larvae are motile and exhibit nictation behaviour, where the animal stands on its tail and waves its head in the air, which is believed to improve dispersal [37]. Thus, dauer larvae are adapted to avoid harsh conditions in both time and space. Further discussion of the dauer larva and ageing can be found in Chap. 3.

Diapause is another common strategy that allows *C. elegans* to arrest development while facilitating dispersal in bad conditions, thereby providing for temporal avoidance of stressful condition. In diapause, development and/or reproduction reversibly arrest but the animals remain otherwise animated. In contrast to dauer, developmental trajectory is not altered, simply delayed. There are three characterized diapause states in *C. elegans*: the L1 diapause, the adult reproductive diapause, and hypoxia-induced diapause. When *C. elegans* hatch in the absence of food, they arrest as L1 that are stress-resistant and can survive for long periods [38]. Like dauer larvae, arrested L1 are resistant to environmental stresses. The arrest of post-embryonic development in L1 diapause is mediated by insulin signalling, which represses TGF-β and DAF-12/NHR signalling [39]. TOR kinase and AMP-activated kinase (AMPK) also play important roles in coordinating metabolism in the L1 diapause [40–42].

The adult reproductive diapause (ARD) is another diapause induced by restricted food availability. The germline of *C. elegans* is exquisitely sensitive to nutrient status, likely due to the fact that progeny production is a huge energetic burden.

Germline development and adult germline proliferation are regulated by both insulin signalling and TOR kinase signalling [43]. Similar to L1 diapause and dauer, when animals are removed from food, germ cells arrest mitotic cycling and meiotic progression halts [44]. An extreme example of this is ARD, in which *C. elegans* deprived of food during the fourth larval stage (L4) enter into a reproductively quiescent state [45, 46]. In these animals, oocyte production is arrested and the germline is degraded, save for a set of protected germline stem cells, and must be regenerated upon refeeding. The NHR *nhr-47* is required to establish ARD [45]. Animals can survive for several weeks in this diapause, suggesting that this state may also promote dispersal. ARD is not engaged in adults; instead, if adult hermaphrodites are removed from food, a behavioural response arrests egg-laying, and embryos held in the uterus continue to develop and hatch – the "bag of worms" phenotype [47]. This facultative vivipary can improve dispersal, as progeny that hatch into conditions with little food will arrest as L1 or dauer [48].

Similar to the situation with starvation, hypoxia (reduced O_2 availability) can also induce a diapause state in *C. elegans. C. elegans* can continue development and reproduction in as little as 5,000 ppm O_2 [49]. However, when exposed to 1,000 ppm O_2 postembryonic development and reproduction reversibly arrest, though animals remain motile [50]. Animals with mutations in the hypoxia-inducible transcription factor, *hif-1*, or *aak-2*/AMPK precociously enter diapause in 5,000 ppm O_2, suggesting that these factors act normally to promote development and reproduction. Unlike ARD, *C. elegans* enter hypoxia-induced diapause at any point during the life cycle. Factors required for developmental arrest in L1 and/or dauer (*daf-16*, *daf-18*) are not required for hypoxia-induced developmental arrest [50]. Embryos exposed to 1,000 ppm O_2 in utero survive, whereas embryos exposed directly to this hypoxic environment die [49, 50]. The embryos in utero enter suspended animation, where cell division and development reversibly arrests, as evidenced by the requirement of the spindle checkpoint protein *san-1* [51]. Suspended animation can also be induced by anoxia (operationally defined as <10 ppm O_2). In suspended animation, all observable cellular activities reversibly arrest including embryonic and postembryonic cell divisions, development, feeding, and movement. The insulin pathway, AMPK, glycogen storage, and glycolytic activity are required for adult *C. elegans* to survive long periods of suspended animation [7, 52–54].

The common theme for all the above avoidance strategies is quiescence. The mechanistic link between quiescence and stress resistance is highly conserved, though not well understood. For example, over 100 different mammalian species hibernate to avoid harsh winter conditions and lack of food [55]. In addition to these stresses, hibernating mammals are resistant to injury and illness. *C. elegans* embryos in suspended animation survive otherwise lethal cold exposure [56]. The stress response pathways that mediate the arrest and recovery from quiescent states are highly enriched for genes that mediate longevity, suggesting a fundamental link between these biological processes.

9.3.2 Anticipation

In some situations, animals can use prior experience or environmental cues to predict upcoming stress, enabling a more rapid and robust response to the imminent changes. A well-studied example of this strategy is preconditioning. In preconditioning a relatively mild stress improves survival upon exposure to a more extreme stress. Hypoxic preconditioning is a well-known example conserved in mammals, where a mild, non-lethal exposure to reduced oxygen availability improves survival upon subsequent exposure to more severe hypoxia. Similarly, *C. elegans* exposed to atmospheres without oxygen (anoxia), for a short time can survive subsequent exposure to long periods of anoxia better than unpreconditioned controls [57]. The effects of hypoxic preconditioning are relatively short-lived, and the mechanistic basis that confers this protection is not clear. However, mutations in the apoptosis factor, *ced-4*, but not other cell death genes, prevent hypoxic preconditioning in these experiments [57]. Hyperosmotic stress can also stimulate a preconditioning response, where animals produce glycerol from glycogen and are resistant to subsequent osmotic stress [58]. Adults exposed to hyperosmotic stress also package glycerol into embryos, which are then resistant to osmotic stress [53]. This maternal protective effect requires insulin signalling, as *daf-2*(*e1370*) mutant animals do not increase glycerol/trehalose provisioning to embryos, which are thereby sensitive to osmotic stress [53]. Preconditioning also improves resistance to heat stress in *C. elegans*, as a short heat-shock increases survival upon subsequent exposure to high temperature [59]. In heat shock, the preconditioning advantage is correlated with the expression of heat-shock proteins and requires the heat-shock transcription factor *hsf-1*. This is an example of hormesis – where induction of a stress response persists even after the stress is removed. The ability for sub-lethal stresses to increase resistance to subsequent stress conditions is highly conserved [60–63].

Epigenetic effects can also enable animals to anticipate stressors to survive, by facilitating environmentally-induced gene expression, and may contribute to effects on lifespan. DNA methylation is not a major source of epigenetic effects, as *C. elegans* do not possess cytosine methyltransferase enzymes; however, adenosine methylation has been recently discovered in *C. elegans*, and it does interact with fertility defects of the histone 3 at lysine 4 (H3K4) demethylase *spr-5* [64]. Instead, epigenetic effects are largely mediated by effects on histone proteins. Loss of function of the ASH-2/trithorax complex, which acts to methylate H3K4, and the H3K4 demethylase RBR-2 increases lifespan in *C. elegans* in a germline dependent manner [65], whereas RNAi of *utx-1*, a H3K27me3 demethylase, increases lifespan in a germline independent manner [66]. It is not clear how stress response pathways are influenced by these epigenetic factors. However, the effects of loss-of-function of the ASH-2 complex on lifespan persist transgenerationally [67], suggesting that information about the environment can be stored and transmitted. The DAF-16 transcription factor has been shown to physically associate with the SWI/SNF chromatin remodelling complex, and this interaction facilitates efficient transcription of *daf-16* target genes in *daf-2* mutant animals [68]. We have recently discovered that

SWI/SNF is also important to maintain an epigenetic memory of exposure to hydrogen sulphide (Fawcett and Miller, unpublished), which increases lifespan and thermotolerance [69].

9.3.3 Compensate or Defend

When stress cannot be avoided or anticipated, stress response pathways are invoked to correct the physiological disturbance and return the system to homeostasis. In each instance, a stress must be sensed and then a physiological response mounted to counteract or correct the damage and restore homeostasis. In the next section we consider a few examples of well-characterized stress responses to highlight these aspects of stress response mechanisms.

9.4 Stress Response Pathways in Action

Although stress response pathways facilitate survival in changing conditions, it is important that these pathways are activated only when stress is encountered. Constitutive activation of stress response pathways can be detrimental, and stress responses are repressed during some developmental stages [70]. For example, inactivation of *daf-2*, the insulin-like receptor, increases stress resistance but also leads to L1 arrest and entry into dauer, and can reduce the rate of reproduction, which would severely reduce fitness. Moreover, some stress response pathways also have important roles in normal development [71–74]. However, when stress is encountered these pathways must be robustly induced. As a result, stress response pathways must be dynamically regulated, often at multiple levels. Although for this discussion we treat different stresses independently, there is much cross-talk and overlap between these stress response pathways. Understanding the molecular basis for interactions between diverse stressors is an exciting area of emerging research.

9.4.1 Thermal Stress

The temperature at which *C. elegans* are raised, similar to many poikilotherm organisms, has a large effect on the ultimate lifespan of the animal. *C. elegans* can be cultured in the lab across a broad range of temperatures from approximately 10–25 °C with a lifespan that is inversely correlated with temperature [8]. At lower temperatures metabolic rate is decreased, leading to the hypothesis that the increased lifespan is simply a “slowing” of normal life processes [75, 76]. However, the lifespan effects of temperature are not just governed by thermodynamics, as there are genetic components which influence the temperature dependence of *C. elegans*

lifespan. For example, the TRP channel, *trpa-1*, is necessary for extended lifespan at low temperatures as well as the decreased lifespan when larval animals are grown at low temperatures [77, 78]. Temperature can also modulate the effects of some lifespan-extending mutations. For example, mutations in the hypoxia-responsive transcription factor *hif-1* have different effects on lifespan at different temperatures [24].

C. elegans has neuronal mechanisms to sense temperature and coordinate behavioural responses to changes in temperature. *C. elegans* thermotax towards the temperature in which they have been cultivated with food and away from high, noxious temperatures [79, 80]. The bilateral AFD amphid neurons are the main thermosensory neurons in *C. elegans*. AFD neurons are activated by both increases and decreases in temperature as small as 0.05 °C [81, 82]. Although AFD is involved in both thermotaxis and the avoidance of noxious temperature (thermonociception), different neural circuits mediate these two behaviours. In thermonociception, AFD neurons activate AIB interneurons, which connect to AFD by electrical junctions, resulting in initiation of backward movement [80]. In thermotaxis, AFD activates a neural circuit including AIY and AIZ interneurons to direct movement towards the preferred temperature [83].

Heat stress impinges on many aspects of cellular physiology. One consequence of thermal stress that contributes to activation of the heat-shock response is the accumulation of unfolded or misfolded proteins [84]. Thermal stress can also induce formation of reactive oxygen species, leading to oxidative damage of cellular components [85–87]. For example, protein carbonylation, an oxidative modification, is enhanced by thermal stress, perhaps because unfolded proteins are more accessible to modification by reactive oxygen species [88]. Upon exposure to thermal stress, cells activate the heat-shock response, which leads to increased expression of heat shock proteins (HSPs) to defend against damage induced by thermal stress. Many HSPs are molecular chaperones, which help to maintain proper protein folding, or promote degradation of damaged proteins (reviewed in [89, 90]).

At the cellular level, the highly conserved HSF-1 transcription factor mediates transcriptional responses to thermal stress to activate the heat-shock response [72]. In non-stressed conditions, HSF-1 binds to HSP-90 and sequesters it in the cytoplasm. Upon heat shock, cellular proteins become unstable and partially unfolded, and these misfolded or unfolded proteins become clients for the protein chaperone HSP-90 (DAF-21 in *C. elegans*), and compete for HSP-90 binding [91, 92]. As a result, HSF-1 is released, transits to the nucleus, and trimerizes. These active HSF-1 trimers bind to heat shock elements in the genome, inducing expression of HSPs. In addition, HSP-70 and HSP-40, two HSF-1 targets, act in a negative feedback loop of HSF-1 activity, binding to HSF-1 and decreasing its activity [93, 94].

Several lines of evidence suggest that HSF-1 modulates organismal ageing. Overexpression of HSF-1 increases lifespan and stress resistance [19], whereas knockdown of *hsf-1* leads to progeroid phenotypes [95]. Moreover, *hsf-1* is required for increased lifespan by reduced insulin/IGF-like signalling and at least one model of dietary restriction [16, 18, 19]. Finally, sublethal heat stress itself can increase

lifespan [9, 96, 97]. The effect of *hsf-1* activation on lifespan is likely a result of increased HSP expression. Overexpression of HSP-16.2 or the chaperone HSP-70 is sufficient to extend lifespan in *C. elegans* [98, 99]. Moreover, variations in expression of the small HSP *hsp-16.2* predict lifespan in an isogenic, wild-type population, with animals that express higher levels living longer [100].

All cells must respond to heat stress, and *hsf-1* is expressed in most, if not all, cells. However, there are also central regulators of the organismal response to thermal stress suggesting that this cellular stress response is not autonomous. Expression of HSPs in response to thermal stress in somatic cells can be blunted in *C. elegans* by ablation of thermosensory AFD neurons [101]. Similarly, ablation of AFD further reduces lifespan at higher temperature [102], suggesting that the thermosensory neurons play a role in integrating the heat-shock response and lifespan. Expression of HSF-1 in neurons increases both lifespan and thermotolerance, but the effects of lifespan require DAF-16 in the periphery whereas increased resistance to thermal stress depends only upon activation of HSF-1 [103]. These experiments indicate that the thermosensory neurons regulate multiple downstream activities to integrate organism-wide responses to different environmental stresses.

9.4.2 Oxidative Stress

Organisms must maintain appropriate redox balance in cells, as this is essential for the oxidation-reduction reactions necessary to maintain life. This involves keeping the cytoplasm reducing, while performing oxidative protein folding in the endoplasmic reticulum. *C. elegans* must accomplish this in a very oxidizing environment, as every cell is exposed to the gaseous environment [104]. The main source of oxidative stress for *C. elegans* in nature is most likely fluctuations in environmental O_2. In the natural environment of *C. elegans*, rotting fruit and compost [105], O_2 levels can fluctuate from normoxia, which we define as 21 % O_2 for the purposes of this discussion, to near anoxia (operationally defined as less than 10 ppm O_2).

9.4.2.1 Environmental Oxidative Stressors

C. elegans avoid both hypoxia (low oxygen) and hyperoxia (high oxygen) [28, 29]. In an O_2 gradient, *C. elegans* migrate to 5–12 % O_2, depending on the steepness of the gradient [29]. At this concentration of O_2, normal aerobic metabolism is maintained [106]. The aerotaxis behaviour to avoid higher O_2 requires the soluble guanylyl cyclase, GCY-35, in the URX, AQR, and PQR sensory neurons and the cGMP-gated TAX-2/TAX-4 channel [29]. URX is activated by increases in O_2 concentration, and is required for slowing and reversal responses to increasing O_2 concentration [107]. URX is not required for behavioural responses to O_2 downshifts, from 21 % to 10 %. Instead, the BAG sensory neurons are activated by O_2

downshifts and are required for increased locomotion and reversals [107]. Different soluble guanylyl cyclases are required for evoked calcium currents upon upshift or downshift of O_2 [107]. Interestingly, the neural circuits that coordinate aerotaxis are modified by prior experience and nutritional status [108–110], which suggests an integration between these distinct stress response modalities. The neural circuits that regulate hypoxia avoidance have not been delineated, but are distinct from the O_2-sensing neurons that mediate avoidance of high O_2.

C. elegans are incredibly tolerant to a broad range of O_2, from anoxia to 100 % O_2 [106], suggesting efficient mechanisms to respond and defend against oxidative damage. Oxidative stress generally causes cellular damage as a result of increased production of reactive oxygen or nitrogen species (ROS or RNS). ROS and RNS are able to oxidize key cellular components such as DNA, lipids, and proteins [111–113]. These damaged cellular components must then be degraded or repaired to maintain cellular function. During aerobic metabolism, up to 1–4 % of O_2 consumed by mitochondria is released as the ROS, superoxide (O_2^-) due to activity of complex III of the electron transport chain [114]. Although these endogenously produced ROS may be damaging, they are also an important cellular signalling molecule [115]. It is only when production of these ROS/RNS is excessive that they cause oxidative damage.

In addition to endogenously produced ROS, environmental toxins can also increase ROS and RNS. Exposure to heavy metals, such as lead, cadmium, chromium, and arsenite, is associated with cellular oxidative damage [116, 117], and UV and heat stress can also increase formation of ROS/RNS [85–87, 118]. Defects in iron homeostasis can also lead to formation of ROS, particularly the highly reactive hydroxyl radical, as a result of Fenton chemistry [118]. There are also several environmental toxins or poisons that increase ROS/RNS load, including the herbicides juglone and paraquat that are commonly used as experimental tools [119].

9.4.2.2 SKN-1/Nrf Coordinates a Transcriptional Response to Oxidative Stress

The primary cellular defence mechanisms against ROS and RNS involve the upregulation of detoxification enzymes. For detoxification of ROS, superoxide dismutase (SOD) converts O_2^- into H_2O_2, which is decomposed to water and O_2 by catalase. H_2O_2 and other peroxides can also be reduced by peroxidase enzymes. Other redox-active compounds encountered, such as xenobiotic compounds or environmental pollutants, are often detoxified by reduction by or conjugation to glutathione (GSH) or UDP-glucuronic acid [119, 120]. Glutathionylation is catalysed by glutathione-S-transferase enzymes (GSTs), and conjugation to UDP-glucuronic acid, or glucuronidation, is catalysed by UDP-glucuronosyltransferases (UGTs). The *C. elegans* genome includes five SOD genes, three catalase genes, 44 genes annotated as GSTs, two GSTK (kappa class) genes, three GSTO (omega class) genes, and 65 genes annotated as UGTs (www.wormbase.org, release WS252).

SKN-1, the *C. elegans* orthologue of mammalian Nrf transcription factors, is a key regulator of the transcriptional response to oxidative stress [121]. Like the mammalian Nrf proteins, SKN-1 has a CNC (cap-n-collar) domain next to a basic region in the DNA binding domain; however, SKN-1 binds to DNA as a monomer whereas Nrf proteins are dimers [122]. In addition to its role in oxidative stress responses, *skn-1* is essential during embryogenesis for specification of the EMS blastomere that gives rise to pharynx and gut [123]. During embryogenesis, SKN-1 is required for the expression of cell fate specification genes [124]. After embryogenesis, SKN-1 is constitutively expressed in the two ASI neurons and is stabilized and accumulates in the nuclei of intestinal cells in response to oxidative stress [125]. Many genes upregulated by SKN-1 are involved in oxidative stress detoxification and clearance, including GSTs, GSH synthesis enzymes, cytochrome P450, SOD and catalases, and UGTs, though the specific set of genes upregulated by SKN-1 depends on if oxidative stress is induced by chemicals (paraquat, tert-butyl hydrogen peroxide, arsenite) or high O_2 [126]. Importantly, even in non-stress conditions SKN-1 regulates the expression of many genes, which have diverse functions including metabolism, protein homeostasis, and detoxification [127]. The diverse transcriptional responses mediated by SKN-1 suggest that it cooperates with various other transcription factors. Consistent with this idea, the conserved mediator subunit MDT-15 is required for induction of some oxidative stress response genes [128].

The mechanism by which oxidative stress regulates SKN-1 activity is not understood, though many aspects of SKN-1 regulation have been established. In mammals, Nrf2 protein stability is regulated by Keap1, a redox-sensitive E3 ubiquitin ligase, such that oxidation of specific cysteine residues of Keap1 promote its association with the cullin Cul3 and promotes degradation of Nrf2 [129–131]. *C. elegans* does not have an orthologue of Keap1. However, SKN-1 protein is targeted for ubiquitination and degraded through interactions with the WD40 repeat protein WDR-23 [132]. Perhaps as a result of this, when the proteasome is disrupted SKN-1 protein accumulates and activates transcription of targets that include proteasome components [133, 134]. In this way, activation of SKN-1 can help to compensate for insults that disrupt proteasome function. Moreover, activation of the 20S proteasome by SKN-1 may also be important for removing oxidatively-damaged proteins [134, 135]. However, unlike Keap1, there is no published evidence that WDR-23 is directly regulated by oxidation.

Many genetic studies have revealed other mechanisms that regulate SKN-1 activity. SKN-1 is negatively regulated by binding to the nucleolar WD40 repeat protein, WDR-46 [136]. WDR-46 is homologous to yeast UTP7, and is involved in rRNA processing [136]. SKN-1 is activated when protein translation is inhibited or by disruption of rRNA processing; however, *cep-1*, the worm orthologue of p53, is only required in response to rRNA processing defects and not for increased SKN-1 activity when protein translation is reduced [133, 136, 137]. SKN-1 is also regulated by reversible protein phosphorylation. The p38 MAPK, PMK-1, phosphorylates SKN-1 leading to nuclear accumulation and activation of gene expression [138]. PMK-1 is activated in response to oxidative stress through the SEK-1/NSY-1 MAPK

cascade [138], but it is not clear if oxidative stress activates the MAPK pathway through a direct or indirect mechanism. An RNAi screen of kinases found four other kinases required for the nuclear accumulation of SKN-1 in response to oxidative stress from exposure to sodium azide: *nekl-2*, *ikke-1*, *mkk-4*, and *pdhk-2* [139]. Depletion of any of these kinases renders animals more sensitive to oxidative stress than wild-type but not as sensitive as *skn-1*(*RNAi*), suggesting redundant activation by these kinases [139]. SKN-1 is also negatively regulated by GSK-3, the glycogen synthase kinase orthologue. GSK-3 phosphorylates SKN-1 at a conserved serine residue and inhibits its nuclear localization and activity in response to oxidative stress [140]. This interaction also occurs in embryogenesis, where GSK-3 is required to inhibit the activity of SKN-1 in the C blastomere [141]. Phosphorylation by GSK-3 requires a priming phosphorylation by the p38 MAPK pathway [140]. This interaction between activating and inhibiting modifications could set a threshold to ensure that SKN-1 is not inappropriately activated.

Although SKN-1 is activated by high O_2, which is associated with increased oxidative damage [127, 142], the HIF-1 transcription factor is more important for adaptation to low O_2, or hypoxia. HIF-1 is the *C. elegans* orthologue of the hypoxia-inducible factor, a conserved bHLH-PAS domain transcription factor that mediates the transcriptional response to hypoxia in metazoans [143–147]. HIF-1 protein is degraded in the presence of O_2 as a result of modification by the EGL-9 prolyl hydroxylase and interaction with the VHL-1 E3 ubiquitin ligase [148]. HIF-1 activity is also increased by ROS/RNS and defects in mitochondrial function, though the mechanistic basis of this interaction is not well understood [27, 149].

9.4.2.3 Oxidative Stress Responses and Ageing

Oxidative stress has been predicted to contribute to ageing phenotypes since Harman proposed the Free Radical/Oxidative Damage Theory of Ageing, which suggests that accumulated oxidative damage resulting from ROS/RNS contributes to cellular dysfunction that drives ageing (reviewed in [150, 151]). Consistent with this idea, protein carbonylation, an oxidative modification, increases with age and is reduced in long-lived *age-1* mutant animals [142]. However, many more observations argue against a role for ROS/RNS or oxidative damage as a driver of ageing. Though many long-lived mutants have increased expression of SODs, the expression of SODs is not generally required for increased lifespan [152]. Moreover, increased lifespan in animals overexpressing SOD-1 is not correlated with reduced oxidative damage of proteins or lipids [153, 154]. Even simultaneous disruption of all five SOD genes does not reduce lifespan, though these animals are dramatically more sensitive to oxidative stress than wild-type controls [155]. Together, these results indicate that superoxide is not a major contributor to lifespan. In fact, low doses of paraquat or arsenite actually increase lifespan of *C. elegans* [27, 156]. Similar studies suggest that peroxides do not significantly drive the ageing process. Overexpression of catalase does not increase lifespan even when SOD-1 is also overexpressed [153], and although deletion of *ctl-2* shortens lifespan, protein

oxidation is actually slowed in *ctl-2* mutant animals [157]. Relatedly, mutations in the peroxiredoxin *prdx-2* shorten lifespan and *prdx-2* mutant animals are sensitive to oxidative stress from exposure to H_2O_2 or heat, but while expression of PRDX-2 increases resistance to oxidative stress it does not increase lifespan [158]. Together, these observations strongly argue against the idea that oxidative damage is a fundamental cause of ageing.

Despite the general lack of correlation between oxidative damage and lifespan, there is ample evidence that oxidative stress response pathways play a role in determining lifespan. However, the relationship between these stress response pathways and lifespan can be complicated. Both SKN-1 and HIF-1 have been shown to modulate lifespan, and also interact with many other longevity-associated mechanisms. It may be that ROS/RNS signalling, rather than oxidative damage, underlies these effects. Increased lifespan from mitochondrial dysfunction is associated with increased production of ROS/RNS and requires *hif-1* [27]. Increased ROS and activation of SKN-1 have also been suggested to contribute to lifespan in some models of dietary restriction [159]. *C. elegans* treated with 2-deoxyglucose, to inhibit glycolysis, are long-lived and this effect is reversed by treatment with the antioxidant N-acetylcysteine, suggesting that ROS/RNS may play a role in this situation [160]. Improved methodologies to measure the highly reactive ROS/RNS and more detailed examination of how changes in ROS/RNS are necessary to understand if there is a direct mechanistic link between ROS/RNS signalling and longevity.

HIF-1 can have both positive or negative effects on longevity (reviewed in [161]). Two groups showed that deletion of *hif-1* increases lifespan; however, the increased lifespan required *daf-16* in one study and was independent of *daf-16* in the other [23, 25]. Other studies have found that the same *hif-1* mutation does not change lifespan [162]. The discrepancies between these experiments could be a result of different assay conditions. Animals with mutations in *hif-1* are very sensitive to changes in environmental conditions. For example, *hif-1* mutants have defects in acclimating to changes in temperature [163], and *hif-1* mutants are long-lived at some temperatures but not others [24]. Animals with mutations in *hif-1* may also have altered responses to nutrient conditions, though this has not been clearly addressed experimentally. Activation of HIF-1 eliminates the effect of food on aerotaxis [164], and *hif-1* is important for increased lifespan in some models of dietary restriction [23]. Other studies have found that activation of *hif-1* can also increase lifespan. This is consistent with observations that hypoxia (less than 2 % O_2) can increase lifespan in a *hif-1*-dependent manner [165, 166]. In normoxia (21 % O_2) reduced function of VHL-1, the E3 ubiquitin ligase required for proteasomal degradation of HIF-1, increases lifespan [162, 167]. The increase in lifespan is related, in part, to increased expression of the *hif-1*-target gene *fmo-2* [168]. Similarly, overexpression of HIF-1 also increases lifespan [25]. In contrast, *egl-9* mutant animals are not long-lived, even though HIF-1 is stabilized and active [23, 25]. This could indicate that pleiotropic functions of *egl-9* counteract the effect of stabilized HIF-1 on lifespan. For example, in addition to its role in promoting degradation of HIF-1 protein, EGL-9 activity represses transcriptional activity of HIF-1 independent of

VHL-1 [169]. Another possibility is that in *egl-9* mutant animals the level of HIF-1 stabilization is so high as to be detrimental, or that there are isoform-specific effects.

In addition to its essential role in embryonic development, *skn-1* is required for normal lifespan. Both RNAi and loss-of-function mutations in *skn-1* decrease lifespan and render animals sensitive to oxidative stressors [125, 127, 138, 139, 170]. Overexpression of SKN-1 and gain-of-function mutations that disrupt the interaction with negative regulator *wdr-23*, or RNAi knockdown of *wdr-23* modestly increase lifespan [132, 171, 172]. However, other gain-of-function mutations in *skn-1* do not increase lifespan [173]. This could be a result of negative effects of too much SKN-1 activity, or effects of different isoforms. There are three distinct isoforms of SKN-1 that act in different tissues and are subject to distinct regulation. The B isoform of SKN-1 is constitutively expressed in the ASI sensory neurons, where it is required for increased mitochondrial respiration and lifespan in response to bacterial-dilution dietary restriction [174]. In the intestine, the A/C isoforms are stabilized in response to oxidative stress [125]. SKN-1 expression has also been detected in neurons, the pharynx, and other tissues, but it is not clear which isoforms are expressed in these tissues [156, 175, 176]. Activation of SKN-1 is also required for extended lifespan of *daf-2* mutants and by reduced TOR signalling [170, 171, 177]. In all of these situations, *skn-1* acts to increase lifespan. In contrast, overexpression of *skn-1* decreases lifespan in hypoxia [168]. Further work to unravel the diverse roles of *skn-1* in different tissues and different conditions is a ripe area of research to reveal novel aspects of how longevity is coordinated. Further discussion of the role of oxidative stress in ageing can be found in Chap. 10.

9.4.3 Protein Folding Stress

The proteostasis network coordinates protein metabolism, including synthesis, folding, quality control, and degradation. Defects in maintaining proteostasis can result in toxic protein aggregation, as is observed in a variety of devastating neurodegenerative diseases. Declining ability to maintain proteostasis occurs early in the ageing process [178]. Many different stresses can cause defects in proteostasis either as a result of inducing protein unfolding or misfolding, disrupting quality control mechanisms, or inhibiting protein degradation pathways. The unfolded protein response (UPR) is the cellular response to stresses that induce protein unfolding or misfolding. There are distinct UPR mechanisms for cytoplasmic proteins, secreted proteins, and mitochondrial proteins.

In the cytoplasm, unfolded proteins can be detected directly by stress response mechanisms, including the heat shock response (detailed above). When unfolded proteins accumulate in the cytoplasm, DAF-21/HSP-90 chaperone engagement with unfolded clients releases HSF-1 to enter the nucleus and induce gene transcription. Expression of the cytoplasmic UPR is also modulated by other transcription factors, though it is not clear whether these are directly responding to unfolded protein. Common cytoplasmic chaperones induced by protein folding stress are the

small HSPs of the HSP-16 family, which bind to unfolded client proteins to prevent aggregation and facilitate refolding. Expression of *hsp-16* is induced by a variety of environmental stressors, presumably due protein folding stress, including exposure to thermal stress, oxidative stress (exposure to hypoxia, juglone, or heavy metals), alcohol exposure, nicotine, pathogenic bacteria, dimethylsulfoxide, and expression of aggregation-prone proteins including human $A\beta_{1\text{-}42}$ or poly-glutamine protein [100, 179–187]. Expression of these and other HSPs help to counteract the effects of the stress and maintain cytoplasmic proteostasis.

For membrane and secreted proteins, folding initiates in the ER. The ER UPR, which is distinct from the cytoplasmic UPR, is activated by protein folding stress from disruptions of the folding environment of the ER or protein maturation and modifications in the Golgi. Defects in ER-associated degradation (ERAD), a quality control mechanism to remove and degrade proteins from the ER, can also lead to accumulation of misfolded proteins in the ER and induce the UPR [188]. In general, those cells that are highly secretory are most sensitive to ER folding stress [189]. Hypoxia induces the ER stress response [190], likely due to a defect in oxidative protein folding. Disulphide bond formation in the ER by ERO-1 requires molecular O_2, so in hypoxia these proteins can no longer fold correctly [191]. Reducing agents such as dithiothreitol (DTT) similarly cause ER folding stress and induce the UPR. Common chemicals produced by bacteria also induce the ER UPR, including tunicamycin, which inhibits N-linked protein glycosylation by GlcNAc phosphotransferase in the ER, thapsigargin, which inhibits SERCA and depletes the ER of calcium, and brefeldin A, which blocks transport of proteins from the ER to Golgi.

The proximal sensors of misfolded proteins in the ER are the transmembrane proteins IRE-1, PEK-1, the *C. elegans* PERK orthologue, and ATF-6. ATF-6 is most important for coordinating the constitutive UPR that is activated during normal development, whereas IRE-1 and PEK-1 are important for the inducible UPR [192]. These pathways are somewhat redundant, as animals with mutations in any one of these genes are viable, though sensitive to ER folding stress. However, double mutant animals die during development, often with severe gut atrophy, suggesting an inability to respond to normal ER stress during development [193, 194]. In unstressed conditions the IRE-1 and PEK-1 kinases bind as monomers to HSP-4/HSP-3, ER resident chaperones homologous to BiP, and are inactive. When unfolded proteins accumulate they are bound by HSP-4, freeing IRE-1 and PEK-1 to homooligomerize and leading to phosphorylation of cytoplasmic targets (reviewed in [195]). In addition to kinase activity, IRE-1 is an endoribonuclease which, when activated, removes a small intron from the *xbp-1* transcript [196]. The spliced *xbp-1* transcript is then efficiently translated, and increased production of the XBP-1 bZIP transcription factor induces expression of ER resident chaperones including *hsp-3* and *hsp-4* [192]. PEK-1 phosphorylates the translation initiation factor eIF2α, which inhibits translation initiation [194]. Together, activation of IRE-1 and PEK-1 reduces both protein folding load, by reducing the synthesis of new proteins, and improving protein folding capacity by increasing chaperone function.

Most mitochondrial proteins are encoded by the nuclear genome and translated in the cytoplasm. These proteins must then be imported into the mitochondria, folded, and then assembled into functional complexes. Any perturbation in mitochondrial protein import or complex assembly can cause protein folding stress in the mitochondria [197–199]. Two chaperones, *hsp-6* and *hsp-60*, are expressed in the mitochondria and induced upon protein folding stress [198]. These chaperones assist in import, folding, and assembly of mitochondrial protein complexes. There are at least two somewhat overlapping modes for activating the mitochondrial UPR (UPR^{MT}), one mediated by the ATFS-1 transcription factor and the other mediated by DVE-1/UBL-5. ATFS-1 is a bZIP transcription factor that has both a mitochondrial and nuclear localization signal [197]. In unstressed conditions, ATFS-1 is imported into the mitochondria and degraded by Lon protease [197]. When the UPR^{MT} is activated protein import into the mitochondria is disrupted by HAF-1, an ATP-binding cassette transporter in the inner mitochondrial membrane, and the cytoplasmic ATFS-1 can then be imported into the nucleus [200]. Full activation of UPR^{MT} also requires the DVE-1 transcription factor in complex with the ubiquitin-like protein UBL-5 [201, 202]. This aspect of UPR^{MT} activation requires the mitochondrial matrix protease CLPP-1 [201]. Together, ATFS-1 and DVE-1/UBL-5 upregulate gene products, including mitochondrial chaperones *hsp-6* and *hsp-60*, that restore proteostasis in the mitochondria. ATFS-1 also limits the expression of nuclear-encoded components of respiratory complex proteins, which reduces the protein folding burden of the mitochondria [203]. In parallel to ATFS-1, the GCN-2 kinase phosphorylates eIF2α, reducing the translation of new proteins and mitochondrial protein folding stress [204]. In sum, these mechanisms counteract mitochondrial stress and improve compartment-specific protein folding capacity.

Activation of the ER UPR and the ability to survive ER stress declines with age [205]. The ER UPR genes *ire-1* and *xbp-1* are required for increased lifespan from decreased insulin/IGF signalling or dietary restriction from bacterial dilution [23, 206], and also for pathogen survival [207, 208]. These data suggest that ER UPR may be an important mechanism to ensure long life. However, the correlation between activation of ER UPR and lifespan is not absolute. Although ubiquitous expression of constitutively active, spliced *xbp-1* restores ER UPR activation late in life, it does not increase lifespan [205]. Curiously, lifespan is increased if expression of spliced *xbp-1* is limited to neurons or the intestine, whereas expression of spliced *xbp-1* in body wall muscle decreases lifespan [205]. The neuronal expression of spliced *xbp-1* that increases lifespan activates the ER UPR nonautonomously in non-neuronal cells [205], but the neuron-derived signal produced in these animals has not been identified.

Genetic perturbation that reduce mitochondrial function increases lifespan [209–214]. These deficiencies also activate UPR^{MT} [197, 199, 215, 216]. Increased lifespan of *isp-1* mutant animals requires *ubl-5*, suggesting that activation of UPR^{MT} is involved in mediating the effects of mitochondrial dysfunction on lifespan [216]. However, *atfs-1* is required for activation of UPR^{MT} but not increased lifespan of *isp-1* mutant animals [199]. Similarly, mitochondrial stress leads to nuclear localization of LIN-65 and a gross chromatin reorganization that is required for DVE-1

nuclear puncta formation, but which is independent of *atfs-1* [217]. These results suggest that DVE-1/UBL-5 may have roles to modulate lifespan that are distinct from activation of UPR^{MT}. Another possibility, which is not mutually exclusive, is that interactions between different tissue types underlie differences between *atfs-1* and *dve-/ubl-5* pathways. Mitochondrial stress in neurons, from RNAi depletion of *cco-1*, leads to non-autonomous activation of UPR^{MT} in peripheral tissues [216]. As of now, it is not known how (or if) nonautonomous activation of UPR^{MT} and ER UPR are related.

9.4.4 Concluding Remarks

Research into fundamental stress response mechanisms in model organisms such as *C. elegans* has begun to reveal basic strategies that can help maintain homeostasis in animals, providing important insight into how stress and lifespan are related. Because of their central importance, stress response pathways are often conserved in humans. Thus, understanding how to manipulate these pathways holds great promise of therapeutic application to reduce morbidity and mortality from a variety of age-associated diseases. This promise will only increase as we learn more of how different stress response pathways are integrated, and how organism-wide responses are coordinated.

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