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Activation of SKN-1 by Novel Kinases in *Caenorhabditis elegans*

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Abstract

Here we use a large-scale RNAi suppression screen to identify additional kinases playing a role in the activation of SKN-1 in response to oxidative stress. The SKN-1 transcription factor specifies cell fate of the EMS blastomere at the four-cell stage in the nematode *Caenorhabditis elegans* and also directs transcription of many genes responding to oxidative stress, including glutathione-S-transferase, NAD(P)H: quinone oxidoreductase, and superoxide dismutase. SKN-1 localizes to the nucleus and directs transcription following exposure to paraquat, heat, hyperbaric oxygen, and sodium azide. Previous studies have identified GSK-3 as an inhibitor of SKN-1 nuclear localization, in the absence of stress, and PMK-1 as an activator of SKN-1 during periods of oxidative stress. Through this screen we have identified four kinases, MKK-4, IKKε-1, NEKL-2, and PDHK-2, which are necessary for the nuclear localization of SKN-1 in response to oxidative stress. Inhibition of two of these kinases results in shorter lifespan and increased sensitivity to stress.

The role of oxidative stress as a factor in aging processes and numerous diseases, including diabetes and cancer, has become the focus of intense study, with many groups examining the cellular responses to oxidative stress. Correct balance of the intracellular redox environment is key because much of intermediary metabolism involves controlled reduction-oxidation reactions; however, an excessive amount of oxidants can cause unwanted damage to important biomolecules including DNA, proteins, and phospholipids, resulting in malfunction and disease [1]. Many of the genes in *Caenorhabditis elegans* involved in aging and longevity have been linked to pathways involving the response to stress and the innate immune response [2, 3]. The DAF-16 transcription factor, for example, is necessary for dauer formation and increased longevity in the worm and is also necessary for resistance to pathogenic bacteria [3]. The MAPK pathway is critically important for oxidative-stress resistance, is involved in anti-fungal and anti-bacterial resistance [2], and is tied to longevity through JNK activity [4, 5]. Unlike higher vertebrates, *C. elegans* possesses only the first line of defense, the innate immune system, comprised of sensory mechanism and anti-pathogen defenses, such as the Toll and p38 MAPK pathways [3]. Therefore, crosstalk between many pathways involved in response to stress, pathogens, and age-related disease is not surprising in the small genome of *C. elegans*.

The SKN-1 transcription factor in *C. elegans* regulates differentiation of mesodermal tissue during development [6]. GSK-3 directly phosphorylates SKN-1 in larval stages and, under

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normal conditions, prevents SKN-1 from accumulating in intestinal nuclei [7]. The SKN-1 protein has been shown to localize to nuclei of intestinal cells in response to oxidative stressors such as paraquat, heat, and sodium azide [8]. In response to oxidative stress, the p38 MAP kinase (PMK-1) directly phosphorylates SKN-1, leading to the translocation of the SKN-1 protein into intestinal nuclei [9]. The SKN-1 transcription factor regulates the expression of the antioxidant and phase II gene *gcs-1* [8] and has been proposed to regulate other antioxidant genes including glutathione-S-transferases (GSTs), NAD(P)H: quinone oxidoreductase (NQO1), and superoxide dismutase (SOD) in response to oxidative stress [8]. Here we describe additional regulatory pathways involved in the regulation of SKN-1.

The development of RNA-interference and the techniques of bacterial feeding RNAi has allowed for large-scale knock-down screens to investigate the function of a large part of the *C. elegans* genome [10]. Such screens have been used for elucidation of interacting pathways, identification of phenotypic variants, and genetic dissection of embryonic development [11–13]. Using a large-scale RNAi screen of predicted kinases encoded by the worm genome and contained in the MRC RNAi library, we have identified genes encoding four additional kinases, which play a role in the activation and nuclear localization of SKN-1 in response to oxidative stress. These kinases are closely related to human genes found in pathways regulating oxidative stress (MKK-4)[14], innate immunity (IKK ϵ -1)[15], cell cycle (NEKL-2)[16], and mitochondrial function (PDHK-2)[17]. We have also investigated the effects of RNAi knockdown of these genes on lifespan and sensitivity to oxidative stress and find that the activity of these proteins is necessary for normal lifespan and survival in oxidative stress conditions.

Materials and Methods

Strains and Culture Methods

All strains were raised on solid NGM plates at 20 °C with *Escherichia coli*, OP50 as a food source, according to standard procedures [18], except as described below. Strains used were: N2 (wild type; CGCb); translational fusion reporter LD001 *Ex007 (skn-1::gfp)* [9]; transcriptional fusion reporter CL2166 *dvIs19 [pAF15 (gst-4::gfp-nls)] III* [19]; deletion mutant VC1089 *mkk-4(ok1545)* X; *gst4::gfp* reporter in a mutant *skn-1* background CL691 *skn-1(zu67)* IV/*DnT1 [unc-?(n754)let-?]* IV;V;*dvIs19 [pAF15 (gst-4::gfp-nls)] III*.

RNA interference

An RNAi library was created of all predicted protein kinases found in the MRC RNAi library, and consolidated by an independent online kinase database[†] [20]. The final kinase RNAi library contained 432 bacterial clones, all of which were then used for the screen described below. To inhibit specific gene function, standard RNAi feeding protocol was followed and empty vector RNAi was used as a control [12]. Eggs were placed on the RNAi bacteria and allowed to grow to adulthood, at which point the animals were harvested for stress treatment or monitored for lifespan. When a candidate gene was found to have an observable biological effect, the relevant RNAi clone was sequenced and its identity thus confirmed.

Sodium Azide Assays

For visualization of extrachromosomal *skn-1::gfp*, LD001 animals were raised on RNAi bacteria and second stage larvae (L2's) from the F₁ generation were exposed to 2% (w/v) sodium azide in S-Basal for 20 minutes. These animals were then placed on 2% agarose pads on a microscope slide, covered with a cover slip, and observed using fluorescent microscopy

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[21]. For visualizing *gst4::gfp*, CL2166 animals were raised on RNAi bacteria and L2 worms from the F₁ generation were exposed to 2% sodium azide in S-Basal for 1.5 hours. Animals were then transferred to solid NGM plates at 20 °C. Four hours after the initial exposure to sodium azide, treated animals were mounted on 2% agarose pads on a microscope slide, covered with a cover slip, and observed using fluorescent microscopy [21]. Digital images were acquired using a Zeiss Axioskop epifluorescence microscope equipped with a digital deconvolution retrofit (Intelligent Imaging Innovations) and were adjusted for brightness and contrast using Adobe Photoshop 6.0 (Adobe Systems, Inc., San Jose, CA, USA). For each replicated screen, five individuals from each RNAi plate were randomly chosen for microscopic observation. A few clones were found to be lethal, and were excluded from the screen.

Lifespan and Sodium Arsenite Assays

N2 gravid adults were placed onto solid NGM plates with RNAi bacteria and allowed to lay eggs for no longer than four hours and then removed. These eggs were raised on the NGM plates with RNAi bacteria at 20 °C. Animals used for lifespan analysis were followed as previously described [22]. Between 60–80 animals were cultured on solid NGM plates with respective RNAi bacteria and transferred every day while egg-laying and every other day afterwards until none remained alive. At the young adult stage, animals involved in the stress tests were transferred to 6 cm petri dishes containing 3.5 mL of 5 mM sodium arsenite in S-Basal and incubated at 20 °C. Survival of animals in this media was assessed at 16, 24, and 36 hours. Survival curves were created and analysis was performed based on number of worms surviving each day.

Statistical Analyses

Mean life span was estimated using the formula: $MLS = \frac{1}{N} \sum_j \frac{x_j + x_{j+1}}{2} * d_j$ where d_j is the number of worms that died in the age interval (x_j, x_{j+1}) and N is the total number of worms. The standard error of the mean life span estimate was calculated using:

$$SE = \sqrt{\frac{1}{N(N-1)} \sum_j \left(\frac{x_j + x_{j+1}}{2} - MLS \right)^2 d_j}$$

Results

Inhibition of SKN-1 Nuclear Localization

Following oxidative stress, SKN-1 is phosphorylated by p38 and translocates into the nucleus. To identify additional protein kinases involved in the activation and subsequent nuclear localization of the SKN-1 protein, we selected 432 clones containing all predicted protein kinases found in the Arhinger RNAi library. *skn-1::gfp* (LD001) worms were grown for one generation on each RNAi bacterial strain. Animals of the F₁ generation at the second larval stage (L2) were then exposed to 2% sodium azide, a potent inducer of SKN-1, causing rapid nuclear localization [8]. Due to the low signal of this SKN-1::GFP reporter strain, this screen had to be conducted by mounting the animals grown on each clone on slides for accurate analysis using fluorescence microscopy. Four RNAi clones were consistently observed to suppress the normal nuclear localization of SKN-1 in response to sodium azide in each of four replicate experiments (Fig. 1): *nekl-2* (ZC581.1, Fig. 1C), *ikke-1* (R107.4, Fig. 1D), *mkk-4* (mitogen-activated protein kinase kinase 4, Fig. 1E), and *pdhk-2*, a worm homolog of pyruvate dehydrogenase kinase 2, (ZK370.5, Fig. 1F). These genes were identified by wormbase as such or identified by sequence homology to human genes.

Effects on Longevity

Since reduced expression of SKN-1 shortens life span [8] we asked about the effects of these four kinases on longevity. The adult lifespan of animals grown on *skn-1* RNAi was significantly shorter than that of animals fed the empty vector control (Fig. 2). The adult lifespan of animals fed RNAi for *nekl-2* and *pdhk-2* kinases was also found to be significantly shorter than those of animals fed empty vector RNAi (Fig. 2). The lifespan of animals fed *ikke-1* and *mkk-4* RNAi was not significantly different from those on empty vector RNAi (Fig. 2). A mutant for *mkk-4* were acquired and tested for lifespan differences with respect to wild-type and *skn-1* mutant animals (Fig. 3). Consistent with RNAi data, the genetic mutant showed neither significantly increased nor decreased lifespan.

Sensitivity to Stress

skn-1 genetic knock-out strains showed increased sensitivity to oxidative stress [8]. Consequently, we decided to explore the possible effects of kinase inhibition on sensitivity to oxidative stress. Exposure to sodium arsenite has been shown to significantly increase ROS production in the intestines of wild-type animals[23]. Using 5 mM sodium arsenite to subject N2 worms to oxidative stress, we measured the survival of animals fed RNAi bacteria for these kinases. Animals fed *skn-1* RNAi were significantly more sensitive to the sodium arsenite as compared to animals fed empty vector control RNAi (Fig. 4). Animals fed *pdhk-2* and *nekl-2* RNAi were also significantly more sensitive to the arsenite stress as compared to empty vector controls (Fig. 4). Consistent with the longevity results, animals fed RNAi against *mkk-4* and *ikke-1* were not significantly more sensitive to arsenite stress (Fig. 4).

gst-4 Induction

The *skn-1* transcription factor has been predicted to turn on a variety of antioxidant response genes [8] and has been shown to regulate the induction of glutathione-S-transferase-4 (*gst-4*) in response to oxidative stress [Kahn N., Rea S., Johnson T.E., *manuscript submitted*]. Using CL2166, a strain which carries a transcriptional fusion of GFP to the *gst-4* promoter [19], we found that unstressed CL2166 animals express GFP only in the hypodermal cells (Fig. 5A). Exposure of CL2166 to 2% sodium azide induces expression of GST-4 as assessed by GFP (Fig. 5B). RNAi against each of the four kinases just identified above partially blocked expression of GST-4 in response to sodium azide (Fig. 5C–F). The expression of GST-4 was also partially blocked by RNAi in response to hyperbaric oxygen, exposure to juglone, and heat stress (data not shown).

Discussion

Through the research presented here, we have discovered four additional kinases which, when inhibited by RNAi, prevent the nuclear localization of SKN-1. This suggests new, unidentified roles for these kinases in SKN-1 oxidative stress response pathways. Interestingly, these four kinases have not been found to function in the same signal transduction pathway. In fact, they have been previously reported to be involved in completely different biological processes: the cell cycle, the p38 mitogen-activated protein kinase cascade, double-stranded RNA recognition and response, and cellular metabolism [7,15,17,24]. Studies describing these pathways have mainly taken place in mammalian cells and tissues with little work performed to describe the roles of these proteins in *C. elegans*.

Inhibition of expression of *nekl-2* is shown in this study to prevent nuclear localization of the SKN-1 protein in response to oxidative stress. This protein is related to the serine/threonine protein kinase Nek8 found in the human genome by a blast match E value of 1.1×10^{-67} . Nek8 was first identified as a novel NIMA-related kinase (Never In Mitosis, gene A) [24]. Nek family proteins have important roles in oocyte differentiation, microtubule formation, and cell cycle

regulation [16]. Research has identified 11 Nek family proteins in humans and four in *C. elegans*. The SKN-1 transcription factor was first identified in *C. elegans* as a necessary component in differentiation of the gut in early embryos [25]. Although the period during which NEKL-2 may be involved in SKN-1 association is not clear, the phenotypic difference between animals fed RNAi against *nekl-2* and those fed the empty vector control is dramatic during oocyte maturation such that many animals were unable to expel eggs from the vulva and instead produced live progeny with the gonads, a condition generally referred to as bagging.

In mammals, the activity of IKK ϵ has been linked to the activity of NF- κ B and interferon regulatory factor 3 (IRF3) [15]. IKK ϵ acts as an inhibitor of NF- κ B activity and activates IRF3 in response to viral invasion of the cell [15]. Both transcription factors, NF- κ B and IRF3, are involved in the innate immune response, specifically viral infection or introduction of double-stranded RNA [15]. Recognition of double-stranded RNA is facilitated through toll-like receptor 3 (TLR3). Recent reports have concluded that activated TLR3 helps to engage the PI3K-AKT pathway in the viral response [26]. RNAi for the *C. elegans* gene R107.4 suppressed the accumulation of SKN-1 in intestinal nuclei in response to oxidative stress. This gene is related to the human IKK ϵ gene by a blast match of $1.1e^{-18}$.

Interestingly, another gene identified in this screen is closely involved in cellular metabolism. *pdhk-2* is a *C. elegans* gene closely related to the human gene for pyruvate dehydrogenase kinase isoform 2 (PDK-2), as identified by a blast match of $4.5e^{-101}$. There are seven human isoforms for pyruvate dehydrogenase kinase which act in different tissues throughout the body [17]. Only one isoform of pyruvate dehydrogenase kinase has been identified in nematodes [27] and none previously described for *C. elegans*. All mammalian PDK isoforms directly phosphorylate the pyruvate dehydrogenase complex in order to inactivate it [26]. Many animals appear to have been made sick after feeding with *pdhk-2* RNAi and exhibited a vulval defect resulting in the expulsion of the intestines through the vulva. Because of its importance in cellular metabolism and energy production, it may not be surprising that a dramatic loss of regulation of the pyruvate dehydrogenase complex results in a shortening of lifespan.

The MAP kinase pathway in *C. elegans* is responsible for stress resistance, immune response, and longevity, as shown in numerous independent studies [4,7,28] and recently in immune function. Previous studies have described the role of p38 MAP kinase in direct phosphorylation of SKN-1 in response to oxidative stress [7] as well as its role in phosphorylation of the SKN-1 mammalian homologue, Nrf2 [29]. The research presented here indicates the involvement of MAP-kinase kinase -4 in SKN-1 nuclear localization and activation in response to oxidative stress. MKK-4 has been shown to activate p38 MAP kinase [14] and activate stress-activated protein kinase 1/c-Jun N-terminal kinase (SAPK1/JNK) isoforms in response to cellular stress [30]. AKT has been reported to directly inhibit MKK-4 activity in response to cellular stress, resulting in decreased downstream activity of JNK [31]. Interestingly, past research has identified glycogen synthase kinase 3 (GSK-3) as a direct substrate of AKT-1 [32]. Phosphorylation of GSK-3 by AKT results in inhibition of GSK-3 activity [32] while GSK-3 has recently been identified as an inhibitor of SKN-1 activation in the absence of stress [7]. In the present study, we show that feeding of *mkk-4* RNAi results in inhibition of SKN-1 nuclear localization in response to sodium azide stress. This is consistent with the model in which MKK-4 phosphorylates PMK-1, which, in turn, phosphorylates SKN-1 in response to oxidative stress.

The link between oxidative stress, aging, and age-related disease theorized for all organisms, has led us to identify pathways involved in mediating the oxidative stress response. It is not unexpected to find that many of the pathways involved in oxidative stress also respond to other types of stress such as xenobiotic responses and double-stranded RNA detection. In invertebrates and other organisms lacking a complex immune system, the pathways of stress

response, insulin-like receptor pathways, and behavioral responses appear to converge and interact to arrest perceived threats to the organism [3]. Past research has demonstrated that deletion of the SKN-1 protein shortens lifespan and increases sensitivity to stress [8]. It is, therefore, not surprising to find pathways involved in innate immunity may also play roles in oxidative stress response in simpler organisms. Activation of cellular defenses in response to oxidative stress and xenobiotics are similarly important to survival of the organism as a whole. Understanding the cellular redox balance and oxidative stress response is key to understanding what causes this balance to be tilted, resulting in the effects of aging and age-related diseases. Lifespan and stress response analyses show that the expression of genes coding for *nekl-2* and *ikke-1* in *C. elegans* is necessary for normal lifespan and survival in oxidative stress conditions.

The minor effect that inhibition of gene expression of both *mkk-4* and *pdhk-2* has on lifespan and stress resistance may indicate the presence of compensatory pathways and mechanisms. Silencing of these kinases through RNAi feeding partially suppressed the induction of GST-4 in response to oxidative stress conditions. This result may be explained by the inability of RNAi to block all SKN-1 function. If enough SKN-1 were able to localize to the nucleus, transcription of GST-4 in response to oxidative stress may occur. A small amount of endogenous SKN-1 nuclear localization may not have been visible using the techniques described above.

The activation of antioxidant genes in response to endogenous oxidative stress has been an area of intense study [33,34]. In vertebrates, this response is directed through the transcription factors NF-E2-related factors, Nrf1 and Nrf2 [34]. Nrf2 (NF-E2-related factor-2) is a basic leucine zipper protein which has been identified and characterized to bind to antioxidant response elements in mammalian cells. Nrf2 has been shown to bind to the ARE with a high affinity as a heterodimer with MafK (musculoaponeurotic fibrosarcoma oncogene family, protein K) [34]. Numerous protein kinases have been implicated in the activation and nuclear localization of Nrf2 [29,35,36]. The pathways involved in the nuclear localization of Nrf2 and activation of ARE genes must be clarified in order to identify the mechanisms by which cells may respond to oxidative stress and aging. In the study presented here, we identify four kinases previously not known to be involved in the SKN-1 stress response. Given the homology of these genes to their human counterparts, our work suggests new pathways that may be involved in the human Nrf2 stress response.

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Fig. 1.

Images of second-larval stage *skn-1::gfp* (LD001) animals, shown at 10X. (A) Four untreated animals raised on empty vector RNAi. (B) Two animals raised on empty vector RNAi and treated with 2% sodium azide. Note localization of GFP in intestinal nuclei. (C&D) Image of anterior intestinal cells of *skn-1::gfp* animal treated with 2% sodium azide shown at 100x magnification without fluorescence (C) and with fluorescence imaging (D). (E–H) Each picture shows four animals treated with 2% sodium azide; one animal was a control raised on empty vector and three were raised on bacteria containing double stranded. (E) *nekl-2* RNAi. (F) *mkk-4* RNAi. (G) *ikke-1* RNAi. (H) *pdhk-2* RNAi.

Fig. 2.

Typical effects of kinase inhibition on lifespan. Values of p represent log rank test for experiment shown, comparing lifespan of animals fed empty vector RNAi and those fed RNAi for the respective kinase. Mean lifespan/p-value: empty vector – 16.7 days; *skn-1* – 15.1 days ($p = 9.6 \times 10^{-5}$); *nekl-2* – 7.9 days ($p = 2.4 \times 10^{-20}$); *ikke-1* – 17.6 days ($p = 0.27$); *mkk-4* – 16.1 days ($p = 0.103$); *pdhk-2* – 13.9 days ($p = 0.74$). N = 60 animals/replicate, replicated three times independently.

Fig. 3.

Typical effects of kinase genetic mutants on lifespan. Values of p represent log rank test for experiment shown, comparing lifespan of wild-type animals and genetic mutants of *mkk-4* and *skn-1*. Mean lifespan/p-values: N2 – 17.8 days; *skn-1* - 14.0 days (8.6×10^{-6}); *mkk-4* (*ok1545*) – 16.5 days (0.64).

Fig. 4.

Typical effects of kinase inhibition on sensitivity to stress. N2 animals fed RNAi for respective kinases exposed to 5 mM sodium arsenite. Values of p represent log rank test comparing survival of animals fed empty vector RNAi and those fed RNAi for respective kinases. *skn-1* – ($p = 2.2 \times 10^{-10}$); *nekl-2* – ($p = 7.3 \times 10^{-76}$); *ikke-1* – ($p = 2.9 \times 10^{-16}$); *mkk-4* – ($p = 3.9 \times 10^{-6}$); *pdhk-2* – ($p = 2.3 \times 10^{-8}$). $N = 50$ animals/replicate, replicated four times independently.

Fig. 5. *gst-4::GFP*. Green fluorescence protein (GFP) images of adult CL2166 transgenic animals shown at 10x. (A) Image of two animals raised on empty vector and untreated. (B) Image of an untreated animal raised on empty vector RNAi adjacent to an animal raised on empty vector RNAi and treated with 2% sodium azide. Notice prominent GFP fluorescence throughout intestines and body. (C) Image of three animals raised on RNAi and treated with 2% sodium azide. One animal raised on empty vector RNAi, one raised on *nekl-2* RNAi, and one raised on *skn-1* RNAi. (D) Image of three animals raised on RNAi and treated with 2% sodium azide. One animal raised on empty vector RNAi, one raised on *mkk-4* RNAi, and one raised on *skn-1* RNAi. (E) Image of three animals raised on RNAi and treated with 2% sodium azide. One animal raised on empty vector RNAi, one raised on *ikke-1* RNAi, and one raised on *skn-1* RNAi. (F) Image of three animals raised on RNAi and treated with 2% sodium azide. One animal raised on empty vector RNAi, one raised on *pdhk-2* RNAi, and one raised on *skn-1* RNAi.