Proteasomal dysfunction activates the transcription factor SKN-1 and produces a selective oxidative-stress response in *Caenorhabditis elegans*

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SKN-1 in the nematode worm *Caenorhabditis elegans* is functionally orthologous to mammalian NRF2 [NF-E2 (nuclear factor-E2)-related factor 2], a protein regulating response to oxidative stress. We have examined both the expression and activity of SKN-1 in response to a variety of oxidative stressors and to down-regulation of specific gene targets by RNAi (RNA interference). We used an SKN-1–GFP (green fluorescent protein) translational fusion to record changes in both *skn-1* expression and SKN-1 nuclear localization, and a *gst-4*–GFP transcriptional fusion to measure SKN-1 transcriptional activity. GST-4 (glutathione transferase-4) is involved in the Phase II oxidative stress response and its expression is lost in an *skn-1*(*zu67*) mutant. In the present study, we show that the regulation of *skn-1* is tied to the protein-degradation machinery of the cell. RNAi-targeted removal of most proteasome subunits in *C. elegans* caused nuclear localization of SKN-1 and, in some cases, induced transcription of *gst-4*. Most intriguingly, RNAi knockdown of proteasome core subunits caused nuclear localization of SKN-1 and induced *gst-4*, whereas RNAi knockdown of proteasome regulatory subunits resulted in nuclear localization of SKN-1 but did not induce *gst-4*. RNAi knockdown of ubiquitin-specific hydrolases and chaperonin components also caused nuclear localization of SKN-1 and, in some cases, also induced *gst-4* transcription. *skn-1* activation by proteasome dysfunction could be occurring by one or several mechanisms: (i) the reduced processivity of dysfunctional proteasomes may allow oxidatively damaged by-products to build up, which, in turn, activate the *skn-1* stress response; (ii) dysfunctional proteasomes may activate the *skn-1* stress response by blocking the constitutive turnover of SKN-1; and (iii) dysfunctional proteasomes may activate an unidentified signalling pathway that feeds back to control the *skn-1* stress response.

Key words: *gst-4*, chaperonin, 3*H*-1,2-dithiole-3-thione (D3T), nuclear factor-E2 (NF-E2)-related factor 2 (NRF2), proteasome, SKN-1.

INTRODUCTION

skn-1 encodes a key transcription factor required for mesodermal development in the nematode worm *Caenorhabditis elegans* [1]. SKN-1, the product of the *skn-1* gene, was also previously shown to have a role in adult worms controlling the acute transcriptional response to oxidative stress [2]. SKN-1 is the functional orthologue of the mammalian protein NRF2 [NF-E2 (nuclear factor-E2)-related factor 2] which controls induction of multiple genes involved in electrophile detoxification and antioxidant stress response [3]. *NRF-2* genes have been identified in many species, including *Caenorhabditis briggsae*, *Drosophila melanogaster* (fruitfly), *Xenopus laevis* (South African clawed frog), *Mus musculus* (mouse) and human (*Homo sapiens*). At the protein level, SKN-1 and human NRF2 share limited sequence identity, with 23 % amino acid homology in the CnC-βZip DNA-binding region and 71 % amino acid homology in the DIDLID [Keap1 (Kelch-like enoyl-CoA hydratase-associated protein 1)-binding] region [2]. Interestingly, SKN-1 and NRF2 both bind AREs (antioxidant response elements) but they employ different mechanisms: NRF2 binds as an obligate heterodimer, whereas SKN-1 recognizes a βZIP half-site [2].

The molecular mechanisms behind NRF2 antioxidant function have been fairly well elucidated: NRF2 complexes with another protein, Keap1, to act as a cytoplasmic oxidative-stress sensor. Under basal conditions, Keap1 functions both as an NRF2 cytosolic repressor [4], and as an adaptor for a cullin 3-based E3 ubiquitin ligase that enhances the ubiquitination and rapid proteasomal degradation of NRF2 [5,6]. A slower, Keap1-independent NRF2 degradation pathway also exists [7]. NRF2 becomes post-translationally up-regulated following exposure to electrophiles and/or ROS (reactive oxygen species) via two distinct mechanisms – conformational alteration and phosphorylation. Oxidative modification of several reactive cysteine residues in Keap1 can induce the Keap1–NRF2 complex to dissociate. Similarly, a variety of stress-activated kinases provoke NRF2 phosphorylation and also lead to disruption of the Keap1–NRF2 complex [8–11]. Once free in the cytoplasm, NRF2 migrates to the nucleus where it interacts with a number of *cis*-acting AREs in the regulatory regions of target genes encoding detoxifying and antioxidative enzymes/proteins to produce a co-ordinated protective response [12,13].

In *C. elegans*, SKN-1 is activated following oxidative stress and, like NRF2 in other species, has been shown to control the induction of the Phase II enzyme GSC-1 (catalytic subunit of 1,3-β-D-glucan synthase) [2]. This control is mediated through a combination of three AREs. Based on these findings, several additional ARE-containing genes were predicted to be direct SKN-1 targets [2], including GST-4 (glutathione transferase-4), which is employed in the present study. Phosphorylation plays a key regulatory role in SKN-1 nuclear localization in worms: under basal conditions, GSK-3 (glycogen synthase kinase-3) inhibits SKN-1 nuclear localization. Following oxidative stress, PMK-1, a p38 MAPK (mitogen-activated protein kinase), phosphorylates

Abbreviations used: ARE, antioxidant response element; D3T, 3*H*-1,2-dithiole-3-thione; GFP, green fluorescent protein; GST, glutathione transferase; Keap1, Kelch-like enoyl-CoA hydratase-associated protein 1; L1 etc., first stage larvae etc.; MAPK, mitogen-activated protein kinase; NRF2, NF-E2 (nuclear factor-E2)-related factor 2; RNAi, RNA interference; ROS, reactive oxygen species.

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SKN-1 and this event is essential for SKN-1 nuclear localization [14]. We have found that four additional kinases also act to control the level of SKN-1 in the cytoplasm. Worm homologues of MKK-4 (MAPK kinase-4), IKKε-1 (inhibitory κB kinase ε-1), NEKL-2 [NEKL-2: NEK (never in mitosis kinase)-like protein 2] and PDHK-2 (pyruvate dehydrogenase kinase-2) are necessary for nuclear localization of SKN-1 in response to oxidative stress. Inhibition of these kinases results in shorter life span and increased sensitivity to stress [15]. The *C. elegans* gene *gst-4* encodes a GST and was originally identified because of its robust induction following paraquat treatment [16]. GSTs form integral components of the Phase II metabolic response that is activated after exposure to xenobiotics and products of oxidative stress [17]. GSTs catalyse conjugation of glutathione to a variety of electrophilic compounds.

In the present study, we extend earlier work and show that a variety of oxidative stress treatments (in addition to hyperbaric O_2 and paraquat), including H_2O_2, sodium azide and the redox cycler juglone, induce *gst-4*, as measured by using a *gst-4*–GFP (green fluorescent protein) reporter [18]. We show that exposure to the chemoprotectant D3T (3*H*-1,2-dithiole-3-thione) similarly induces *gst-4*. In all cases, we show that *gst-4* transcription is mediated by SKN-1. We also ask if these various oxidative stressors affect SKN-1 at a transcriptional or protein level. Previous studies in mammals have shown that proteasome components are up-regulated by antioxidants in an NRF2-dependent manner [19]. Because proteasomal degradation helps maintain homoeostasis, we wanted to explore the possibility that SKN-1 expression and/or activity might be regulated via a feedback loop with proteasomes. In order to study this phenomenon fully, we constructed several RNAi (RNA interference) sublibraries using all available RNAi clones in the Ahringer Genomic RNAi Library that target proteasome subunits, ubiquitin-specific hydrolases and chaperonins. We found that inhibition of almost all subunits of the proteasome results in *skn-1* activation. Surprisingly, only core proteasomal subunits, and not regulatory proteasomal subunits, activate *gst-4* transcription. Inhibition of several ubiquitin-specific hydrolases also results in *skn-1* activation, yet in only a subset of cases is *gst-4* transcription induced. Finally, for chaperonins, we observed a pattern of *skn-1*-dependent *gst-4* induction in the absence of any observable *skn-1* activation. The results of the present study indicate that regulation of *skn-1* in *C. elegans* is integrally tied to the protein-maintenance machinery of the cell, and especially to the function of the proteasome.

MATERIALS AND METHODS

Strains

Three transgenic reporter strains were used for the present study: the transcriptional fusion reporter CL2166 *dvIs19* [*pAF15 (gst-4*–GFP-*nls)*] III [20]; the translational fusion reporter LD007 *Is007* (*SKN-1*–GFP) [2]; and the *gst-4*–GFP reporter in a mutant *skn-1* background – CL691 *dvIs19* [*pAF15 (gst-4*–GFP-*NLS)*] III; *skn-1*(*zu67*) IV/*nT1* [*unc-?(n754) let-?*] IV;V.

Animal maintenance

Strains of *C. elegans* were maintained at 20 °C and grown on nutrient agar plates spread with *Escherichia coli* – either freshly grown OP50 or HT115 containing RNAi plasmid constructs. Synchronous populations were initiated either from egg lays obtained from unstarved gravid adults, or by collecting first stage larvae (L1) from unstarved mixed populations {via gravity separation over four consecutive 50 ml S-Basal buffer [5.85 g of NaCl, 1 g of K_2HPO_4, 6 g of KH_2PO_4 and 1 ml of cholesterol (5 mg/ml in ethanol) in 1 litre] washes}. All experimental observations were recorded at the L4/young adult stage of development.

Oxidative stress tests

The following compounds and conditions were used: D3T at 0.001 % in 0.1 % DMSO for 24 h, hyperbaric oxygen exposure (>99 % O_2) at 40 lbf/in^2 (1 lbf/in^2 = 6.9 kPa) for 6–8 h at 20 °C in an airtight steel chamber [18], H_2O_2 at 10 mM for 20 min, 2 % (w/v) sodium azide for 15 min and juglone at 225 μM for 15 min.

Feeding RNAi

Bacteria containing plasmid constructs engineered to make double-stranded RNA (RNAi clones) were grown in LB (Luria–Bertani)–ampicillin (100 μg/ml) in sterile 96-well microtitre plates or in sterile 15 ml polypropylene tubes for 16 h at 37 °C, with moderate shaking, and then spotted on to a fresh NGM (nutrient growth medium) worm agar plate containing 100 μg/ml ampicillin plus 1 mM IPTG (isopropyl β-D-thiogalactoside). Bacterial lawns were grown at room temperature (22 °C) for at least 36 h before L1 worms of the desired strain (~100 animals per plate) were tested. Animals were allowed to grow on their respective RNAi at 20 °C to the L4/young adult stage and then examined to find clones that caused spontaneous induction of GFP (induction) or clones that blocked GFP expression under inducing conditions (suppression). Induction conditions for suppression screens were as follows: for *gst-4*–GFP, exposure to an oxygen-rich atmosphere (>99 % O_2 in an airtight plastic container) for 24 h at 20 °C; and for *skn-1*–GFP, treatment with 2 % (w/v) sodium azide for 1 h. GFP 'induction' was detected using fluorescence microscopy; RNAi 'suppression' blocked GFP induction. Each set of RNAi clones included positive controls known to cause reporter induction in the respective reporter strain. All plates were scored blind with respect to RNAi clone identity. All clones mentioned in the present paper were sequence-verified. RNAi screens were conducted at least twice. Individual RNAi clones that caused reporter induction or suppression were independently re-tested a minimum of three times. Typically, RNAi efficacy within a population ranged from ~90 % to complete penetration. This level of variability is expected given the often incomplete knockdown of target genes using the bacterial feeding RNAi method [21]. All RNAi targets reported in Tables 2–5 represent genes which, when deleted, resulted in strongly penetrant and highly reproducible phenotypes.

Fluorescent microscopy

GFP fluorescence was detected in worms exposed to stress or RNAi treatment via a Zeiss Axioskop retrofitted with deconvolution capabilities and a digital fluorescent camera containing a Sedat (Quad) filter set. GFP fluorescence was collected using an FITC 490 nm/528 nm absorption/emission filter pair (peak transmission with a 20 nm/38 nm band pass). For animals of the same strain, all images were collected using identical exposure settings at both ×100 and ×400 magnification. In almost all instances, assessment periods following each treatment exceeded 6 h, the minimum time required to reliably detect *gst-4*–GFP expression. In some cases, animals were monitored over a 48-h period. For juglone, animals were examined at 15 and 30 min intervals after treatment, for a minimum of 4 h.

Table 1 GFP reporter induction following oxidative stress

Activation refers to nuclear translocation, increased cytosolic expression and/or the appearance of GFP-enriched gut granules. Treatment-induced phenotypes were highly penetrant within each genotype (almost 100 %), but the level of reporter activation/induction varied depending on the stressor: '+++', '++' and '–' represent strong, moderate and no activation/induction of GFP respectively.

Stressor	*skn-1*–GFP activation	*gst-4*–GFP induction	*gst-4*–GFP; *skn-1(zu67)* induction
D3T	++	+++	–
Hyperbaric oxygen	++	+++	–
H_2O_2	++	+++	–
Sodium azide	++	+++	–
Juglone	–	+++	–

RESULTS

gst-4 is induced by oxidative stressors

A reporter strain (CL2166), containing *gst-4*–GFP, showed marked transgene induction following treatment with hyperbaric oxygen (Table 1; Figures 1a and 1b). Induction was easily discernible, with GFP brightly visible in the nuclei of the intestine and body wall muscle, in the pharynx and head, and in the cytoplasm of most tissues. The induction of *gst-4*–GFP was so strong that the normal GFP background glow found in untreated animals was almost indiscernible in some comparisons. These results are consistent with previous findings [20]. An identical pattern of *gst-4*–GFP reporter induction was also observed when animals were treated with several other oxidative stressors (Figure 1), including H_2O_2, sodium azide and juglone (direct oxidants), as well as the chemoprotectant D3T (an indirect antioxidant) [17].

gst-4 induction is blocked in a mutant *skn-1* background

gst-4 induction requires *skn-1* activity: when *gst-4*–GFP was crossed into an *skn-1(zu67)* mutant background, no induction of the reporter was observed following exposure of worms to D3T, hyperbaric oxygen, H_2O_2, sodium azide or juglone (Table 1 and Figure 1). The only fluorescence observed in treated animals was the background glow present in the strain under control conditions.

SKN-1 is activated by oxidative stressors

We monitored the effects of oxidant exposure on the activation of the *skn-1* stress-response system using a previously described GFP translational reporter fused to SKN-1 [2]. D3T, hyperbaric oxygen, H_2O_2 and sodium azide each led to the activation of SKN-1–GFP (Table 1 and Figure 2). 'Activation' was visible in a variety of ways, including discrete relocalization of GFP from the cytoplasm to the nucleus, increased cytosolic fluorescence, and the accumulation of GFP-bearing gut granules. At present, we are uncertain of the exact nature of these granules. Surprisingly, SKN-1–GFP did not appear to be activated by the redox cycling compound juglone, even though the same treatment induced *gst-4*–GFP expression in an *skn-1*-dependent manner. The length of time animals were exposed to each kind of oxidant varied: 15–20 min for H_2O_2, sodium azide and juglone; 6–8 h for hyperbaric oxygen; and 24 h for D3T. It is likely, therefore, that activation of SKN-1–GFP reflects changes both at the protein and the transcriptional level.

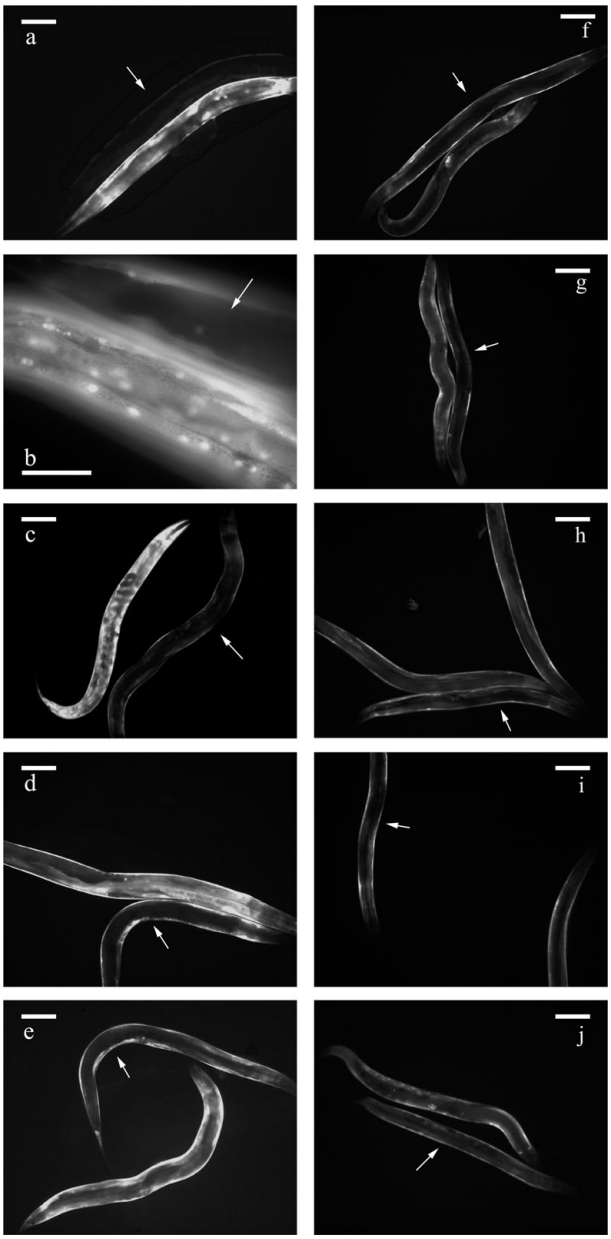

Figure 1 Oxidant-mediated induction of *gst-4*–GFP is *skn-1*-dependent

(**a**–**e**) Transgenic worms containing a *gst*-4–GFP transcriptional fusion were exposed to oxidant, then reporter induction was monitored using fluorescence microscopy. (**a**, **b**) Hyperbaric oxygen (100 % O_2, 40 lbf/in^2, 24 h); (**c**) 10 mM H_2O_2 (20 min); (**d**) 2 % (w/v) sodium azide (15 min); (**e**) 225 μM juglone (15 min). (**f**–**j**) The *gst*-4–GFP transcriptional fusion was crossed into an *skn-1(zu67)* mutant background. Animals were exposed to oxidant and the effect on reporter induction again recorded using fluorescence microscopy. (**f**) 0.001 % D3T (24 h); (**g**) hyperbaric oxygen (100 % O_2, 40 lbf/in^2, 24 h); (**h**) 10 mM H_2O_2 (20 min); (**i**) 2 % (w/v) sodium azide (15 min); (**j**) 225 μM juglone (15 min). Removal of *skn-1* blocked oxidant-dependent induction of *gst-4*–GFP. In each panel, an untreated, control worm (arrow) is shown for comparison. Low constitutive expression of the reporter is detectable in hypodermal cells. Scale bars, 100 μm.

skn-1 is activated following proteasomal disruption

In mammals, most of the proteasomal subunits are under the control of NRF2 action [19]. In *C. elegans*, six subunits of the proteasomal machinery contain multiple, SKN-1-binding AREs in their promoter regions (*pbs-1, pbs-5, rpn-1, rpn-2* and *rpn-6*). We sought to determine whether proteasomal dysfunction can signal activation of the *skn*-1 stress-response system in a

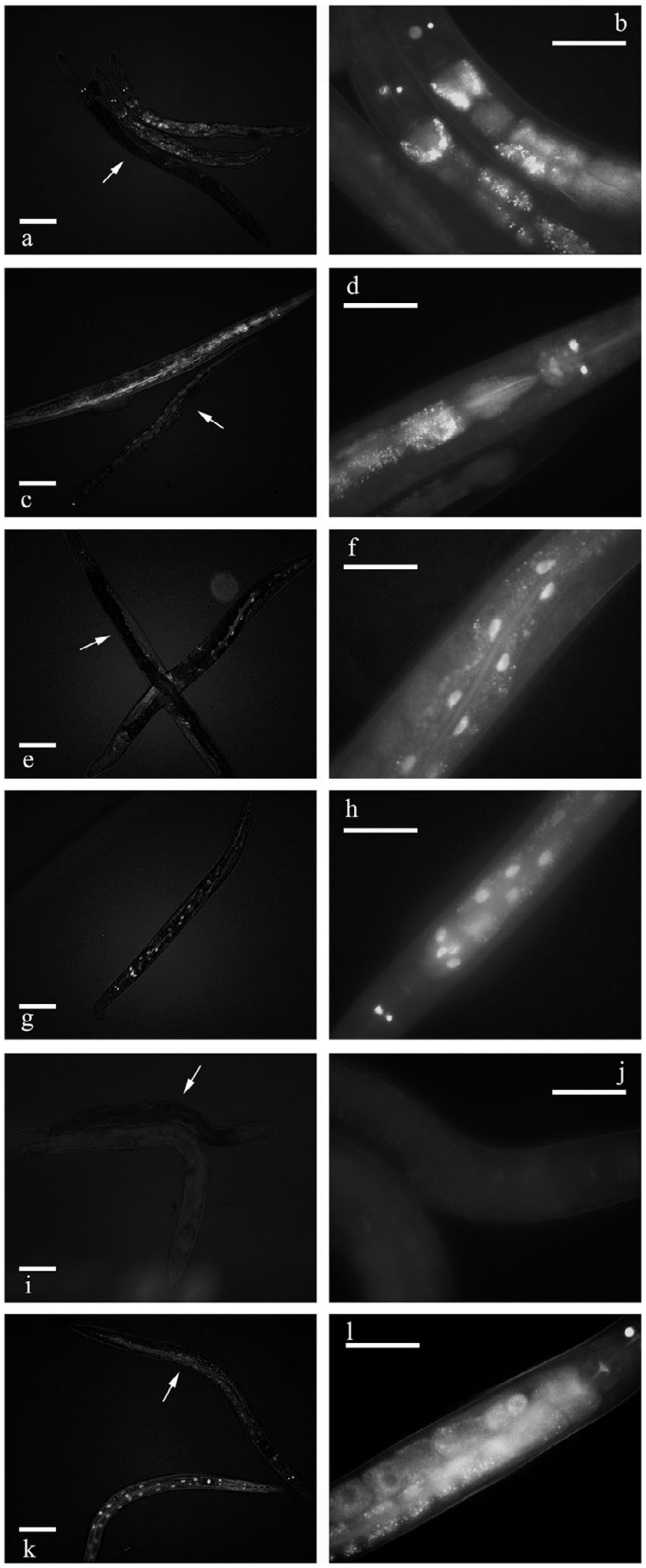

Figure 2 Oxidant-mediated induction of SKN-1–GFP

Transgenic worms containing an SKN-1–GFP translational fusion were exposed to oxidant, then reporter induction was monitored using fluorescence microscopy. (**a**, **b**) 0.001 % D3T (24 h); (**c**, **d**) hyperbaric oxygen (100 % O_2, 40 lbf/in^2, 24 h); (**e**, **f**) 10 mM H_2O_2 (20 min); (**g**, **h**) 2 % (w/v) sodium azide (15 min); (**i**, **j**) 225 μM juglone (15 min); (**k**, **l**) 225 μM juglone (15 min) followed by 2 % (w/v) sodium azide (15 min) after a 24 h recovery period. For each condition, an untreated, control worm is shown for comparison (left panels, arrow). Panels on the right are high-magnification images of images on the left. Scale bars, 100 μm.

reciprocal feedback interaction. The SKN-1–GFP reporter strain (LD007) was fed RNAi targeting components of the proteasome (including the α and β core subunits and multiple regulatory subunits). Knockdown of almost all subunits caused SKN-1–GFP to be activated (Table 2). 'Activation' was defined as nuclear relocalization, increased cytosolic fluorescence and/or the accumulation of GFP-containing gut granules. Examples of SKN-1–GFP animals activated by RNAi are shown in Figure 3.

gst-4 is induced by loss of proteasomal core subunits but not by loss of proteasomal regulatory subunits

Increased nuclear localization of SKN-1, and/or increased cytoplasmic levels of SKN-1, do not necessarily reflect SKN-1 transcriptional competency. To test the effect of proteasomal dysfunction on SKN-1 transcriptional activity, *gst*-4–GFP reporter worms were exposed to proteasomal RNAi. We observed that loss of proteasome α and β core subunits, and not loss of proteasomal regulatory subunits generally, induced *gst-4* expression (Figures 3k and 3l and Table 2).

gst-4 induction occurs without detectable activation of *skn-1* following removal of the chaperonin complex

We next assessed the role of chaperonins in regulation of the *skn-1* stress response. RNAi targeting chaperonin subunits led to a pattern of *gst-4* activation without recordable *skn-1* activation (Table 3). *gst-4* induction was nonetheless dependent on *skn-1*, since *gst-4*–GFP showed no inducibility in an *skn-1(zu67)* genetic background following exposure to chaperonin RNAi (Table 3).

skn-1 is activated following loss of ubiquitin hydrolases, but *gst-4* induction is variable

RNAi knockdown of multiple ubiquitin hydrolases caused *skn-1* to be constitutively activated in all cases that we examined (Table 4). Loss of some of these activities caused induction of *gst-4*. There appears to be no obvious correlation between *gst-4* induction and target gene function. Both the ubiquitin hydrolase RNAi- and the proteasome RNAi-studies present cases where SKN-1 can be relocalized to the nucleus, or cytosolically up-regulated without causing a corresponding induction of *gst-4* (Figure 3f and Table 4).

RNAi suppression screens reveal deubiquitination machinery is necessary for *skn-1* activation

Our previous experiments were designed only to detect aberrant reporter induction following RNAi-mediated removal of proteasome, chaperonin and deubiquitination target genes. We therefore asked if removal of these same genes by RNAi could instead block activation of the normal *skn-1* stress response under conditions where it should be induced. RNAi knockdown of proteasomal subunits or chaperonin subunits was unable to suppress either *skn-1* or *gst-4* induction following treatment with sodium azide (1 h) or 100 % oxygen (24 h) respectively (results not shown). RNAi suppression of components of the deubiquitination machinery, on the other hand, revealed that loss of five ubiquitin hydrolases suppressed the nuclear localization and/or cytoplasmic activation of *skn-1* following acute sodium azide treatment (Table 5). However, no RNAi treatment targeting these ubiquitin-processing enzymes blocked *gst-4* induction. This difference in suppressibility may reflect the substantially longer

Table 2 GFP reporter induction following proteasomal RNAi

Activation refers to nuclear translocation, increased cytosolic expression and/or the appearance of GFP-enriched gut granules. RNAi-induced phenotypes were highly penetrant (>90 %) within each treatment. Level of reporter activation/induction varied between stressors: '+++', '++', '+' and '–' represent strong, moderate, weak and no activation/induction of GFP respectively. reg., regulatory.

Gene	Ahringer ID	Description	*skn-1*::GFP activation	*gst-4*::GFP induction	*gst-4*::GFP; *skn-1(zu67)* induction
D1054.2	V-7M06	*pas-2*, proteasome component C3	++	++	–
C36B1.4	I-4G04	*pas-4*, proteasome A-type submit	+++	++	–
F25H2.9	I-5G02	*pas-5*, proteasome ζ chain	+++	+++	–
CD4.6	V-4G08	*pas-6*, protease	–	+++	–
K08D12.1	IV-1F23	*pbs-1*, (20S proteasome)	++	++	–
C47B2.4	I-6H19	*pbs-2*, proteasome A- and B-type	+++	++	–
Y38A8.2	II-4K15	*pbs-3*, peptidase	+++	+++	–
T20F5.2	I-1N17	*pbs-4*, proteasome β-subunit	+	–	–
K05C4.1	I-7E10	*pbs-5*, proteasome A- and B-type	+++	++	–
C02F5.9	III-4K04	*pbs-6*, proteasome component C5	+++	+++	–
F39H11.5	I-4E20	*pbs-7*, yeast NIP80 protein like	+++	–	–
T22D1.9	IV-3K24	*rpn-1*, proteasome reg. particle	+	–	–
C30C11.2	III-4D09	*rpn-3*, proteasome reg. particle	++	–	–
F10G7.8	II-3P14	*rpn-5*, proteasome reg. particle	++	–	–
F57B9.10	III-3N05	*rpn-6*, proteasome reg. particle	++	–	–
F49C12.8	IV-4D24	*rpn-7*, proteasome reg. particle	++	–	–
R12E2.3	I-1F10	*rpn-8*, proteasome reg. particle	++	–	–
B0205.3	I-5M08	*rpn-10*, proteasome reg. particle	++	–	–
K07D4.3	II-3L07	*rpn-11*, proteasome reg. particle	+++	+++	–
ZK20.5	II-7N16	*rpn-12*, proteasome reg. particle	++	++	–

test period used to detect *gst-4* versus *skn-1* induction. Very small amounts of nuclear-localized and transcriptionally competent SKN-1 may be sufficient to activate *gst*-4–GFP after extended periods. Consistent with this idea, *gst-4*–GFP induction was blocked for all RNAi clones, in all three suppression screens, when present in the *skn-1(zu67)* mutant background.

DISCUSSION

Oxidative stress has been implicated as a contributor to aging and age-related diseases because the oxidized forms of many proteins, lipids and other biomolecules have been shown to increase during the course of these processes [22,23]. Indeed, there is abundant evidence that oxidized forms of various biomolecules are involved in cancer, cardiovascular disease and neurodegenerative disorders [24–26]. To defend against damage caused by oxidizing compounds, cells have developed a well-conserved mechanism that can recognize and respond to a variety of electrophilic agents. At the heart of this regulation is the transcription factor NRF2 (in mammals) and SKN-1 (in *C. elegans*) [2,12,27–29]. By inducing an array of ARE-controlled genes, NRF2 and SKN-1 co-ordinately activate a battery of antioxidant and detoxifying enzymes that limit electrophile levels and reduce oxidative stress [13,30–32]. Among these inducible genes are the 26S proteasome components and the Phase II drug-metabolizing enzyme GST [19,32].

A variety of chemical and physiological treatments have been employed to induce oxidative stress in *C. elegans* [33,34]. We show here that most of these standard tests cause recognizable activation of SKN-1, the main electrophile-sensing regulator of AREs in *C. elegans*. For all oxidative stressors that we tested, activation of the ARE-containing gene *gst-4* was detected. SKN-1 activity was essential for *gst-4* activation, since in no instance after exposure to oxidative stress could *gst-4* be induced if animals lacked a functional *skn-1* gene. Unexpectedly, the redox cycling oxidant juglone caused no apparent activation of *skn-1*. Nevertheless, juglone induced *gst-4* and paradoxically this response required *skn-1*, since *gst*-4 activation was blocked in a mutant *skn-1* background. Two interpretations may explain this observation: juglone might not be an oxidative stressor in *C. elegans*; instead, it may be seen by cells as a xenobiotic and induce *gst-4* through alternate pathways in which overt *skn-1* activation is not visible. Alternatively, it is possible that some oxidative stress responses do not require an SKN-1 cytoplasmic response, but nevertheless still require nuclear localization of SKN-1.

In eukaryotic cells, most proteins are degraded by the ubiquitin/proteasome pathway, a complex enzymatic system that is responsible for protein quality control, antigen processing, signal transduction, cell cycle control, cell differentiation and apoptosis [35]. The 26S proteasome is a proteolytic machine, which degrades protein targets that have been marked by the covalent addition of polyubiquitin chains. The 26S proteasome is made up of different subcomplexes: a barrel-shaped 20S core containing proteolytic sites sequestered inside, and two 19S cap-like structures that execute regulatory functions such as substrate recognition, substrate unfolding and substrate translocation. The 20S core complex is composed of four rings: two inner rings, each composed of seven β-subunits, sandwiched beneath two outer rings, each composed of seven α-subunits. The 19S cap is composed of a base and a lid that are linked to each other via a regulatory particle subunit. The base is composed of a ring of different ATPase subunits, which interlock the α rings on both ends of the core 20S particle, plus additional non-ATPase subunits. The lid is composed of different, non-ATPase subunits (reviewed in [35]).

It is evolutionarily adaptive that the genes encoding the proteasomal complex should be co-ordinately up-regulated by oxidative stress: as more proteins become damaged by ROS there will be an increased need for protein degradation. We show here that dysfunction of the proteasome, and of other components of the cell's protein degradation machinery, activates *skn-1*, and in some cases also induces the ARE-containing gene, *gst-4*. The results suggest that proteasomes and ubiquitins are complexed in a feedback loop with SKN-1. The 20S

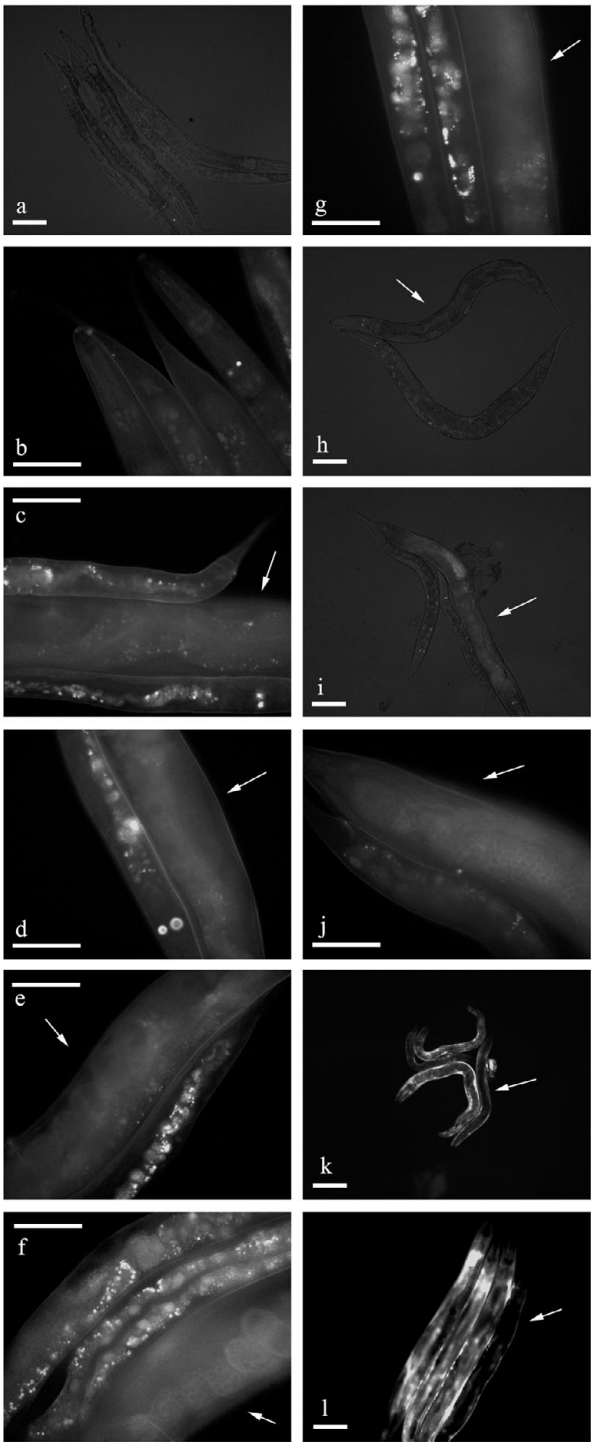

Figure 3 Removal of the proteasomal or deubiquitination machinery induces SKN-1–GFP and *gst-4*–GFP

Transgenic worms containing an SKN-1–GFP translational fusion (**a**–**j**), or a *gst-4*–GFP transcriptional fusion (**k**, **l**), were exposed, from the time of hatching, to RNAi against components of the proteasome or ubiquitin-specific hydrolases. Reporter induction was measured when vector-fed, control animals reached the fourth larval/young adult stage. Many RNAi treatments inhibited or delayed larval development. Representative fluorescence images of selected RNAi treatments are shown (see also Tables 2 and 4). (**a**, **b**) Vector control RNAi; (**c**) F57B9.10 (proteasome); (**d**) F49C12.8 (proteasome); (**e**) K08D12.1 (proteasome); (**f**) C04E6.5 (ubiquitin hydrolase); (**g**) R12E2.3 (proteasome); (**h**) T06D8.8 (proteasome); (**i**, **j**) Y110A7A.14 (proteasome); (**k**) F25H2.9 (proteasome); (**l**) ZK20.5 (proteasome). T06D8.8 and Y110A7A.14 did not alter SKN-1–GFP relative to vector control and are not included in Table 2. Vector-fed, control worms are included in (**c**–**l**) for comparison (arrow). Scale bars, 100 μm.

proteasome can directly degrade oxidatively damaged proteins, without assistance from the ubiquitination machinery, through direct recognition of oxidatively induced hydrophobic patches [36]. Increased expression of proteasome components following oxidative stress, and especially the 20S catalytic core, therefore presumably acts to facilitate the removal of oxidatively damaged proteins. These considerations make the differences we observed in *gst-4* induction by proteasomal RNAi even more interesting: loss of almost any proteasomal component activated SKN-1, yet only loss of the proteasomal core components generally induced *gst-4*. This difference might also be interpreted as suggesting that proteasomal regulatory subunits play additional roles in permitting SKN-1 to attain transcriptional competency (see below).

Many polyubiquitinated proteins are degraded by the proteasomes. Increased expression of ubiquitinating enzymes could therefore theoretically also increase the removal of damaged proteins following oxidative stress. Similarly, blockage of deubiquitination activity could likewise increase the removal of oxidatively damaged proteins. Unexpectedly, we observed that RNAi-mediated knockdown of several deubiquitinating enzymes led, in all cases, to *skn-1* nuclear localization and/or cytosolic up-regulation. Interestingly, though, only in some of these instances was ARE-dependent activation of *gst-4* detected. Taken together, all our observations support the notion that there are multiple pathways connecting proteasomal activity and the cellular ubiquitination machinery to the *skn-1* stress-response system. Our findings suggest that cross-talk occurs between these protein-regulating systems – with SKN-1 controlling proteasomal subunit expression and, reciprocally, proteasomal activity controlling SKN-1 nuclear localization and/or transcriptional competency.

The chaperonins comprise a family of high-molecular-mass chaperones that co-assemble into a two-ringed chamber to assist in nascent protein folding. The chaperonin complex [also called TriC (TCP-1 ring complex)] functions in an ATP-dependent manner and it has been estimated that $\sim$10 % of newly synthesized proteins interact with it [37]. The chaperonin complex is unique in its ability to fold certain proteins that cannot be folded by more simple chaperone systems. In the present study, we observed that RNAi-mediated removal of any one of six of the eight chaperonin subunits in *C. elegans* (the other two subunits were not tested), induced *gst-4* in an *skn-1*-dependent manner, although without any discernible relocalization or change in *skn-1* expression level. These results suggest that chaperonin disruption might cause a signal to be sent from the cytoplasm to *gst-4* in the nucleus via a route independent of cytosolic SKN-1. This response is similar to what was observed using juglone. Alternatively, in both cases – chaperonins and juglone – it is possible that the cytoplasmic response of SKN-1 was just too small to be observed. This idea, however, begs the question of how and why some stress stimuli cause a tiny activation of *skn-1* while others produce a very large activation. Furthermore, we are also left pondering whether conditions, in addition to nuclear translocation, are required to make SKN-1 transcriptionally competent to permit ARE induction. One such possibility that might explain why loss of the chaperonin complex induces *gst-4* expression without obvious alteration of *skn*-1 activity is that low levels of SKN-1 may be present at AREs constitutively, but they are kept in an inactive state by repressor proteins. Chaperonins may be required for the efficient folding of such repressors and hence their loss would naturally lead to ARE activation. Support for this idea comes from recent studies showing the co-repressor SMRT (silencing mediator for retinoic acid receptor and thyroid-hormone receptor) can bind and inactivate NRF2 [38].

Table 3 GFP reporter induction following chaperonin RNAi

Activation refers to nuclear translocation, increased cytosolic expression and/or the appearance of GFP-enriched gut granules. RNAi-induced phenotypes were highly penetrant (>90 %) within each treatment. Level of reporter activation/induction varied between stressors: '+++', '++' and '–' represent strong, moderate and no activation/induction of GFP respectively.

Gene	Ahringer ID	Description	*skn-1*::GFP activation	*gst-4*::GFP induction	*gst-4*::GFP; *skn-1(zu67)* induction
T05C12.7	II-6C15	Chaperonin TCP-1, subunit 1 (α)	–	+++	–
T21B10.7	II-6O24	Chaperonin TCP-1, subunit 2 (β)	–	+++	–
K01C8.10	II-6G17	Chaperonin TCP-1, subunit 4 (δ)	–	+++	–
C07G2.3	III-2C10	Chaperonin TCP-1, subunit 5 (ε)	–	++	–
F01F1.8	III-3G13	Chaperonin TCP-1, subunit 6 (ζ)	–	++	–
T10B5.5	V-1N06	Chaperonin TCP-1, subunit 7 (η)	–	+++	–

Table 4 GFP reporter induction following ubiquitin-specific protease RNAi

Activation refers to nuclear translocation, increased cytosolic expression and/or the appearance of GFP-enriched gut granules. RNAi-induced phenotypes were highly penetrant (>90 %) within each treatment. Level of reporter activation/induction varied between stressors: '+++', '++', '+', and '–' represent strong, moderate, weak and no activation/induction of GFP respectively.

Gene	Ahringer ID	Description	*skn-1*::GFP activation	*gst-4*::GFP induction	*gst-4*::GFP; *skn-1(zu67)* induction
K08B4.5	IV-3I03	Similar to ubiquitin C-terminal hydrolase	++	–	–
F29C4.5	IV-1C15	*duo-2*, ubiquitin-specific protease	++	–	–
Y106G6H.12	I-5A16	*duo-3*, ubiquitin C-terminal hydrolase	+++	–	–
F46E10.8	V-4F02	*ubh-1*, ubiquitin C-terminal hydrolase	+	–	–
C08B11.7	II-5N18	*ubh-4*, thiolesterase (ubiquitin C-terminal hydrolase)	++	–	–
H34C03.2	IV-3G06	Ubiquitin C-terminal hydrolase	++	–	–
T27A3.2	I-2J12	Ubiquitin C-terminal hydrolase	++	–	–
T05H10.1	II-5N24	Ubiquitin C-terminal hydrolase	++	–	–
K02C4.3	II-5P18	Ubiquitin C-terminal hydrolase	++	–	–
C34F6.9	X-5K20	Ubiquitin C-terminal hydrolase	++	–	–
K09A9.4	X-7K21	Ubiquitin C-terminal hydrolase	+++	–	–
F37B12.4	II-6D15	Ubiquitin C-terminal hydrolase	++	+++	–
H19N07.2	V-7H21	Ubiquitin C-terminal hydrolase	++	+++	–
C04E6.5	V-4B03	Similar to ubiquitin C-terminal hydrolase	++	++	–
R10E11.3	III-5A10	Ubiquitin-specific processing protease	++	++	–
K08B4.5	IV-3103	Ubiquitin C-terminal hydrolase	++	++	–

Table 5 Suppression of GFP reporter inducibility by ubiquitin-specific protease RNAi

Activation refers to nuclear translocation, increased cytosolic expression and/or the appearance of GFP-enriched gut granules. RNAi-induced phenotypes were highly penetrant (>90 %) within each treatment. Level of reporter activation/induction varied between stressors: '+++', '++' and '–', represent strong, moderate and no activation/induction of GFP respectively.

Gene	Ahringer ID	Description	*skn-1*::GFP activation	*gst-4*::GFP induction	*gst-4*::GFP; *skn-1(zu67)* induction
K08B4.5	IV-3I03	Similar to ubiquitin C-terminal hydrolase	++	+++	–
F38B7.5	V-7J06	*duo-1*, ubiquitin C-terminal hydrolase	++	+++	–
F29C4.5	IV-1C15	*duo-2*, ubiquitin-specific protease	++	+++	–
Y106G6H.12	I-5A16	*duo-3*, ubiquitin C-terminal hydrolase	++	+++	–
F46E10.8	V-4F02	*ubh-1*, ubiquitin C-terminal hydrolase	++	+++	–
C08B11.7	II-5N18	*ubh-4*, thiolesterase (ubiquitin C-terminal hydrolase)	++	+++	–
H34C03.2	IV-3G06	Ubiquitin C-terminal hydrolase	–	+++	–
T27A3.2	I-2J12	Ubiquitin C-terminal hydrolase	–	+++	–
T05H10.1	II-5N24	Ubiquitin C-terminal hydrolase	++	+++	–
K02C4.3	II-5P18	Ubiquitin C-terminal hydrolase	–	+++	–
C34F6.9	X-5K20	Ubiquitin C-terminal hydrolase	++	+++	–
K09A9.4	X-7K21	Ubiquitin C-terminal hydrolase	++	+++	–
F37B12.4	II-6D15	Ubiquitin C-terminal hydrolase	–	+++	–
H19N07.2	V-7H21	Ubiquitin C-terminal hydrolase	++	+++	–
C04E6.5	V-4B03	Similar to ubiquitin C-terminal hydrolase	++	+++	–
R10E11.3	III-5A10	Ubiquitin-specific processing protease	–	+++	–
K08B4.5	IV-3103	Ubiquitin C-terminal hydrolase	++	+++	–

The current model for NRF2 function does not explain our RNAi data for *skn-1* in *C. elegans*. Based on what is known regarding the function of NRF2, we would expect the same response from all perturbations of protein degradation: build-up of damaged or misfolded proteins should cause metabolic stress and ultimately oxidative damage, in turn activating SKN-1 to induce AREs. Instead, we have observed that only selective components of the protein degradation system activate AREs via *skn*-1. Specifically, RNAi of proteasome core components generally induces *gst-4*; however, RNAi of proteasome regulatory units does not. Also, among deubiquitinating enzymes, *skn-1* activation by RNAi does not necessarily result in the activation of *gst-4*. Additionally, chaperonins do not visibly activate *skn-1*, but nevertheless cause induction of *skn-1*-mediated AREs. Clearly, a more complex model for *skn-1* regulation in *C. elegans* is required. Our results indicate that there are several stress-response mechanisms regulating *skn-1* activation remaining to be elucidated.

Previous revelations regarding the complex functional roles of the ubiquitin–proteasome system in proteolysis, protein unfolding, translocation and transcription point towards a potentially complex relationship between the proteasomes and NRF2/SKN-1. Because mutations in 19S ATPases affect transcription [39,40], and 19S ATPases have been found on promoter DNA *in vivo* [41,42], there is the possibility that *C. elegans* proteasomal regulatory particles play a required role in the transcriptional regulation of AREs. Perhaps when the 19S proteasome subunit was made dysfunctional by RNAi in worms, transcriptional initiation of AREs, such as those in *gst-4*, was impeded. Another consideration involves the observation that ubiquitin plays a direct and dynamic role in gene regulation: ubiquitin-mediated proteolysis, monoubiquitination and deubiquitination have each been implicated in the transcription of different genes [43–45]. In addition, the association between chromatin and 19S ATPase requires the ubiquitination of histone H2B [42]. Thus RNAi knockdown of proteasome-regulatory components and of some deubiquitinating enzymes may prevent a proteolytic activity necessary for transcription of *gst-4*. While these hypotheses might help to explain our observations for RNAi against the proteasome and ubiquitin proteases, they do not fully address the unusual results seen using RNAi against chaperonin subunits. Future work is needed to clarify the mechanisms involved in the complex regulation of the NRF2/SKN-1 antioxidant stress response.

We thank Dr Christopher Link (Institute for Behavioral Genetics, University of Colorado, Boulder, CO, U.S.A.) for reagents, construction of CL691 and constructive criticism on the manuscript before its submission. Financial support was provided by the National Institute of Aging and the Polis Foundation (S.L.R. and T.E.J.) and the Ellison Medical Foundation (T.E.J.).

REFERENCES

1 Bowerman, B., Eaton, B. A. and Priess, J. R. (1992) Skn-1, a maternally expressed gene required to specify the fate of ventral blastomeres in the early *C. elegans* embryo. Cell **68**, 1061–1075
2 An, J. H. and Blackwell, T. K. (2003) SKN-1 links *C. elegans* mesendodermal specification to a conserved oxidative stress response. Genes Dev. **17**, 1882–1893
3 Kwak, M. K., Wakabayashi, N., Itoh, K., Motohashi, H., Yamamoto, M. and Kensler, T. W. (2003) Modulation of gene expression by cancer chemopreventive dithiolethiones through the Keap1–Nrf2 pathway. Identification of novel gene clusters for cell survival. J. Biol. Chem. **278**, 8135–8145
4 Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., Igarashi, K., Engel, J. D. and Yamamoto, M. (1999) Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. Genes Dev. **13**, 76–86
5 Itoh, K., Wakabayashi, N., Katoh, Y., Ishii, T., O'Connor, T. and Yamamoto, M. (2003) Keap1 regulates both cytoplasmic–nuclear shuttling and degradation of Nrf2 in response to electrophiles. Genes Cells **8**, 379–391
6 McMahon, M., Itoh, K., Yamamoto, M. and Hayes, J. D. (2003) Keap1-dependent proteasomal degradation of transcription factor Nrf2 contributes to the negative regulation of antioxidant response element-driven gene expression. J. Biol. Chem. **278**, 21592–21600
7 McMahon, M., Thomas, N., Itoh, K., Yamamoto, M. and Hayes, J. D. (2004) Redox-regulated turnover of Nrf2 is determined by at least two separate protein domains, the redox-sensitive Neh2 degron and the redox-insensitive Neh6 degron. J. Biol. Chem. **279**, 31556–31567
8 Zhang, D. D. and Hannink, M. (2003) Distinct cysteine residues in Keap1 are required for Keap1-dependent ubiquitination of Nrf2 and for stabilization of Nrf2 by chemopreventive agents and oxidative stress. Mol. Cell. Biol. **23**, 8137–8151
9 Numazawa, S., Ishikawa, M., Yoshida, A., Tanaka, S. and Yoshida, T. (2003) Atypical protein kinase C mediates activation of NF-E2-related factor 2 in response to oxidative stress. Am. J. Physiol. Cell Physiol. **285**, C334–C342
10 Kang, K. W., Lee, S. J., Park, J. W. and Kim, S. G. (2002) Phosphatidylinositol 3-kinase regulates nuclear translocation of NF-E2-related factor 2 through actin rearrangement in response to oxidative stress. Mol. Pharmacol. **62**, 1001–1010
11 Bloom, D. A. and Jaiswal, A. K. (2003) Phosphorylation of Nrf2 at Ser40 by protein kinase C in response to antioxidants leads to the release of Nrf2 from INrf2, but is not required for Nrf2 stabilization/accumulation in the nucleus and transcriptional activation of antioxidant response element-mediated NAD(P)H:quinone oxidoreductase-1 gene expression. J. Biol. Chem. **278**, 44675–44682
12 Ishii, T., Itoh, K., Takahashi, S., Sato, H., Yanagawa, T., Katoh, Y., Bannai, S. and Yamamoto, M. (2000) Transcription factor Nrf2 coordinately regulates a group of oxidative stress-inducible genes in macrophages. J. Biol. Chem. **275**, 16023–16029
13 Talalay, P., Dinkova-Kostova, A. T. and Holtzclaw, W. D. (2003) Importance of phase 2 gene regulation in protection against electrophile and reactive oxygen toxicity and carcinogenesis. Adv. Enzyme Regul. **43**, 121–134
14 An, J. H., Vranas, K., Lucke, M., Inoue, H., Hisamoto, N., Matsumoto, K. and Blackwell, T. K. (2005) Regulation of the *Caenorhabditis elegans* oxidative stress defense protein SKN-1 by glycogen synthase kinase-3. Proc. Natl. Acad. Sci. U.S.A. **102**, 16275–16280
15 Kell, A., Ventura, N., Kahn, N. and Johnson, T. E. (2007) Activation of SKN-1 by novel kinases in *Caenorhabditis elegans*. Free Radical Biol. Med. **43**, 1560–1566
16 Tawe, W. N., Eschbach, M. L., Walter, R. D. and Henkle-Duhrsen, K. (1998) Identification of stress-responsive genes in *Caenorhabditis elegans* using RT–PCR differential display. Nucleic Acids Res. **26**, 1621–1627
17 Fahey, J. W. and Talalay, P. (1999) Antioxidant functions of sulforaphane: a potent inducer of phase II detoxication enzymes. Food Chem. Toxicol. **37**, 973–979
18 Link, C. D. and Johnson, C. J. (2002) Reporter transgenes for study of oxidant stress in *Caenorhabditis elegans*. Methods Enzymol. **353**, 497–505
19 Kwak, M. K., Wakabayashi, N., Greenlaw, J. L., Yamamoto, M. and Kensler, T. W. (2003) Antioxidants enhance mammalian proteasome expression through the Keap1–Nrf2 signaling pathway. Mol. Cell. Biol. **23**, 8786–8794
20 Link, C. D., Cypser, J. R., Johnson, C. J. and Johnson, T. E. (1999) Direct observation of stress response in *Caenorhabditis elegans* using a reporter transgene. Cell Stress Chaperones **4**, 235–242
21 Rea, S. L., Ventura, N. and Johnson, T. E. (2007) Relationship between mitochondrial electron transport chain dysfunction, development and life extension in *C. elegans*. PLoS Biol. **5**, e259
22 Shringarpure, R. and Davies, K. J. (2002) Protein turnover by the proteasome in aging and disease. Free Radical Biol. Med. **32**, 1084–1089
23 Kumar, G. N., Houtz, R. L. and Knowles, N. R. (1999) Age-induced protein modifications and increased proteolysis in potato seed-tubers. Plant Physiol. **119**, 89–100
24 Miller, J. A. (1970) Carcinogenesis by chemicals: an overview – G.H.A. Clowes memorial lecture. Cancer Res. **30**, 559–576
25 Halliwell, B., Gutteridge, J. M. and Cross, C. E. (1992) Free radicals, antioxidants, and human disease: where are we now? J. Lab. Clin. Med. **119**, 598–620
26 Halliwell, B. (2001) Role of free radicals in the neurodegenerative diseases: therapeutic implications for antioxidant treatment. Drugs Aging **18**, 685–716
27 Itoh, K., Chiba, T., Takahashi, S., Ishii, T., Igarashi, K., Katoh, Y., Oyake, T., Hayashi, N., Satoh, K., Hatayama, I. et al. (1997) An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. Biochem. Biophys. Res. Commun. **236**, 313–322
28 Venugopal, R. and Jaiswal, A. K. (1996) Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD(P)H:quinone oxidoreductase1 gene. Proc. Natl. Acad. Sci. U.S.A. **93**, 14960–14965

29 Motohashi, H., O'Connor, T., Katsuoka, F., Engel, J. D. and Yamamoto, M. (2002) Integration and diversity of the regulatory network composed of Maf and CNC families of transcription factors. Gene **294**, 1–12
30 Primiano, T., Sutter, T. R. and Kensler, T. W. (1997) Antioxidant-inducible genes. Adv. Pharmacol. **38**, 293–328
31 Rushmore, T. H., Morton, M. R. and Pickett, C. B. (1991) The antioxidant responsive element. Activation by oxidative stress and identification of the DNA consensus sequence required for functional activity. J. Biol. Chem. **266**, 11632–11639
32 Friling, R. S., Bensimon, A., Tichauer, Y. and Daniel, V. (1990) Xenobiotic-inducible expression of murine glutathione S-transferase Ya subunit gene is controlled by an electrophile-responsive element. Proc. Natl. Acad. Sci. U.S.A. **87**, 6258–6262
33 Johnson, T. E., de Castro, E., Hegi de Castro, S., Cypser, J., Henderson, S. and Tedesco, P. (2001) Relationship between increased longevity and stress resistance as assessed through gerontogene mutations in *Caenorhabditis elegans*. Exp. Gerontol. **36**, 1609–1617
34 Cypser, J. R. and Johnson, T. E. (2002) Multiple stressors in *Caenorhabditis elegans* induce stress hormesis and extended longevity. J. Gerontol. A Biol. Sci. Med. Sci. **57**, B109–B114
35 Collins, G. A. and Tansey, W. P. (2006) The proteasome: a utility tool for transcription? Curr. Opin. Genet. Dev. **16**, 197–202
36 Davies, K. J. (2001) Degradation of oxidized proteins by the 20S proteasome. Biochimie **83**, 301–310
37 Spiess, C., Meyer, A. S., Reissmann, S. and Frydman, J. (2004) Mechanism of the eukaryotic chaperonin: protein folding in the chamber of secrets. Trends Cell Biol. **14**, 598–604
38 Ki, S. H., Cho, I. J., Choi, D. W. and Kim, S. G. (2005) Glucocorticoid receptor (GR)-associated SMRT binding to C/EBPbeta TAD and Nrf2 Neh4/5: role of SMRT recruited to GR in GSTA2 gene repression. Mol. Cell. Biol. **25**, 4150–4165
39 Swaffield, J. C., Bromberg, J. F. and Johnston, S. A. (1992) Alterations in a yeast protein resembling HIV Tat-binding protein relieve requirement for an acidic activation domain in GAL4. Nature **357**, 698–700
40 Rubin, D. M., Coux, O., Wefes, I., Hengartner, C., Young, R. A., Goldberg, A. L. and Finley, D. (1996) Identification of the gal4 suppressor Sug1 as a subunit of the yeast 26S proteasome. Nature **379**, 655–657
41 Gonzalez, F., Delahodde, A., Kodadek, T. and Johnston, S. A. (2002) Recruitment of a 19S proteasome subcomplex to an activated promoter. Science **296**, 548–550
42 Ezhkova, E. and Tansey, W. P. (2004) Proteasomal ATPases link ubiquitylation of histone H2B to methylation of histone H3. Mol. Cell **13**, 435–442
43 Dennis, A. P., Lonard, D. M., Nawaz, Z. and O'Malley, B. W. (2005) Inhibition of the 26S proteasome blocks progesterone receptor-dependent transcription through failed recruitment of RNA polymerase II. J. Steroid Biochem. Mol. Biol. **94**, 337–346
44 Lipford, J. R., Smith, G. T., Chi, Y. and Deshaies, R. J. (2005) A putative stimulatory role for activator turnover in gene expression. Nature **438**, 113–116
45 Zhang, Y. (2003) Transcriptional regulation by histone ubiquitination and deubiquitination. Genes Dev. **17**, 2733–2740

Received 16 April 2007/20 August 2007; accepted 23 August 2007
Published as BJ Immediate Publication 23 August 2007, doi:10.1042/BJ20070521