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Condition-adapted stress and longevity gene regulation by *Caenorhabditis elegans* SKN-1/Nrf

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Summary

Studies in model organisms have identified regulatory processes that profoundly influence aging, many of which modulate resistance against environmental or metabolic stresses. In *Caenorhabditis elegans,* the transcription regulator SKN-1 is important for oxidative stress resistance and acts in multiple longevity pathways. SKN-1 is the ortholog of mammalian Nrf proteins, which induce Phase 2 detoxification genes in response to stress. Phase 2 enzymes defend against oxygen radicals and conjugate electrophiles that are produced by Phase 1 detoxification enzymes, which metabolize lipophilic compounds. Here, we have used expression profiling to identify genes and processes that are regulated by SKN-1 under normal and stress–response conditions. Under nonstressed conditions SKN-1 upregulates numerous genes involved in detoxification, cellular repair, and other functions, and downregulates a set of genes that reduce stress resistance and lifespan. Many of these genes appear to be direct SKN-1 targets, based upon presence of predicted SKN-binding sites in their promoters. The metalloid sodium arsenite induces *skn-1*-dependent activation of certain detoxification gene groups, including some that were not SKN-1-upregulated under normal conditions. An organic peroxide also triggers induction of a discrete Phase 2 gene set, but additionally stimulates a broad SKN-1-independent response. We conclude that under normal conditions SKN-1 has a wide range of functions in detoxification and other processes, including modulating mechanisms that reduce lifespan. In response to stress, SKN-1 and other regulators tailor transcription programs to meet the challenge at hand. Our findings reveal striking complexity in SKN-1 functions and the regulation of systemic detoxification defenses.

Key words: aging; *Caenorhabditis elegans*; detoxification; insulin signaling; oxidative stress; SKN-1.

Introduction

Living organisms are subjected to stress caused by reactive oxygen species (ROS) or electrophiles that are derived from metabolism of various compounds. Cellular damage caused by oxidative stress has been implicated in conditions that include diabetes, atherosclerosis, many neurodegenerative syndromes, and aging (Droge, 2002). It is important to understand how organisms defend themselves against this damage at the systemic level. For example, several transcription factors that promote resistance to free radicals have been associated with extended longevity in model organisms, including worms, flies, and mice (Lithgow & Walker, 2002; Kenyon, 2005; Guarente, 2007; Tullet *et al.*, 2008).

Eukaryotes defend themselves from toxic or reactive compounds through a three-phase detoxification system (Xu *et al.*, 2005; Sarkadi *et al.*, 2006). During Phase 1, lipophilic endobiotics or xenobiotics are solubilized through modification by enzymes, such as cytochrome P450s (CYPs) and short-chain dehydrogenases/reductases (SDRs). This process allows these compounds to be excreted, but may also produce damaging reactive compounds. The Phase 2 enzymes defend cells against such compounds, as well as ROS. They encompass a diverse group of enzymes that metabolize free radicals, repair cellular structures, or directly conjugate xenobiotics and peroxidized lipids, including glutathione-*S*-transferases (GSTs) and UDP-glucuronosyl/glucosyl transferases (UGTs). In Phase 3, conjugated toxins are pumped out of the cell by ATP-binding cassette (ABC) or other transporters (Sarkadi *et al.*, 2006). How these systems contribute to the functions of different tissues, how they are regulated in the context of an organism, and how this regulation might be adapted to different stress scenarios are all important questions.

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Current data suggest that Phase 2 genes may be regulated as a co-ordinated network. In mammals, many Phase 2 genes are induced directly by the Nrf1 and Nrf2 (NF-E2-related factor) proteins (Nguyen *et al.*, 2003; Kobayashi & Yamamoto, 2006). In the cell types examined thus far Nrf proteins are predominantly cytoplasmic, but in response to stress they accumulate in nuclei and upregulate Phase 2 gene expression. Mice that lack Nrf2 are sensitive to ROS and other toxic insults, but it is problematic to evaluate how a complete lack of Nrf proteins affects the intact mouse because *Nrf1−/−; Nrf2−/−* mice embryos die by day 10 (Leung *et al.*, 2003).

As the Phase 2 network is broadly conserved (Jasper, 2008), it is possible to employ simpler model organisms to study its regulation and functions. In the nematode *Caenorhabditis elegans*, the Nrf ortholog SKN-1 inducibly regulates expression of candidate Phase 2 genes in the intestine, the digestive system equivalent, and *skn-1* mutants are highly sensitive to oxidative stress (An & Blackwell, 2003; An *et al.*, 2005; Inoue *et al.*, 2005). SKN-1 accumulates in intestinal nuclei in response to stress and is inhibited from doing so constitutively by mechanisms that include phosphorylation by glycogen synthase kinase-3 and the conserved insulin/IGF-1-like signaling (IIS) pathway (An *et al.*, 2005; Tullet *et al.*, 2008). In *C. elegans*, IIS is initiated by binding of insulin-like peptides to the receptor DAF-2, which leads eventually to activation of the downstream IIS kinases AKT-1/2 and SGK-1. These kinases phosphorylate and inhibit SKN-1 in parallel to the FOXO transcription factor DAF-16 (Tullet *et al.*, 2008), which regulates genes involved in numerous biological processes, including stress resistance (Murphy *et al.*, 2003; Kenyon & Murphy, 2006; Oh *et al.*, 2006; Dong *et al.*, 2007; McElwee *et al.*, 2007; Samuelson *et al.*, 2007). It is still unknown whether SKN-1 might simply regulate a suite of Phase 2 genes, or is involved more broadly in control of stress defense or other genes.

Multiple lines of evidence implicate SKN-1 in *C. elegans* longevity. For example, reductions in IIS delay aging and increase stress resistance in diverse organisms (Kenyon, 2005). While it is well established that in *C. elegans* these benefits of reduced IIS require DAF-16, it has been shown recently that SKN-1 also contributes to these effects (Tullet *et al.*, 2008). In addition, SKN-1 delays aging under normal conditions, at least in part through its action in the intestine (An & Blackwell, 2003; Tullet *et al.*, 2008). Finally, *skn-1* is required for lifespan extension by calorie restriction (CR), a condition that promotes longevity in all eukaryotes tested thus far (Bishop & Guarente, 2007b). This last SKN-1 function is mediated by its expression in the two ASI neurons (Bishop & Guarente, 2007b), which sense or regulate food intake (You *et al.*, 2008). These observations indicate that SKN-1 has important functions under nonstressed as well as stress conditions. It remains to be determined whether SKN-1 regulates similar sets of genes under normal and stress–response conditions, and how these genes influence stress resistance and longevity.

Here, we have used expression profiling to investigate how SKN-1 influences *C. elegans* gene expression under normal conditions, and in response to two different sources of oxidative stress, the metalloid sodium arsenite (As) and *tert*-butyl hydrogen peroxide (*t*-BOOH). Arsenite is a highly toxic trivalent form of the environmentally pervasive metalloid arsenic. It attacks thiol groups on glutathione and other polypeptides, and stimulates ROS production (Hughes, 2002). The stable organoperoxide *t*-BOOH attacks cellular proteins and lipids, and is also scavenged by glutathione (Mathews *et al.*, 1994). *skn-1* mutants are sensitive to each of these stresses (An *et al.*, 2005; Inoue *et al.*, 2005). We find that under normal conditions SKN-1 regulates expression of numerous genes, many of which may be direct targets. These genes are involved in processes that include detoxification and stress resistance, lysosome and proteasome function, metabolism, and cell-surface recognition. Interestingly, SKN-1 also suppresses expression of many genes that decrease stress resistance and lifespan, including the insulin-like peptide *ins-7* and the IIS pathway kinase *pdk-1*. Treatment with As results in activation of a particular group of SKN-1 dependent detoxification genes. By contrast, *t*-BOOH treatment also mobilizes a broad SKN-1-independent stress response. Some functional clusters of genes are regulated by SKN-1 specifically under normal or particular stress conditions, indicating that unknown signals interact with SKN-1 to restrict its activities. Our findings identify a complex set of processes that are regulated by SKN-1 under normal conditions, and reveal that SKN-1 acts together with other regulators in specialized responses to exogenous stresses.

Results

We used oligonucleotide microarrays to compare expression profiles of worms that had been treated with RNA interference (RNAi) against *skn-1* [*skn-1(–)*], or control (*gfp*) RNAi [*skn-1(+)*] from hatching (Fig. 1). We examined synchronized L4 stage larvae, in which stress robustly induces intestinal expression of the SKN-1 target gene *gcs-1* (An & Blackwell, 2003). To investigate how SKN-1 responds to stress, worms were exposed to As or *t*-BOOH or incubated under the corresponding control conditions [Nematode Growth Medium (NGM) agar plates or M9 liquid media, respectively]. In applying stress-inducing agents, we titrated the concentrations used and the time of exposure so that *gcs-1* was induced comparably (Supporting Fig. S1), and at least 95% of the animals consistently survived the treatment (not shown).

SKN-1 regulates stress-related and other genes under normal conditions

We first searched for genes that are regulated by SKN-1 under normal (nonstressed) conditions, by comparing the expression profiles of *skn-1(+)* and *skn-1(–)* wild-type (N2) animals that served as controls for our stress-treatment experiments (Fig. 1, red arrow). We compared these seven sets of *skn-1(+)* and *skn-1(–)* samples using hierarchical clustering (Eisen *et al.*, 1998) and statistical analysis of microarrays (SAM) (Tusher *et al.*, 2001). In performing SAM, we adjusted the delta value to 1.023, resulting

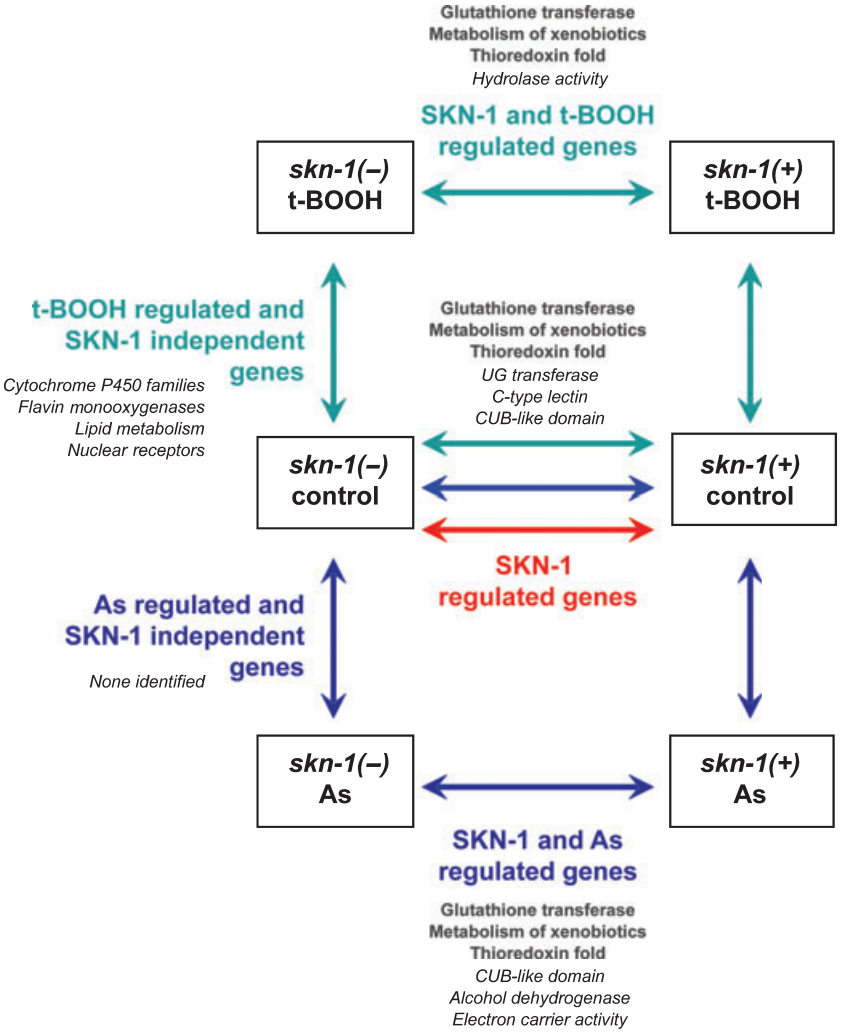

Fig. 1 Identification of SKN-1-regulated genes. mRNA samples were generated under the indicated conditions, with *skn-1(–)* referring to *skn-1* RNAi and *skn-1(+)* to RNAi control. Pairs of samples designated by arrows were compared on Agilent 4 × 44 oligonucleotide microarrays to identify genes that are regulated by SKN-1 under normal conditions (red arrow), and in response to treatment with Arsenite (As) (dark blue arrows), or *tert*-butyl hydroperoxide (*t*-BOOH) (teal arrows). Genes that are regulated by SKN-1 under normal conditions were identified by both SAM and hierarchical clustering (red arrow). *skn-1*-dependent and -independent genes that respond to As or *t*-BOOH stress were identified by hierarchical clustering. While the As-induced response seemed to be entirely dependent upon *skn-1*, *t*-BOOH induced both *skn-1*-dependent and independent gene sets (see text). Some gene categories that we identified as being prominent in stress- and SKN-1-upregulated gene sets are listed in bold, with those that were over-represented in only one or two sets indicated in italics.

in an expected false positive rate of 1.7%. These analyses identified 233 genes for which expression was significantly reduced in *skn-1(–)* animals (SKN-1-upregulated genes; Fig. 2a; Table 1; Supporting Table S1). The extent of this SKN-1-upregulated profile was unexpected, because under nonstressed conditions SKN-1 is seen at relatively low levels in intestinal nuclei (An & Blackwell, 2003). We also identified 63 genes for which expression was increased in *skn-1(–)* animals, indicating that they are downregulated by SKN-1 (SKN-1-downregulated genes; Fig. 3a; Table 2; Supporting Table S2). As an independent test of these results, we assessed the relative levels of representative SKN-1-regulated mRNAs in *skn-1(+)* and *skn-1(–)* animals using quantitative (q)RT-PCR. These results concurred with our microarray experiments for both SKN-1-upregulated (13 of 13) and -downregulated (10 of 10) genes (Supporting Tables S3 and S4).

We investigated the functions of SKN-1-upregulated genes by using the Database for Annotation, Visualization and Integrated Discovery (DAVID) to analyze the statistical representation of functional gene categories, as defined by gene ontology (GO) terms or predicted protein domains. The GO categories for glutathione transferase, xenobiotic metabolism, thioredoxin fold, and UGT were highly over-represented among the SKN-1-upregulated genes (Fig. 2d), 19% of which overall are involved in detoxification or stress responses (Fig. 2b; Supporting Table S1). In addition, numerous Phase 2 genes were among the SKN-1-upregulated genes with the highest SAM scores, including the known SKN-1 target *gst-4* (Kahn *et al.*, 2008; Tullet *et al.*, 2008), other GSTs (*gst-10, -13, -38, F56A4.4, C02D5.3*), and UGTs (*ugt-16, K04A8.10*) (Table 1). The SKN-1-upregulated genes also included some known or predicted Phase 1 detoxification genes [aldo/keto reductase proteins (*C07D8.6, T08H10.1*), a CYP (*cyp-14A.1*), and multiple SDRs (*dhs-8, C55A6.6, C55A6.7, F20G2.1, F20G2.2, R08H2.1, F25D1.5*), Supporting Table S1] (McElwee *et al.*, 2007). In addition, short chain dehydrogenase was one of the most highly enriched GO terms in this gene set (Fig. 2d). We conclude that SKN-1 not only controls expression of numerous Phase 2 detoxification genes, but also upregulates some Phase 1 genes.

The SKN-1-upregulated genes also included gene groups that represent other biological processes, some of which are stress related. We identified numerous genes involved in protein folding or degradation, some of which have lysosomal functions

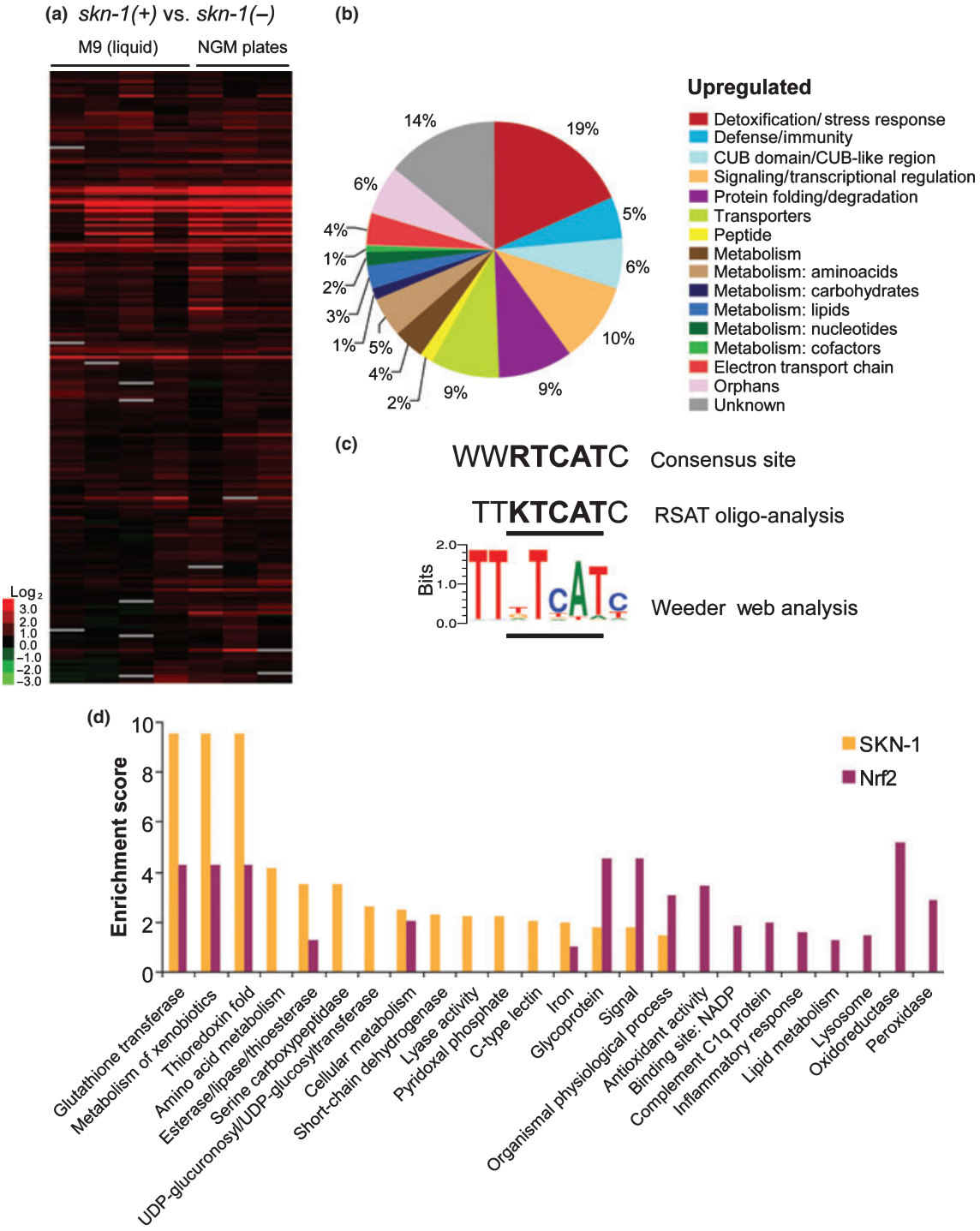

Fig. 2 Genes that are up-regulated by SKN-1 under normal conditions. (a) Hierarchical clustering of *gfp* control [*skn-1(+)*] and *skn-1(–)* RNAi samples that were analyzed on microarrays (seven sample sets in total). Incubation conditions under which these samples were obtained are indicated. A representative subset of SKN-1-upregulated genes is shown. (b) Representation of functional group categories among the 233 SKN-1-upregulated genes that were identified by hierarchical clustering and SAM (Supporting Table S1). Genes were classified according to their molecular or biological function, based upon GO terms. The CUB/CUB-like group was classified by the presence of these motifs (Blanc *et al.*, 2007). The following broad categories were created by combining GO-terms: detoxification/stress response, defense/immunity, signaling/transcriptional regulation, and protein folding/degradation. (c) Enrichment of SKN-1-binding motifs at SKN-1-upregulated genes. RSAT and Weeder Web were used to identify novel sequence motifs that are over-represented in the predicted promoters of SKN-1-up-regulated genes, as defined by the 2 Kb or less of intergenic sequence upstream of each ORF. The consensus identified by Weeder Web is represented by WebLogo (Crooks *et al.*, 2004). (d) Enrichment of functional gene categories among SKN-1-upregulated genes, compared to a set of Nrf2-upregulated genes. Highly represented GO terms are graphed for SKN-1-upregulated genes, and for Nrf2-dependent genes that were identified by expression profiling of primary cortical astrocytes from $Nrf2^{-/-}$ and $Nrf2^{+/+}$ mice under nonstressed conditions (Lee *et al.*, 2003a).

Sequence name	Gene name	KOG title or protein domain	Score
K08F4.7	*gst-4*	Glutathione *S*-transferase	15.351
C32H11.4		Cub-like domain/Cub-like region	8.778
C32H11.12	*dod-24*	Cub-like domain/Cub-like region	8.711
ZK1058.6	*nit-1*	Carbon–nitrogen hydrolase	8.506
Y45G12C.2	*gst-10*	Glutathione *S*-transferase	7.765
K10D11.1	*dod-17*	Cub-like domain/Cub-like region	7.664
C32H11.3		Cub-like domain/Cub-like region	7.342
Y102A11A.3		RNAi causes Ste and Sck	7.268
T26C5.1	*gst-13*	Glutathione *S*-transferase	7.087
F56D5.3		NADH:flavin oxidoreductase/12-oxophytodienoate reductase	6.667
F56A4.4		Glutathione *S*-transferase	6.551
K10C2.3		Aspartyl protease	6.349
F23B2.12	*pcp-2*	Hydrolytic enzymes of the alpha/beta hydrolase fold	6.166
F55G11.2		Cub-like domain/Cub-like region	6.153
C35B1.5		Thioredoxin, nucleoredoxin and related proteins	5.811
ZK896.4		Cub-like domain/Cub-like region	5.625
T25B6.2		M13 family peptidase, neprilysin, metallopeptidase	5.568
C55A6.7		Predicted short chain-type dehydrogenase	5.455
C09B8.4		Protein of unknown function DUF829	5.444
ZC443.6	*ugt-16*	UDP-glucuronosyl and UDP-glucosyl transferase	5.366
H20E11.3		Cub-like domain/Cub-like region	5.354
F14D7.6		Predicted transporter/transmembrane protein	5.331
K11H12.4		Protein of unknown function DUF274	5.087
Y32F6A.5		Serine carboxypeptidases	5.028
K04A8.10		UDP-glucuronosyl and UDP-glucosyl transferase	4.922
B0041.6	*ptps-1*	6-pyruvoyl tetrahydrobiopterin synthase	4.906
K10H10.2		Cystathionine beta-synthase and related enzymes	4.905
F32G8.6	*cat-4*	GTP cyclohydrolase I	4.814
F01D5.3		Secreted surface protein	4.602
C07D8.6		Aldo/keto reductase family proteins	4.545
C02D5.3		Glutathione *S*-transferase	4.471
F35E8.8	*gst-38*	Glutathione *S*-transferase	4.434
M03F8.4		Protein of unknown function DUF23	4.411
F 08G5.6		Cub-like domain/Cub-like region	4.344

Table 1 SKN-1-upregulated genes identified under nonstressed conditions

The top 35 genes for which expression was differentially decreased in *skn-1(RNAi)* animals under nonstressed conditions (SKN-1-upregulated genes), as ranked by SAM score. These and the other SKN-1-upregulated genes we identified (233 total) are listed by functional group in Table S1.

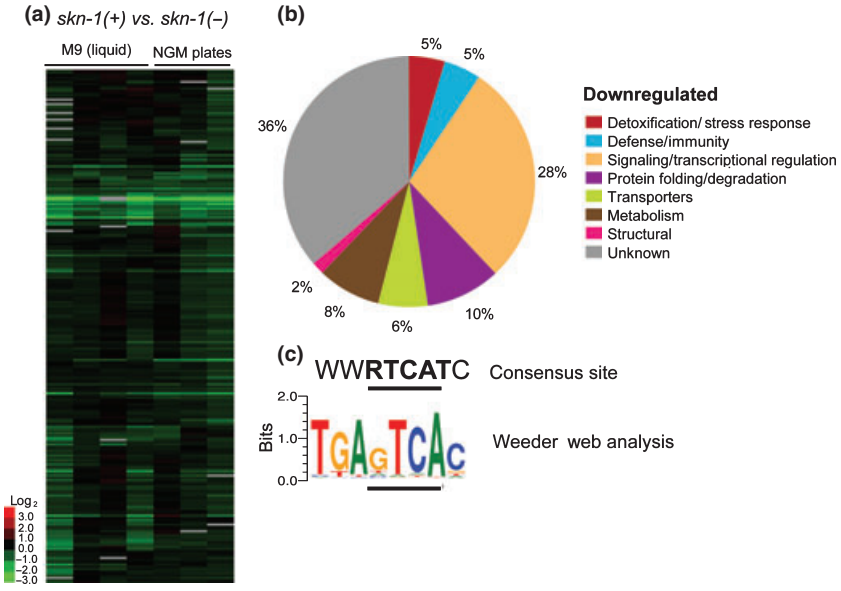

Fig. 3 Genes that are down-regulated by SKN-1 under normal conditions. (a) Hierarchical clustering (pictured) and statistical analysis of microarrays analysis of seven sample sets identified a set of 63 SKN-1-downregulated genes (Supporting Table S2). (b) Representation of functional gene groups among the SKN-1-downregulated genes, analyzed as in Fig. 2b. (c) Enrichment of SKN-1 binding motifs in SKN-1-downregulated genes, analyzed as in Fig. 2c.

Table 2 SKN-1-downregulated genes identified under nonstressed conditions

Sequence name	Gene name	KOG title or protein domain	Score
F42G2.4	*fbxa-182*	Protein containing an F-box motif	–9.335
T26F2.2		Uncharacterized protein	–6.858
C31B8.4		Uncharacterized protein	–6.611
Y39B6A.24		Aspartyl protease	–6.555
ZC196.4		Protein of unknown function DUF713	–6.389
C17H1.7		Uncharacterized protein	–6.218
Y47H9C.1		Protein of unknown function DUF274	–6.137
C06E1.1		Protein involved in membrane traffic (YOP1/TB2/DP1/HVA22 family)	–6.065
Y69A2AR.12		Uncharacterized protein	–5.562
F15B9.6		Uncharacterized protein	–5.402
Y75B8A.32		Predicted DNA-binding protein	–5.093
F15D3.8		Uncharacterized protein	–5.031
F47H4.8	*fbxa-188*	Protein containing an F-box motif	–5.009
M01G12.12	*rrf-2*	Putative RNA-directed RNA polymerase QDE-1 required for posttranscriptional gene silencing and RNAi	–4.913
K08H10.1	*lea-1*	Protein that is predicted to be hydrophilic and heat-resistant	–4.785
Y46G5A.20		Uncharacterized protein	–4.639
Y6E2A.4		Protein of unknown function DUF713	–4.611
Y41C4A.11		Vesicle coat complex COPI, beta' subunit	–4.424
F45E4.1	*arf-1.1*	GTP-binding ADP-ribosylation factor Arf1	–4.408
F47H4.10	*skr-5*	SCF ubiquitin ligase, Skp1 component	–4.021
C54G6.5	*spp-17*	Saposin-like Protein family	–4.009
Y43C5A.3		Uncharacterized protein	–3.989
B0024.4		Protein of unknown function DUF274	–3.929
W03D2.6		C-type lectin	–3.826
F58B3.3	*lys-6*	*N*-acetylmuraminidase/lysozyme	–3.758
Y51B9A.9		Jun-N-terminal kinase (JNK)	–3.546
C06E4.8		Uncharacterized protein	–3.527
F23F12.3		Synaptic vesicle transporter SVOP and related transporters (major facilitator superfamily)	–3.474
F25B3.5		Uncharacterized protein	–3.464
F58F9.7		Pristanoyl-CoA/acyl-CoA oxidase	–3.452
C01G6.7		Acyl-CoA synthetase	–3.428
ZK1251.2	*ins-7*	Insulin-like peptides	–3.423
C45B11.3	*dhs-18*	Reductases with broad range of substrate specificities	–3.295
F02C12.5	*cyp-13B1*	Cytochrome P450 CYP3/CYP5/CYP6/CYP9 subfamilies	–3.276
C04F12.1		Translation initiation factor 2C (eIF-2C) and related proteins	–3.263

The top 35 genes for which expression was differentially increased in *skn-1(RNAi)* animals under nonstressed conditions (SKN-1-downregulated genes), as ranked by SAM score. These and the other SKN-1-downregulated genes we identified (63 total) are listed by functional group in Table S2.

(Fig. 2b; Supporting Table S1). Among the latter group were vacuolar H+ATPases that translocate protons into lysosomes and other organelles (*vha-2*, *vha-6*, *vha-8*, *vha-16,* and *vha-17*), serine carboxypeptidases, and the ortholog of LYST (T01H10.8), which has been implicated in the lysosomal storage disease Chediak–Higashi syndrome (Kaplan *et al.*, 2008). We also identified many genes that encode transporters for metals, small molecules, ions, or water (Fig. 2b; Supporting Table S1). Many SKN-1-upregulated genes are involved in metabolic processes (Fig. 2b,d; Supporting Table S1). These include cystathionine beta-synthase and cystathionine beta-lyases, which convert homocysteine to cysteine, a precursor of glutathione (Banerjee & Zou, 2005). Two prominent groups of SKN-1-upregulated genes are involved in cell surface processes (C-type lectins and CUB-like domain proteins) (Fig. 2b,d; Table 1; Supporting Table S1). In other species, the CUB domain has been implicated in cell-surface functions, such as complement activation, tissue repair, axon guidance, inflammation, and receptor-mediated endocytosis (Blanc *et al.*, 2007).

SKN-1 binds preferentially *in vitro* to the consensus WWTRTCAT (W = A/T, R = G/A), and upregulates *gcs-1* by interacting with this motif (Blackwell *et al.*, 1994; An & Blackwell, 2003). The underlined RTCAT motif is most critical for binding affinity and specificity, because SKN-1 directly contacts

the RTCA base pairs in the major groove (Rupert *et al.*, 1998; Kophengnavong *et al.*, 1999). The WWTRTCAT motif should occur randomly only once every 2048 bp in the genome, but 49% of the SKN-1-upregulated genes (115/233) contained two to six copies of this element within their putative promoters, as defined by 5′ intergenic sequence up to 2 kb (Supporting Table S1; Supporting Fig. S2). An analysis of SKN-1-upregulated gene promoters for novel over-represented sequences identified a motif that is similar to the canonical SKN-1 consensus [TTDTCATC, (D = A/G/T); Fig. 2c; see Experimental Procedures], and in many instances corresponds to the same element within these putative promoters (data not shown). This novel motif, which is more restrictive than the WWTRTCAT consensus (1/10923 bp randomly), was present in 110 (47%) of the SKN-1-upregulated gene promoters (≤ 2 kb) (Supporting Table S1; Supporting Fig. S2). Taken together, the data indicate that SKN-1 may directly control the expression of many of the SKN-1-upregulated genes we identified.

The functional parallels between SKN-1 and mammalian Nrf proteins (An & Blackwell, 2003) predicts that these proteins should regulate similar categories of genes. Accordingly, many of the GO terms that we identified in SKN-1-upregulated genes were also enriched in a set of Nrf2-dependent genes that were identified in murine primary cortical astrocytes (Lee *et al.*, 2003a), including glutathione transferase, xenobiotic metabolism, and other stress response groups (Fig. 2d). In addition, UGTs were identified in two sets of stress-induced Nrf2-dependent genes (Kwak *et al.*, 2003; Lee *et al.*, 2003a). Interestingly, the SKN-1- and Nrf2-upregulated genes also included some GO term groups that did not overlap (Fig. 2d). Apparent *C. elegans* homologs exist for more than half of the Nrf2-regulated genes in the GO terms that were unique to Nrf2 (not shown), suggesting that their lack of detectable regulation by SKN-1 might derive from tissue- or organism-specific differences.

In contrast to the SKN-1-upregulated genes, no particular biological function predominated among the 63 genes that were down-regulated by SKN-1 under normal conditions (Fig. 3b; Supporting Table S2). Two of these genes function in the IIS pathway. *ins-7* encodes an insulin-like peptide and DAF-2 agonist (Murphy *et al.*, 2003), and *pdk-1* encodes a kinase that functions downstream of DAF-2 to activate the AKT-1/2 and SGK-1 kinases, which inhibit DAF-16 and SKN-1 through phosphorylation (Paradis & Ruvkun, 1998; Hertweck *et al.*, 2004; Tullet *et al.*, 2008). Other SKN-1-downregulated genes encode regulatory proteins that function in signaling, ubiquitin-mediated proteolysis, or gene regulation, including four that may be involved in RNAi [a putative RNA-directed RNA polymerase (RRF-2) and the Argonaute-related proteins PPW-1, SAGO-2, and C04F12.1 (Yigit *et al.*, 2006)]. Of 14 genes that had been identified as downregulated by Nrf2 in cortical astrocytes under normal conditions (Lee *et al.*, 2003a), only four have apparent *C. elegans* homologs (not shown). The SKN-1-downregulated genes did not include any of these four genes, a group that is too small for a conclusive comparison.

Multiple copies of the canonical *in vitro* SKN-1 binding site WWTRTCAT were present within predicted promoters at 17 SKN-1-downregulated genes (27%), including *ins-7* (Supporting Fig. S2; Supporting Table S2), suggesting that many of these genes might be repressed directly by SKN-1. In addition, within these 63 promoters we identified the novel motif TGAGTCAC (Fig. 3c), which may be a variant of the canonical motif. Interestingly, only 10 of 233 SKN-1-upregulated genes (4.3%) displayed this new motif, compared to 31% of the SKN-1 downregulated genes (Supporting Fig. S2, Table S2), suggesting that it might mediate transcriptional inhibition by SKN-1.

SKN-1 mediates the transcriptional response to Arsenite

We next examined how SKN-1 contributes to stress responses, first by investigating its role in the As response (Fig. 1, dark blue arrows). Hierarchical clustering identified 118 genes that are upregulated in a *skn-1*-dependent manner upon As exposure (As/SKN-1-dependent genes), but did not detect any genes that were down-regulated by SKN-1 in response to As, or induced by As independently of SKN-1 (Fig. 4a; Supporting Table S5). A qRT-PCR analysis confirmed the *skn-1*-dependence of a set of our As/SKN-1-dependent genes, supporting the microarray data (Supporting Table S6). An analysis of the predicted promoters of these As/SKN-1-dependent genes for novel shared motifs identified essentially the same elements we had earlier detected in the SKN-1-upregulated gene promoters (Figs 2c and 4b; Supporting Table S5), suggesting that a high proportion of these genes are likely to be direct SKN-1 targets. The bulk of the transcriptional response to As therefore appears to be mobilized by SKN-1.

When we compared the As/SKN-1-dependent genes to the SKN-1-upregulated genes we had identified under normal conditions, we found that the majority of the As transcriptional response (83 genes) was common to both sets (Fig. 4c). These gene sets also shared many prominent GO terms, including glutathione transferase, thioredoxin fold, lyase activity, and short-chain dehydrogenase (Fig. 4d). The SKN-1-upregulated genes that were identified under normal and As-induction conditions also differed in important respects. Within the GO terms that these gene sets had in common, the As/SKN-1-dependent set included potentially important As-specific genes such as *hmt-1*, an ABC-type transporter that is critical for *C. elegans* heavy metal tolerance (Vatamaniuk *et al.*, 2005). In addition, the As/SKN-1-dependent genes included some new GO terms, such as alcohol dehydrogenase, as well as a new set of genes involved in glutathione synthesis (Supporting Table S5). Importantly, these As-induced genes also lacked some GO terms that were prominent among the SKN-1-upregulated genes we had identified under normal conditions (i.e. UGTs and C-type lectins) (Fig. 4d). We conclude that the response to As does not consist of simply a broad induction of the genes that are upregulated by SKN-1 in the absence of stress, but instead involves induction of particular sets of those genes, along with additional targets.

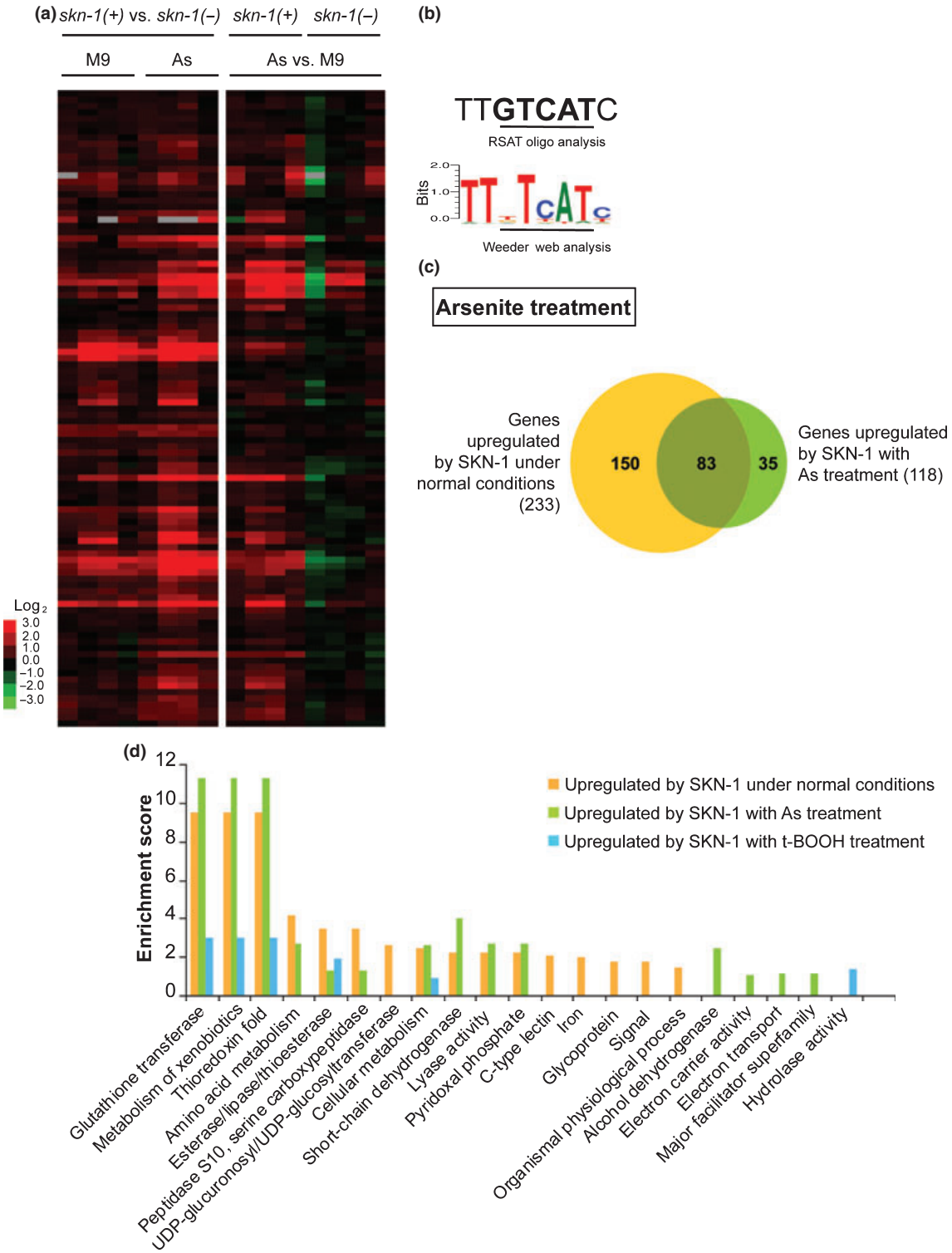

Fig. 4 SKN-1 regulation of overlapping gene groups under normal and Arsenite (As) stress conditions. (a) Hierarchical clustering of genes that are differentially regulated in response to As treatment (four sample sets, see Experimental Procedures). Genes were identified that are As-upregulated and *skn-1*-dependent, but none were identified that are As-upregulated and *skn-1*-independent. A subset of the genes identified by hierarchical clustering is shown. (b) Enrichment of SKN-1-binding motifs in As and SKN-1-upregulated genes, analyzed as in Fig. 2c. (c) Venn diagram showing overlap among genes that were upregulated by SKN-1 under normal and As stress conditions. (d) Comparison of SKN-1-upregulated genes identified under normal, As-treatment, and *t*-BOOH-treatment conditions, grouped by GO terms. Note that some GO terms are overrepresented among only one or two of these gene groups.

We also used hierarchical clustering across the As-treated SKN-1 (+) and (–) samples and their controls (dark blue arrows, Fig. 1) to identify genes that were upregulated by SKN-1 under both normal and As-exposed conditions, but were not upregulated by As treatment (As-independent; Supporting Table S7). As would be predicted, these new genes did not include any of our As-/SKN-1-dependent genes, and included few or no UGTs, C-type lectins, or alcohol dehydrogenases, groups that were prominent among SKN-1-upregulated genes under either normal or As-treated conditions, but not both (Fig. 4c; Supporting Tables S1, S5 and S7). This further supports the idea that As treatment activates distinct subsets of SKN-1 target genes. In addition, by analyzing samples obtained under nonstressed and As-treatment conditions simultaneously, this analysis detected many SKN-1-regulated genes that we had not identified in our study of *skn-1(+)* and *skn-1(–)* control samples only, including 15 genes that encode proteasome subunits (Supporting Table S7). Proteasome genes have been implicated as Nrf2 targets (Kwak *et al.*, 2003), suggesting that the regulation of these genes by SKN-1/Nrf proteins is conserved.

SKN-1-dependent and -independent responses to *tert*-butyl hydroperoxide

To test further the notion that SKN-1 upregulates particular subsets of its target genes in response to stress, we investigated how SKN-1 contributes to the transcriptional response to *t*-BOOH treatment, again using hierarchical clustering (Fig. 1, teal arrows; Fig. 5a). Here, in striking contrast to the effects of As treatment, we observed that *t*-BOOH not only upregulates a set of SKN-1-dependent genes, but also induces a broad SKN-independent response (Fig. 5b; Supporting Tables S8, S9 and S10).

Only a minority of the *t*-BOOH response appeared to require *skn-1*, as *skn-1* RNAi impaired induction of only 64 (22%) of the 285 *t*-BOOH-upregulated genes we detected. Interestingly, we had previously identified only 12 (19%) of the SKN-1-dependent *t*-BOOH-induced genes as being SKN-1-upregulated under normal conditions (Fig. 5b). The other 52 SKN-1-dependent *t*-BOOH-induced genes encompassed some gene classes or GO terms that were not detected under either normal or As-induced conditions (i.e. BTB/POZ-like, casein kinase, hydrolase activity) (Supporting Tables S1, S5 and S8; Fig. 5c). Analysis of the *t*-BOOH-induced SKN-1-dependent gene promoters for novel motifs identified an element that is consistent with the SKN-1 *in vitro* consensus and was not over-represented at SKN-1-independent *t*-BOOH-induced genes (TKTCATCA, Fig. 5a), suggesting that many of these genes might be direct SKN-1 targets.

We identified a much larger number of genes that were up- or down-regulated by *t*-BOOH under both control and *skn-1* RNAi conditions (referred to as SKN-1-independent). One hundred nine genes were downregulated by *t*-BOOH, including many metabolism genes (Supporting Table S10). Importantly, the 221 genes that were upregulated by *t*-BOOH independently of *skn-1* encompassed many functional groups that were not prominent in the As-induced set. They included a greater number of Phase 1 detoxification genes (CYP450 enzymes and other monooxygenases), nuclear hormone receptors, additional signaling or transcription regulators, and many lipid metabolism genes (Fig. 5c; Supporting Table S9).

It is unlikely that the striking differences between the As and *t*-BOOH responses simply reflect different stress 'levels', because our stress conditions were adjusted to comparable *gcs-1* induction and resulted in only a residual frequency of death (Supporting Fig. S1; see Experimental Procedures). Furthermore, under conditions where As treatment resulted in substantially greater toxicity than *t*-BOOH, a representative SKN-1-independent, *t*-BOOH-upregulated gene (*fmo-2*) was induced by *t*-BOOH but not As (Supporting Fig. S3). A qRT-PCR analysis showed that many *t*-BOOH-upregulated genes were induced more robustly in a predicted null *skn-1* mutant than in N2, thereby confirming their independence from *skn-1* and suggesting that their induction is stronger when SKN-1-mediated stress defenses are impaired (Supporting Fig. S4). Surprisingly, we had previously identified some SKN-1-independent *t*-BOOH-upregulated genes as being SKN-1-dependent under normal or As-induction conditions (including *gst-14* and *gst-39*; Supporting Tables S1, S5 and S9). A qRT-PCR analysis confirmed that *gst-14* and *gst-39* were induced by *t*-BOOH in the absence of SKN-1, although their induction was more robust in N2 (Supporting Fig. S4).

Taken together, the data demonstrate that the organismal transcriptional response to *t*-BOOH is more complex than the As response. The *t*-BOOH response involves induction of particular Phase 2 genes by SKN-1, along with a broad *skn-1*-independent response that includes upregulation of some Phase 2 genes that were *skn-1*-dependent under As-induction or normal conditions.

SKN-1 downregulates genes that decrease stress resistance or lifespan

The SKN-1-regulated gene profiles that we identified under normal and stress conditions were surprisingly complex, suggesting that SKN-1 not only responds acutely to stress, but also may regulate many genes under normal conditions that could be important for stress resistance and longevity. To test this idea, we investigated how genes that are regulated by SKN-1 under normal conditions influence the organism's capacity for stress resistance. We first examined how six SKN-1-upregulated genes affect As resistance, by inhibiting their expression using RNAi (Fig. 6a). These genes were selected from among those that showed the most statistical significance by SAM (Table 1). They each encoded known stress-defense enzymes, with the exception of the CUB-like genes *C32H11.3* and *C32H11.4*. RNAi of each gene that we tested decreased As resistance but did not impair movement or fertility of control animals (Fig. 6a; not shown), suggesting that many SKN-1-upregulated genes contribute to stress resistance. In no case did knockdown of these genes compromise As resistance comparably to *skn-1* RNAi (Fig. 6a), consistent with the idea that SKN-1 coordinates many defense mechanisms.

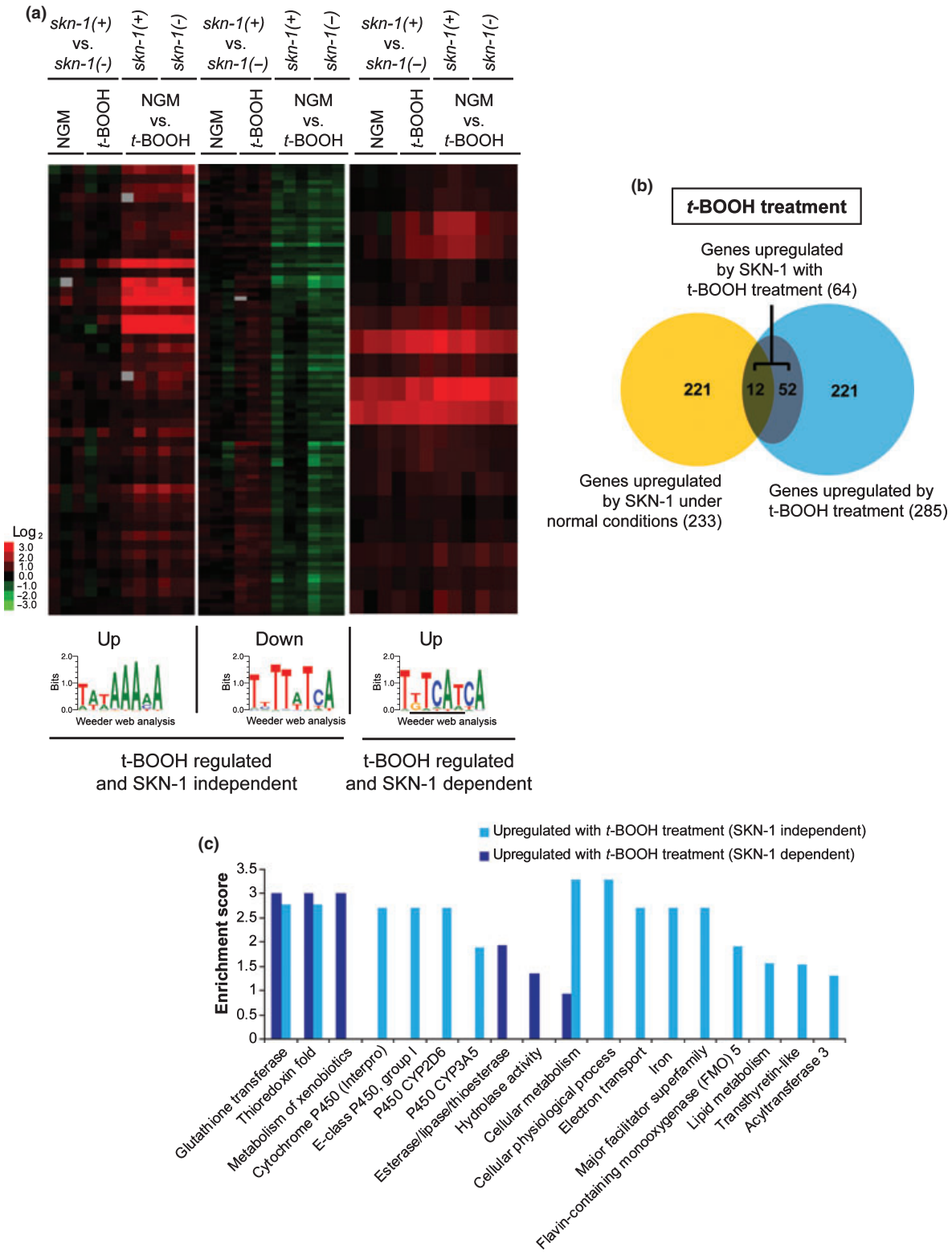

Fig. 5 SKN-1-dependent and -independent responses to an organoperoxide. (a) *tert*-butyl hydrogen peroxide (*t*-BOOH) treatment affects regulation of *skn-1*-dependent and *skn-1*-independent gene programs. Hierarchical clustering identified genes that are up- or down-regulated in response to *t*-BOOH treatment, and unaffected by *skn-1* RNAi [*skn-1(–)*] (SKN-1-independent genes). A subset of the genes identified from three sample sets by hierarchical clustering is shown, along with motifs that were identified as being over-represented in their predicted upstream promoters (determined as in Fig. 2c). Nematode Growth Medium corresponds to normal conditions (see Experimental Procedures). (b) Venn diagram of genes that were upregulated by SKN-1 under normal conditions and *t*-BOOH treatment. (c) Genes that were upregulated by *t*-BOOH treatment (Supporting Tables S8 and S9), graphed as in Fig. 4d. GO terms that are over-represented among *t*-BOOH-induced SKN-1-upregulated and SKN-1-independent genes are compared.

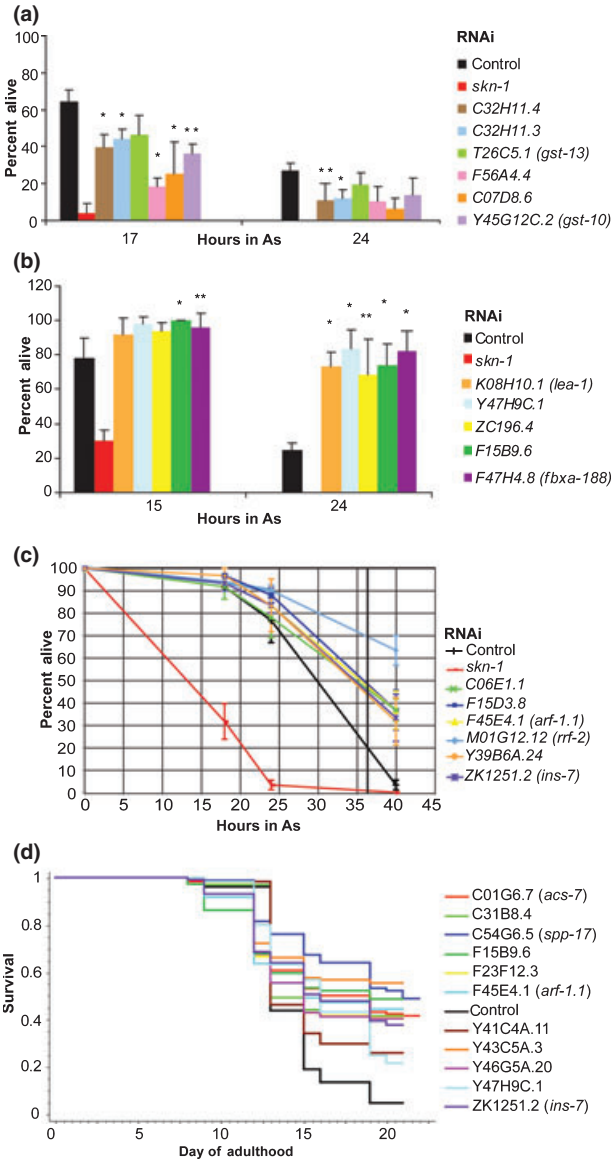

Fig. 6 SKN-1-regulated genes influence oxidative stress resistance and lifespan. (a) Many SKN-1-upregulated genes promote oxidative stress resistance. SKN-1-upregulated genes (Supporting Table S1) were knocked down by RNAi, then survival of young adults (8–9 h) was assayed at the indicated times after introduction into 4 mM As. A representative experiment is shown in which five wells of ten worms each were examined. Error bars indicate the SEM, and *P*-values (Student's *t*-test) indicate comparison to control RNAi. $*P \leq 0.0008$; $**P \leq 0.008$ (Student's *t*-test). (b) Many SKN-1-downregulated genes reduce oxidative stress resistance. Resistance to As was analyzed after RNAi of the indicated genes (Supporting Table S2) as in (a). Other experiments and analyses of additional genes are described in (c) and Fig. S5 (Supporting information). $*P \leq 0.0008$; $**P \leq 0.008$ (Student's *t*-test). (c) Analysis of As resistance in young adults (2–6 h). Experimental and control RNAi worms were placed in 5 mM As, then the fraction surviving was counted 16, 24, and 40 h later. Results are presented as a graph from which we calculated the approximate fraction of animals in each set that were alive when 20% of the control animals were still surviving (black vertical line). A comparison of this fraction to control is plotted in Fig. S5 (Supporting information). Six samples of ten worms each were examined for every condition. *P*-value of fraction alive compared to control at 20% control survival is < 0.05 for all genes shown (Student's *t*-test performed across samples). Error bars = SEM. (d) Many SKN-1-downregulated genes decrease lifespan. A set of SKN-1-downregulated genes was analyzed for effects on longevity using a feeding RNAi longevity assay in RNAi-sensitive *rrf-3(pk1426)* worms at 20 °C. Genes for which RNAi extended lifespan significantly in 3/3 trials ($P < 0.01$, log-rank) are diagrammed, with data from a single trial shown (Supporting Table S11b, Experiment 2). Control is empty RNAi feeding vector L4440. Data and statistical analyses for all experiments and genes tested are provided in Table S11a–c and Table S12 (Supporting information).

We also asked how 22 of the SKN-1-downregulated genes affect stress resistance. One possible model is that these genes might protect against stress under normal conditions, and were upregulated after *skn-1* RNAi as a secondary defensive response to stress resulting from SKN-1 loss. Alternatively, if these SKN-1-downregulated genes are actively repressed by SKN-1, through SKN-1 either acting directly at their promoters or triggering a repressive signal, they might be predicted to decrease stress resistance. Consistent with the latter model, the SKN-1-downregulated genes *pdk-1* and *ins-7* (Supporting Table S2) each act in the IIS pathway to *decrease* lifespan (Paradis & Ruvkun, 1998; Murphy *et al.*, 2003). These examples suggested that other SKN-1-downregulated genes might also decrease stress resistance or lifespan. Accordingly, As resistance was increased consistently in young adult animals after RNAi knockdown of 15 of 22 SKN-1-downregulated genes that we tested, including *ins-7* (Fig. 6b,c and Supporting Fig. S5). The observation that these SKN-1 downregulated genes act to decrease stress resistance suggests that they might be actively repressed by SKN-1.

We next examined how 26 of the SKN-1-downregulated genes affect lifespan under nonstressed conditions. These genes were selected among those with the most significant SAM scores (Supporting Table S2). RNAi knockdown of 11 of these 26 genes significantly extended lifespan in each of three independent experiments, ($P < 0.01$; log-rank) (Fig. 6d; Supporting Table S11a–c, Table S12). These 11 genes included *ins-7*, along with ten other genes that are newly identified here as longevity-affecting genes, as none had been shown previously to influence lifespan (WormBase). This number is probably an underestimate, as RNAi of several additional genes extended lifespan in two of three trials (Table 3). Of the 15 genes for which RNAi increased As stress resistance (Supporting Fig. S5), seven were associated with increased lifespan in all three of our longevity trials, and five in two of these trials (Supporting Table S2). We conclude that under normal conditions SKN-1 inhibits many genes that reduce stress resistance and/or longevity (Fig. 7).

Discussion

SKN-1 is required for oxidative stress resistance and has important functions in the absence of acute stress, as it promotes longevity under normal, reduced IIS, and CR conditions (An & Blackwell, 2003; Bishop & Guarente, 2007a; Tullet *et al.*, 2008). Here we show that under nonstressed conditions SKN-1 upregulates numerous detoxification genes, along with other genes

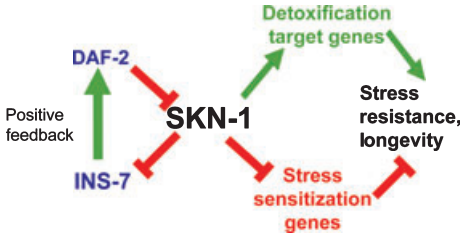

Fig. 7 A model for SKN-1 functions under normal conditions. A positive feedback interaction with *ins-7* and the DAF-2 pathway is featured. SKN-1 upregulates many genes that promote detoxification and stress resistance, and also downregulates genes that decrease stress resistance, lifespan, or both. Among the SKN-1-downregulated genes are both *ins-7* and *pdk-1* (not shown), each of which promotes DAF-2 pathway signaling (see text). The DAF-2 pathway in turn inhibits SKN-1 (Tullet *et al.*, 2008).

with functions that may be related to stress defenses. We also found that SKN-1 inhibits genes that reduce stress resistance and longevity. Finally, we observed that SKN-1 induces discrete target gene groups in response to stress, and that some stresses activate SKN-1-independent stress defense mechanisms in parallel. Many of the genes we identified in each SKN-1-dependent gene set are likely to be direct SKN-1 targets, as suggested by the prevalence of SKN-1 binding sites in their promoters. Our results reveal a notable degree of complexity in SKN-1 functions and *C. elegans* stress responses.

Multiple SKN-1 functions under normal conditions

Many of the 233 genes that we identified as SKN-1-upregulated under normal conditions are involved directly in stress-related processes (Table 1; Supporting Table S1). These included GST, UGT, and other Phase 2 genes that are involved in conjugation of toxic compounds, ROS metabolism, or glutathione production (Fig. 2b,d; Supporting Table S1). Interestingly, some of these GSTs might be involved in functions besides detoxification. For instance, the most highly upregulated SKN-1 target (*gst-4*, Table 1) seems to function not only as a GST, but also as a glutathione-dependent prostaglandin D synthase (Kubagawa *et al.*, 2006). Besides Phase 2 genes, we also detected significant representation of Phase 1 (SDR, CYP) and Phase 3 (Transporter) genes, indicating that SKN-1 plays a broad role in systemic detoxification. Interestingly, our SKN-upregulated gene sets did not include superoxide dismutase (SOD) or catalase genes, and we found previously that *sod-3* is upregulated independently of *skn-1* in the context of reduced IIS (Tullet *et al.*, 2008). Taken together, our data suggest that SKN-1 does not regulate a primary response to endogenously produced superoxide or hydrogen peroxide, and instead promotes detoxification, cellular repair, and activity of the many antioxidant and stress-defense systems that depend upon glutathione.

Numerous SKN-1-upregulated genes are involved in lysosomal or proteasomal functions (Supporting Table S1, S5 and S8; see Results), suggesting that SKN-1 may promote recycling of damaged cellular components. RNAi knockdown of many proteasome component genes has been shown to result in accumulation of SKN-1 in nuclei, through an unknown mechanism (Kahn *et al.*, 2008). Together with this observation, our results suggest the existence of a feedback mechanism whereby SKN-1 might limit its own activity by upregulating proteasome gene expression.

Other genes we identified implicate SKN-1 in additional activities. Many SKN-1-upregulated genes encode cell-surface proteins, including CUB-like proteins (Fig. 2b; Supporting Table S1). Some CUB-like genes are also regulated by p38 MAPK signaling, which is important for SKN-1 function in the intestine (Inoue *et al.*, 2005; Troemel *et al.*, 2006). RNAi of the CUB-like genes *C32H11.3* and *C32H11.4* modestly reduced As resistance (Fig. 6a), indicating that some CUB-like proteins affect stress resistance. SKN-1-upregulated genes are involved in additional diverse molecular functions that include transcription, signaling, ubiquitination, and metabolism (Fig. 2b,d; Supporting Table S1), indicating that SKN-1 is involved in a complex group of processes. It will be interesting to elucidate which of these processes might indirectly affect detoxification or stress resistance.

It seems likely that the SKN-1-regulated genes we identified under normal and other conditions primarily reflect SKN-1 functioning in the intestine, as opposed to the ASI neurons, because SKN-1 expression is more prominent in the intestine (An & Blackwell, 2003). In addition, we reduced SKN-1 expression by RNAi, which works comparatively poorly in neurons (Timmons *et al.*, 2001). We were therefore very surprised to find that SKN-1 controls so many genes under normal conditions, because SKN-1 is present in intestinal nuclei at comparatively low levels in the absence of stress (An & Blackwell, 2003). It is even possible that we might have underestimated the breadth of SKN-1 activity, because these analyses of whole worms could have missed some genes that are regulated by SKN-1 in only subsets of tissues. Importantly, most of the individual genes that were controlled by SKN-1 under normal conditions were not upregulated in As or *t*-BOOH stress responses (Figs 2, 4 and 5), arguing against the idea that the SKN-1-dependent gene activity detected under normal conditions derives simply from animals being mildly 'stressed'. We conclude that under normal conditions SKN-1 is important for fine-tuning of genes involved in many stress related and other functions.

Many of the functional categories that are characteristic of SKN-1-upregulated genes (GST, UGT, SDR, CYT, CUB domain, drug transporters) are also prominent among *C. elegans* or yeast genes that depend upon the general mRNA transcription factor MDT-15 (MED-15) (Taubert *et al.*, 2008; Thakur *et al.*, 2008). MDT-15 is a subunit of Mediator, a large multiprotein complex that must be brought to promoters for transcription to initiate. MDT-15 is required for function of the transcription regulators SBP-1 (SREBP) and NHR-49 (PPARα), which are critical for lipid homeostasis and metabolic regulation (Taubert *et al.*, 2006; Yang *et al.*, 2006). Those two regulators seem to activate transcription at least in part by binding to MDT-15, and thereby recruiting Mediator to promoters. MDT-15 is also required for xenobiotic defense and has been proposed to co-ordinate multiple transcriptional responses to food, toxins, and other ingested

materials (Taubert *et al.*, 2008). Our results suggest the intriguing model that SKN-1 might interact functionally or physically with MDT-15 to regulate some detoxification genes.

SKN-1-dependent suppression of stress-sensitization and anti-longevity genes

It was striking that SKN-1 downregulates numerous genes under nonstressed conditions, and that in many cases RNAi of these genes increased stress resistance and/or lifespan (Fig. 6; Supporting Table S2 and Table 3). Many of these SKN-1-downregulated genes contain predicted SKN-1 binding sites in their putative promoters, predicting that some might be repressed directly by SKN-1 (Supporting Table S2). This seems surprising, because SKN-1 is a powerful activator of transcription (Walker *et al.*, 2000). However, other examples have been identified of transcription regulators that seem to function as both activators and repressors, including DAF-16 (Murphy *et al.*, 2003). An important implication of our findings is that the previously described functions of SKN-1 in promoting stress resistance and longevity (An & Blackwell, 2003; Bishop & Guarente, 2007b; Tullet *et al.*, 2008) might be attributable not only to SKN-1 upregulating stress defense and other genes, but also to its inhibiting genes that have the opposite effect.

The stress-sensitization and anti-longevity genes we identified among the SKN-1-downregulated genes are involved in diverse functions (Supporting Table S2). For example, *lea-1* decreases As resistance (Fig. 6b) but is predicted to protect against dessication (Browne *et al.*, 2002). Perhaps adaptations to some conditions are not beneficial in the setting of stresses that would activate a SKN-1 response. Other SKN-1-downregulated genes that reduce stress resistance or lifespan encode regulatory proteins, including an F-box protein (FBXA-188) and the predicted SCF ubiquitin ligase component SKR-5 (Fig. 6b,c; Table 3). It is particularly noteworthy that SKN-1 downregulates genes that encode the IIS pathway kinase PDK-1 and the DAF-2 agonist INS-7, each of which had previously been shown to reduce longevity (Paradis & Ruvkun, 1998). INS-7 coordinates IIS and DAF-16 activity among tissues (Murphy *et al.*, 2003, 2007). DAF-16 inhibits *ins-7* expression in a positive feedback loop, thereby relieving negative regulation of itself by IIS. Our results indicate that SKN-1 and INS-7 are involved in a similar feedback loop that could magnify the effects of upregulating IIS on the one hand, or either DAF-16 or SKN-1 on the other (See model in Fig. 7).

SKN-1 functions analogously to DAF-16 in three intriguing ways. First, both proteins are inhibited directly by IIS (Tullet *et al.*, 2008). Second, our new results reveal that SKN-1, like DAF-16, down-regulates multiple mechanisms that reduce stress resistance or longevity (Murphy *et al.*, 2003) (Fig. 7). Why would such mechanisms exist, and why would they respond to SKN-1? Perhaps it is advantageous to hold some stress defense mechanisms in check under normal conditions; for example, enzymes that metabolize endobiotics or free radicals could have profound effects on hormonal and cell signaling pathways. Third, like SKN-1, DAF-16 also upregulates many stress resistance genes, as indicated by transcription profiling and proteomics performed under conditions of reduced IIS (Murphy *et al.*, 2003; Dong *et al.*, 2007; McElwee *et al.*, 2007), comparative genomics and bioinformatics (Lee *et al.*, 2003b), and chromatin immunoprecipitation studies (Oh *et al.*, 2006). DAF-16 upregulates many CYP and other Phase 1 genes (Murphy *et al.*, 2003; McElwee *et al.*, 2007), and one analysis suggests that some GSTs are upregulated by DAF-16 and are associated with IIS regulation in other species (McElwee *et al.*, 2007). In addition, we observed earlier that SKN-1 and DAF-16 together increase activity of particular GST genes in the context of a *daf-2* mutant (Tullet *et al.*, 2008). In the future, it will be interesting to elucidate the extent to which SKN-1 and DAF-16 might function cooperatively under particular conditions.

Customized *skn-1*-dependent and -independent responses to stress

How animals respond to metabolic or environmental stresses and how these responses are regulated are fundamentally important questions. By analyzing the transcriptional responses of *C. elegans* to As and *t*-BOOH we have obtained new insights into SKN-1 functions, its role in these stress responses, and how *C. elegans* adapts to stresses. For example, it was striking that only a subset of SKN-1-responsive genes were upregulated by each of these stresses. Multiple GO terms that were prominent among SKN-1-upregulated genes under normal conditions were not represented among the As or *t*-BOOH induced genes (i.e. UGT, C-type lectin), and new GO terms appeared among the SKN-1-dependent genes that were upregulated by As (i.e. alcohol dehydrogenase) and *t*-BOOH (hydrolase) (Fig. 4d). The apparent specificity of these responses indicates that SKN-1 does not simply regulate its target genes in tandem in response to stress levels. Instead, SKN-1 must integrate multiple signals, so that in response to a given stimulus some genes are induced and others are left unaffected. Our results suggest that mammalian Nrf proteins may have a similarly complex set of functions that might not be apparent from analyses of single cell types or tissues.

Another interesting observation was that while the entire As response we detected required *skn-1*, *t*-BOOH stimulated a *skn-1*-independent response that included induction of large numbers of Phase 1 detoxification, nuclear receptor, and lipid metabolism genes, along with upregulation of some genes that were *skn-1*-dependent under other conditions (Fig. 5b,c; Supporting Table S9). As these findings were obtained with *skn-1* RNAi (Fig. 1), it is impossible to establish that all of these genes were induced by *t*-BOOH independently of *skn-1*. However, this was true for each of the seven genes that we analyzed in a predicted null *skn-1* mutant (Supporting Fig. S4), indicating that *t*-BOOH induces a broad *skn-1* independent response. Interestingly, RNAi knockdown of the 2-Cys peroxiredoxin *prdx-2* results in *skn-1*-independent activation of the SKN-1 target gene *gcs-1* (Olahova *et al.*, 2008), further supporting the idea that some signals induce Phase 2 genes independently of *skn-1*.

Table 3 Summary of lifespan effects of SKN-1 downregulated genes

		Expt 1		Expt 2		Expt 3	
Gene	Gene name	% of control	*P*-value	% of control	*P*-value	% of control	*P*-value
C54G6.5**	*spp-17*	121.7	< 0.0001	125.2	< 0.0001	119.3	< 0.0001
C01G6.7**	*acs-7*	112.6	< 0.0001	116.1	< 0.0001	121.4	< 0.0001
Y43C5A.3**		121.7	< 0.0001	114.0	< 0.0001	113.6	0.006
F25B3.5*		126.6	< 0.0001	113.3	< 0.0001	110.9	0.0209
F23F12.3**		116.1	< 0.0001	113.3	0.0002	125.1	< 0.0001
Y47H9C.1**		113.3	< 0.0001	112.6	< 0.0001	119.8	< 0.0001
ZK1251.2**	*ins-7*	111.2	0.0006	112.6	< 0.0001	122.3	< 0.0001
B0024.4*		120.3	< 0.0001	110.5	< 0.0001	103.4	0.5417
F58F9.7*		118.2	< 0.0001	110.5	< 0.0001	112.6	0.0151
F58B3.3	*lys-6*	116.8	< 0.0001	110.5	0.0128		
F02C12.5*	*cyp-13B*	127.3	< 0.0001	109.1	< 0.0001	110.4	0.039
C31B8.4**		116.8	< 0.0001	109.1	< 0.0001	121.6	< 0.0001
F15B9.6**		125.2	< 0.0001	108.4	< 0.0001	122.7	< 0.0001
ZC196.4*		101.4	0.898	108.4	0.0003	120.6	0.0001
Y46G5A.20**		130.8	< 0.0001	107.7	0.0001	120.0	< 0.0001
F45E4.1**	*arf-1.1*	111.2	0.0002	107.7	< 0.0001	117.5	0.0008
F47H4.8*	*fbxa-188*	117.5	< 0.0001	107.0	0.0208	120.0	0.0002
F15D3.8*		112.6	< 0.0001	107.0	0.0087	100.0	
Y41C4A.11**		121.7	< 0.0001	105.6	0.0005	123.4	< 0.0001
Y6E2A.4		113.3	< 0.0001	105.6	0.8425		
C17H1.7*		124.5	< 0.0001	104.9	0.0004	109.9	0.0435
F47H4.10*	*skr-5*	116.1	< 0.0001	104.2	0.0079		
Y51B9A.9		104.2	0.0858	104.2	0.0106		
Y39B6A.24		112.6	< 0.0001	102.8	0.0508		
K08H10.1*	*lea-1*	120.3	< 0.0001	97.2	0.867	112.9	0.007
M01G12.12	*rrf-2*	114.0	< 0.0001	93.7	0.6139		

For details of lifespan assay conditions and analysis see Experimental Procedures, Table S11, and Table S12.
% of control refers to the mean lifespan. Functional information for these genes is available in Table 2.
*Significant ($P < 0.01$) in two trials.
**Significant ($P < 0.01$) in all three trials.

Together, our findings suggest that the transcriptional responses to oxidative stresses may be highly specific, and adapted to the challenge faced by the organism.

Why would the responses to As and *t*-BOOH be so different? Arsenite is a metalloid that attacks thiols, depletes glutathione, and induces ROS formation, whereas *t*-BOOH is a stable lipid soluble peroxide that attacks lipids and proteins. Perhaps the As-induced genes represent a 'simpler' response to stress arising from excess ROS or a need for glutathione-related defenses. We speculate that the more complex *t*-BOOH response could additionally involve stress from phospholipid damage, or a global response to lipophilic toxins. Further supporting the notion that *C. elegans* stress responses are 'tailored', the list of gene groups induced by acrylamide is very similar to our As list (GSTs, UGTs, SDRs, glutathione metabolism), but also includes some distinct categories (collagens, major sperm proteins) (Hasegawa *et al.*, 2008). *Caenorhabditis elegans* could prove to be valuable for elucidating signals that lie upstream of different stress responses, and the role of individual tissues in mobilizing these signals and defending the organism against stress.

Our results demonstrate that SKN-1 plays a number of roles besides inducing Phase 2 detoxification genes, and that multiple factors influence its transcriptional output under normal and stress conditions. It will now be important to delineate how its regulation of detoxification and regulatory genes contributes to the effects of SKN-1 on longevity under normal and reduced IIS conditions, and to identify how SKN-1 acts in different tissues to influence regulation of these genes at the organismal level.

Experimental procedures

Caenorhabditis elegans growth and RNAi for microarray experiments

Caenorhabditis elegans were maintained on NGM and *Escherichia coli* OP50 as described (Brenner, 1974). For microarray experiments, a synchronous population of wild-type (N2) animals was obtained by hypochlorite treatment of embryos. Synchronized L1 larvae were placed at 20 °C on *E. coli* HT115 that expressed either *skn-1* or control dsRNA for 46 h, until they reached the L4 stage. For As exposure, worms were incubated for 30 min in 5 mM Sodium Arsenite (Sigma-Aldrich, St. Louis, MO, USA) in M9 medium, or in M9 alone. Worms were exposed to *t*-BOOH (12 mM; Sigma-Aldrich) for 1 h on NGM plates, or incubated on NGM control plates. In each case, worms were then allowed to recover for 1 h on OP50-seeded NGM plates. These stress treatment conditions were established by titrating As or *t*-BOOH concentrations and incubation times, and scoring for induction of the SKN-1 target gene reporter *gcs-1::GFP*

(Supporting Fig. S1) (An & Blackwell, 2003). Under the conditions used for microarray analysis, this reporter was induced robustly in the intestine, but for each stress tested worms that appeared sick or dead were observed at only a low frequency (0–5% across samples).

The *skn-1* RNAi plasmid consisted of a full length SKN-1c isoform cDNA subcloned into pPD129.36 (gift of A. Fire). The control plasmid was pPD128.110 (gift of A. Fire) which contains the GFP gene flanked by T7 promoters (Timmons *et al.*, 2001). RNAi was performed by feeding as described (Kamath & Ahringer, 2003).

RNA preparation and microarray data collection

For each microarray experiment, total RNA was isolated from 50 000 animals using Trizol (Invitrogen, Carlsbard, CA, USA). cDNA was synthesized and linearly amplified from 325 ng RNA using the Low RNA Input Linear Amplification Kit (Agilent, Santa Clara, CA, USA), and labeled with Cy3- or Cy5-CTP (Perkin Elmer, Waltham, MA, USA). A dye swap analysis was performed for each set of biological replicate samples. Samples were fragmented according to Agilent protocols and hybridized overnight at 60 °C to Agilent-015061: *C. elegans* oligonucleotide Microarray 4 × 44 arrays (covering 21 481 genes). Array scanning was performed using a DNA Microarray Scanner (Agilent) at 5 μm resolution. The output image was processed by Feature Extractor (Agilent) and normalized for dye bias by linear correction using rank consistent probes. Prior to hierarchical clustering, values from spots on the microarray that represented the same gene were averaged to a single value. Spots flagged by the Feature Extractor software as having red and green intensities well above background were omitted. Finally, genes that did not have an observed absolute value of 0.4 for the log(base2) ratio of red/green intensities for at least one array were omitted, as were any genes that lacked information for > 20% of the arrays. After filtering, the remaining genes were submitted for downstream analysis. Raw microarray data will be available via the Princeton University MicroaAray database: http://puma.princeton.edu/.

Hierarchical clustering and SAM analysis

Average linkage gene clustering was performed with an uncentered correlation similarity metric using Cluster (Eisen *et al.*, 1998; de Hoon *et al.*, 2004). One-class analysis in SAM (Tusher *et al.*, 2001) was performed to identify genes that had statistically significant changes in expression regardless of the magnitude of change.

Promoter analysis

We analyzed up to 1.5 kb of intergenic sequence upstream of SKN-1-regulated genes for the presence of novel regulatory elements. Sequence elements that were statistically over-represented in these regions were identified using Regulatory Sequence Analysis Tools oligo-analysis (Thomas-Chollier *et al.*, 2008) and Weeder (Pavesi *et al.*, 2004), in each case specifying an oligonucleotide length of eight bases. We later searched directly for these novel consensus elements and the consensus *in vitro* SKN-1 binding site (Blackwell *et al.*, 1994) within up to 2 kb of upstream intergenic sequences. WebLogo (Crooks *et al.*, 2004) was used to display consensus motifs.

Gene ontology analysis

WormBase gene names were converted to NCBI Protein Gene Info (GI) numbers using WormMart (Schwarz *et al.*, 2006), then analyzed using DAVID (Dennis *et al.*, 2003). Functional clusters of SKN-1-regulated genes were identified using DAVID's Functional Annotational Clustering tool, with the exception of the CUB-like domain genes and other annotated open reading frames that lacked a GI entry. The Enrichment Score was used to predict whether representation of a gene group among SKN-1-regulated genes was biologically significant. The Enrichment Score of a cluster of genes or GO terms derives from the geometric mean (in negative log scale) of the *P*-values for members of that cluster. If the geometric mean of the *P*-values = $1e^{-10}$, then the Enrichment Score is 10. These *P*-values correspond to the probability that the members of the cluster are present together randomly in the gene list.

Lifespan analysis

In analyses of lifespan under normal conditions the first day of adulthood was defined as $t = 0$, standard Kaplan–Meier survival curves were generated from the data, and the log-rank (Mantel-Cox) method was used to test the null hypothesis (StatView). These analyses were performed using the RNAi-sensitive strain *rrf-3(pk1426)* (Sijen *et al.*, 2001). In two assays (Experiments 1 and 2), $n > 100$ worms (see Supporting Table S11 for *n*) were transferred at L4 to 100 mm HGM plates (1 mM IPTG, 100 μg mL^{-1} carbenicillin, and 50 μM FUdR, 20 °C) that had been inoculated with the indicated RNAi bacteria. Live/dead counts were made approximately every other day. The experiments were terminated upon contamination after approximately 3 weeks, and the surviving animals were censored from the assay on that day. A third assay (Experiment 3) was also performed using *rrf-3(pk1426)* worms at 20 °C, but in this case, ~75 eggs (see Supporting Table S11c for *n*) were transferred onto 6 × 60 mm NG plates (1 mM IPTG, 100 μg mL^{-1} carbenicillin) that had been inoculated with the indicated RNAi bacteria. Worms were transferred to fresh RNAi plates every 4 days, and animals that were missing, exploded, or bagged were censored from the data on the day of the event.

Stress resistance assays

For stress assays, N2 or *rrf-3(pk1426)* worms that had been arrested at L1 were grown for 48–55 h at 20 °C on either

RNAi or control bacteria. RNAi clones were obtained from published libraries (Kamath & Ahringer, 2003; Rual *et al.*, 2004). RNAi was performed essentially according to Protocol 2 in (Kamath *et al.*, 2000). Worms were then placed in 4 or 5 mM Sodium Arsenite (in M9) and periodically tested for survival, with three to six wells of ≥ 10 worms each examined in each experimental measurement. Worms were prodded with a platinum wire and scored as dead if they displayed no pharyngeal pumping or movement. Control wells of M9 always displayed 100% survival for all time points examined. The assays represented in Fig. S3 (Supporting information) were carried out on NGM agar plates containing either As or *t*-BOOH.

qRT-PCR

Stress- and control-treated worms were collected as for the microarray analysis samples. RNA was isolated and purified using Tri Reagent (Sigma). cDNA was synthesized using Superscript III Reverse Transcriptase (Invitrogen). SYBR GreenR (Invitrogen) real time PCR was performed in an ABI 7700 machine in duplicate and the data was analyzed using the comparative C_t method with the exception of data in Fig. S3b (Supporting information), which were analyzed by normalization to a standard curve. Relative mRNA levels were normalized to *act-1* mRNA levels, and calculated from at least three biological replicates. Primers were designed to be intron-spanning, with sequences available upon request.

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Note added in proof

While this manuscript was under review, it was reported online that *skn-1* is required for a substantial proportion of the transcriptional response to hyperbaric oxygen (Park SK, Tedesco PM, and Johnson TE, *Aging Cell*, Accepted Article). Several of these potential SKN-1 target genes overlapped with those identified here. A contemporaneous study identified many of our SKN-1-dependent genes as being induced in an age-dependent manner by the oxygen-generating stressor juglone (Pryzbysz, *et al.*, *Mech. Aging Dev.* (2009) **130**, 357–369).

Author contributions

Conceived and designed the experiments: RPO, JPA, KD, CTM, TKB. Performed the experiments: RPO, JPA, KD, JL, JA. Analyzed the data: RPO, JPA, KD, JL, CTM, TKB. Wrote the paper: RPO, JPA, CTM, TKB.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Induction of the SKN-1 target *gcs-1* promoter by As and *t*-BOOH.

Fig. S2 Motif occurrence in SKN-1-regulated gene promoters.

Fig. S3 Stress responses are specific to stress type.

Fig. S4 qRT-PCR analysis of *t*-BOOH-induced genes.

Fig. S5 Increased oxidative stress resistance after inhibition of SKN-1-downregulated genes.

Table S1 Genes upregulated by SKN-1 under normal conditions.

Table S2 Genes downregulated by SKN-1 under normal conditions.

Table S3 qRT-PCR analysis of genes upregulated by SKN-1 under normal conditions.

Table S4 qRT-PCR analysis of genes downregulated by SKN-1 under normal conditions.

Table S5 Genes upregulated by SKN-1 and As stress.

Table S6 qRT-PCR results for SKN-1-dependent and-independent stress responsive genes.

Table S7 Genes upregulated by SKN-1 independently of As treatment.

Table S8 Genes upregulated by SKN-1 under *t*-BOOH stress.

Table S9 Genes upregulated by *t*-BOOH (SKN-1-independent).

Table S10 Genes downregulated by *t*-BOOH (SKN-1-independent).

Table S11 (a) Lifespan effects of SKN-1-downregulated genes (Experiment 1); (b) lifespan effects of SKN-1-downregulated genes (Experiment 2); (c) Lifespan effects of SKN-1-downregulated genes (Experiment 3).

Table S12 Counting and censoring data for lifespan experiments 1 and 2.

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